Carboxyl Ester Lipase Cofractionates with Scavenger Receptor BI in Hepatocyte Lipid Rafts and Enhances Selective Uptake and Hydrolysis of Cholesteryl Esters from HDL₃*

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Cholesteryl esters are selectively removed from high density lipoproteins by hepatocytes and steroidogenic cells through a process mediated by scavenger receptor BI. In the liver, this cholesterol is secreted into bile, primarily as free cholesterol. Previous work showed that carboxyl ester lipase enhanced selective uptake of cholesteryl ether from high density lipoprotein by an unknown mechanism. Experiments were performed to determine whether carboxyl ester lipase plays a role in scavenger receptor BI-mediated selective uptake. When added to cultures of HepG2 cells, carboxyl ester lipase cofractionated with scavenger receptor BI and [³H]cholesteryl ether-labeled high density lipoprotein in lipid raft fractions of cell homogenates. Confocal microscopy of immunostained carboxyl ester lipase and scavenger receptor BI showed a close association of these proteins in HepG2 cells. The enzyme and receptor also cofractionated from homogenates of mouse liver using two different fractionation methods. Antibodies that block scavenger receptor BI function prevented carboxyl ester lipase stimulation of selective uptake in primary hepatocytes from carboxyl ester lipase knockout mice. Heparin blockage of cell-surface proteoglycans also prevented carboxyl ester lipase stimulation of cholesteryl ester uptake by HepG2 cells. Inhibition of carboxyl ester lipase activity in HepG2 cells reduced hydrolysis of high density lipoprotein-cholesterol esters ~40%. In vivo, hydrolysis was similarly reduced in lipid rafts from the livers of carboxyl ester lipase-null mice compared with control animals. Primary hepatocytes from these mice yielded similar results. The data suggest that carboxyl ester lipase plays a physiological role in hepatic selective uptake and metabolism of high density lipoprotein cholesteryl esters by direct and indirect interactions with the scavenger receptor BI pathway.

The cardioprotective effects of high density lipoproteins (HDLs) are well known to derive from their contributions to cholesterol efflux from cells of peripheral tissues and the transport of this cholesterol to the liver where it is removed from the circulation and eliminated from the body. The process of reverse cholesterol transport (Refs. 1–6; for review, see Ref. 4). During transport, much of this cholesterol is carried in esterified-form in the hydrophobic core of HDL particles (7). Transfer of cholesteryl esters (CEs) and cholesterol from HDL to liver parenchymal cells (hepatocytes) occurs by selective removal of core lipids with the primary HDL particle (phospholipid, free cholesterol, apolipoprotein A-I), returning to the circulation undegraded, the process commonly called selective uptake (8–10).

In recent years, our understanding of the mechanisms underlying the internalization and subsequent metabolism of HDL cholesterol has greatly increased, although several steps remain incompletely understood. It is now clear that the class B, type I scavenger receptor (SR BI) is the plasma membrane receptor that mediates selective uptake (6, 11, 12, 14–17). SR BI has high affinity for apolipoprotein A-I, and interaction of this protein with the receptor is important to the selective uptake process (18, 19). Recent reports appear to confirm the model suggested several years ago (9, 10) in which HDL particles undergo a retroendocytosis process facilitated by SR BI during which lipids are separated from the holoparticle for delivery to the bile canaliculus, and the particle itself is resorbed (20, 21).

The majority of HDL CEs absorbed by the liver via the selective uptake mechanism are hydrolyzed to free cholesterol before being secreted into bile (17). Although it is known that this hydrolysis occurs in an extralysosomal (22, 23) membrane compartment (24), the cholesterol esterase responsible has not been identified. A recent study showed that HDL CE hydrolysis is achieved by a different enzyme in different tissues (24). There are four cholesterol esterase enzymes in hepatocytes with the potential to serve this function. Hormone-sensitive lipase has cholesterol esterase activity, but in vitro studies (24) and analyses of knockout mice (25, 26) suggest this enzyme hydrolyzes HDL CE in testes and adrenal glands but not liver. A microsomal cholesterol esterase that is biochemically distinct from other esterases has been purified from hepatocytes, but its location on the luminal face of rough endoplasmic reticulum is not consistent with a role in HDL CE hydrolysis (27). Similarly, the well-characterized hepatic neutral cholesterol ester hydrolase (28, 29) is located in the cytosol and, thus, is also not a good candidate for HDL CE hydrolysis after selective uptake.

Carboxyl ester lipase (CEL, also known as bile salt-stimu-

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The abbreviations used are: HDL, high density lipoprotein; CE, cholesteryl ester; SR BI, scavenger receptor class B, type I; CEL, carboxyl ester lipase; PBS, phosphate-buffered saline; HSPG, heparin-sulfate proteoglycan; TLC, thin layer chromatography; MVB, multivesicular bodies; CURL, compartment for uncoupling of receptor and ligand; RRC, receptor recycling compartment; CEF, caveolae-enriched fraction; KO, carboxyl ester lipase knockout; WT, wild type; TDC, taurodeoxycholate.

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lated lipase or BSSL) is another cholesterol esterase present in the liver (30) with the capacity to hydrolyze HDL CE. CEL is abundant in pancreatic juice and in the milk of most mammals, and its role in lipid digestion and absorption is well studied (Refs. 31–35; for review, see Refs. 36 and 37). In addition, CEL is made and secreted by hepatocytes (38), endothelial cells (39), and macrophages (40), and the enzyme is present at low levels in plasma (41, 42). A role for CEL in HDL metabolism was suggested by studies that showed increased selective uptake of cholesteryl ether by HepG2 cells when CEL was added to the medium (43). In addition, cell fractionation studies showed the presence of CEL in a retromicrosome fraction prepared from rat liver endosomes (44).

The purpose of the current study was to test the hypothesis that CEL affects selective uptake by the SR BI pathway and contributes to hydrolysis of HDL-derived cholesteryl esters. Using multiple approaches CEL is shown to colocalize with SR BI in lipid rafts and to enhance selective uptake of HDL CE by the SR BI pathway. Results also show decreased hydrolysis of HDL CE in CEL knockout mice and in cultured hepatocytes lacking CEL activity.

**EXPERIMENTAL PROCEDURES**

**HDL Isolation and Radiolabeling—**HDL (1.125 < p < 1.21) was isolated from recently expired human plasma obtained from the local blood bank (Foxworth Blood Center, Cincinnati, OH) by standard methods of sequential centrifugation using KBr to adjust solution density (45). KBr was removed by extensive dialysis against phosphate-buffered saline (PBS). If necessary, the HDL fraction was concentrated using Aquacide II (Calbiochem-Novabiochem) for several hours and re-dialyzed. Radiolabeled lipids were added to HDL according to the potato starch method described by Glass et al. (8). Briefly, 2 mg of HDL (protein mass) was lyophilized onto 25 mg of potato starch (Sigma-Aldrich) in a siliconized glass tube. This mixture was vortexed thoroughly, chilled in a dry ice/ethanol bath, and lyophilized until the starch was powder-dry. [3H]Cholesteryl oleoyl ether or ester (2–5 μCi/mg of HDL protein, 30–60 Ci/mmol, Amersham Biosciences) was added to the dry HDL/starch mixture in 500 μl of heptane and vortexed thoroughly. The sample was incubated for 1 h at room temperature with periodic vortexing. The heptane was evaporated under nitrogen, and the HDL was solubilized in 1 ml of PBS. The HDL/starch mixture was incubated overnight at 37 °C with gentle agitation, after which the starch was pelletted along with the unincorporated label, and the supernatant containing the reconstituted HDL was removed. This supernatant was filtered through a 1.0-μm syringe filter, and the specific activity was determined. In one experiment (experiment 1, Table I) HDLₙ was labeled by cholesteryl ester transfer protein-mediated transfer of radio-labeled cholesteryl oleate from donor liposomes followed by reisolation of HDL, as described (22). Protein concentration of isolated HDL was determined by the modified Lowry method of Markwell et al. (46). Cholesterol was determined by enzymatic methods using kits from Wako Chemicals (Richmond, VA). When warranted by agarose gel analysis) HDL was repurified by isopycnic centrifugation in KBr as above or in self-forming gradients of iodixanol/PBS exactly as described (47).

**Radioiodination of CEL and HDL—**Human CEL (purified from milk, a generous gift from Dr. Ron Jandacek) or HDLₙ was labeled with 125I using the IODO-Beads® iodination reagent from Pierce according to manufacturer’s instructions. In a typical reaction 100 μg of protein was reacted with 1.0 μCi of Na125I. Protein was separated from unincorporated 125I with a 1.0-ml Sephadex G-25 (Amersham Biosciences) column followed by extensive dialysis against PBS to remove residual free iodine. Protein was determined by the Lowry method (48).

**Cell Culture Selective Uptake Assays—**The culture conditions used for the human hepatoma cell line, HepG2, were those of Li et al. (43). For selective uptake experiments cells were plated on 80-mm plates, and experiments were performed 5–7 days after plating, when the cells reached confluence. On the day of the experiment, fresh medium containing 0.1–2 mtcholestrol (concentration varied with experiment according to each figure) or 0.5 μm taurodeoxycholate and the radiola-beled HDL (2–5 × 10⁶ dpm/dish) was added to the cells, and incubation was carried out for 4 h at 37 °C in the presence or absence of 125I-labeled CEL. Plates were then washed 2 times with fresh medium and incu-

bated with serum-free medium for 30 min at 37 °C. After removing the “wash” medium plates were chilled on ice, and 1 ml of ice-cold homogenization buffer (0.25% sucrose, 10 mM Tris·HCl, pH 7.4, 1% protease inhibitor mixture (Sigma)) was added to each. Cells were removed by scraping and disrupted with several strokes of a tight-fitting Dounce homogenizer. The cell homogenate was overlaid on a preformed density gradient of 10–26% iodixanol (OptiPrep™, Sigma). The gradient was centrifuged at 110,000 × g for 3 h at 4 °C in a Beckman SW40 rotor, and 1-ml fractions were harvested from the top by pipette and stored at −80 °C until analyzed. The protein content of the fractions was determined using the MicroBCA™ protein assay reagent kit from Pierce. Appropriate concentrations of iodixanol were included in standard curves and blanks to correct for its slight interference with the assay. When investigating the possible involvement of cell surface proteo-
glycans in the selective uptake process, the HepG2 cells were pre-

treated for 3 h at 37 °C in medium containing 10% lipoprotein-deficient serum with or without heparin at 100 μg/ml, a concentration that specifically blocks interactions with heparin-sulfate proteoglycans (HSPOG™) (49, 50) but not binding to lipoprotein receptors (50, 51). After 3 h, 10% lipoprotein-deficient serum medium containing 0.2 μm tauro-

cholate and HDL [3H]CE was added to the cells with or without CEL (30 μg/ml). The incubation, wash, and fractionation of the cells were performed as described above. The lipid raft fractions for each experimenta-
tal group were analyzed by thin layer chromatography (TLC) to determine the total cholesterol incorporated from the HDL [3H]CE during selective uptake.

**Western Blot Analysis—**The proteins in each volumes of density gradient fractions from HepG2 or mouse liver cell homogenates were separated by electrophoresis through an 8% polyacrylamide, 0.1% so-
dium dodecyl sulfate gel, and the resolved proteins were electroblotted onto nylon-supported nitrocellulose (Osmonics, Westborough, MA). Af-
ter transfer, SR BI protein was detected with a specific primary anti-
body (Novus Biologicals, Littleton, CO) and the ECF Western blotting Kit (Amersham Biosciences). Chemiluminescence was measured with a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**TLC of Lipids—**Lipids were isolated from gradient fractions by ex-
tracting 2 times with 2 volumes of chloroform:methanol (2:1). The solvents were evaporated under nitrogen, and the lipid raft fractions were dissolved in 30 μl of chloroform and then spotted onto silica gel G TLC plates that were pre-dried for 30 min at 80 °C. Cholesterol and cho-

lesteryl ester standards were loaded separately on the same plate. Lipids were resolved with a mobile phase of petroleum ether:ethyl ether:acetic acid (300:60:1). Lipids were visualized with iodine vapor, and free cholesterol and cholesteryl esters were scraped from the plate and quantitated by scintillation spectrometry.

**Immunohistochemistry and Confocal Microscopy—**For immunohisto-
chemistry, HepG2 cells were grown on gelatin-coated (1%) glass cover slips in 60-mm culture dishes. All other growth conditions were the same as described above. Immunostaining was done 3 days after plating when cells were ~60% confluent. At this time, CEL was added to the cells exogenously in 2 ml of fresh medium, and the cells were incubated for 30 min at 37 °C. The medium was then removed, and the cells were rinsed with PBS to remove unbound CEL. Cells were fixed by sequential treatment with 5% formalin in PBS, 5% formalin, 10% methanol in PBS, and 30% methanol in PBS for 1 min each and were then blocked with 1% bovine serum albumin in PBS for 30 min. Antibody stocks were centrifuged for 30 min at 150,000 × g in a Beckman Airfuge to remove aggregates and reduce background before being diluted with 1% bovine serum albumin in PBS for use. Primary antibodies were diluted 1:500, and all other reagents were diluted 1:100 as recommended by the suppliers. All incubations were for 30 min at room temperature and were followed by one brief PBS washes. Because both primary antibodies were raised in rabbits, spe-
cific reagents from Jackson Immunoresearch Laboratories (West Grove, PA) that were pre-selected by the manufacturer for the absence of cross-reactivity were used. Cells were first incubated with the CEL-specific antibody (43) followed by the Fab fragment of a goat anti-rabbit IgG antibody (Jackson Immunoresearch) and then rhodamine-conjugated mouse anti-goat IgG antibody. Cells were next incubated with the SR BI-specific antibody (Novus Biologicals) and, finally, with fluores-

csein isothiocyanate-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch). The coverslip was inverted, cell side down, onto a mini-

magnification 100× objective of ProLong® antifade reagent (Molecular Probes, Eugene, OR) and was allowed to dry. Cells were then mounted with 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei and examined using a Leica TCS 4D microscope/SCANeware system (Heidelberg, Germany) equipped with an Omnichrome krypton-argon laser (Chino, CA). Excitation was performed with the 488- and 568-nm argon/krypton laser lines simultaneously. Emission was de-
cells were incubated for 4 h at 37 °C with \([^{3}H]\)cholesteryl ether-labeled HDL, in the presence (w/o) or absence (w) of exogenous CEL (60 μg/ml). Medium contained 2 mM taurocholate to stimulate CEL activity. Cells were washed, and lipids were extracted as described under “Experimental Procedures,” and cellular \([^{3}H]\)cholesteryl ether was determined by scintillation spectrometry. Data are the average ± S.D. (p < 0.05) of duplicate experiments with three dishes of cells per group.

In other experiments lipid rafts and other endosome fractions were prepared from mouse liver essentially as described by Pol et al. (52). Livers were isolated and homogenized, and debris was removed as just described. The initial supernatant was subsequently centrifuged at 3,500 × g for 15 min and 12,000 × g for 20 min. This third supernatant was diluted with Percoll™ (Amersham Biosciences) and 0.25 M sucrose to give a final density of 1.07 g/ml. Density marker beads (Amersham Biosciences) were added to the solution, and it was centrifuged for 45 min at 29,000 × g and 4 °C. The Percoll™ gradient was harvested down to the 1.052 g/ml density marker, and the solution that was removed was diluted with 3 parts 0.15 M NaCl. Endosomes from this fraction were collected onto a 2.5 M sucrose cushion by centrifugation at 10,000 × g for 15 min. The supernatant from this second spin was overlaid on a preformed 10–26% iodixanol gradient and centrifuged at 110,000 × g for 3 h. The resulting gradient was collected in 1-ml fractions from the top by pipette.

To assay HDL CE uptake and hydrolysis, hepatocytes were concentrated as above and ∼0.5 × 10^6 cells were plated per well in twenty-four-well culture plates. Growth medium (0.3–0.4 ml) was supplemented with taurocholate at a final concentration of 0.2 mM. Cells were pretreated for 1 h at 37 °C with or without SR B1 blocking antibodies (54, Novus Biologicals) diluted 1:100 in medium, then incubated with HDL [^{3}H]CE (1–3 × 10^6 dpm/well) with or without 30 μg/ml CEL for 1.5 h. Cells were homogenized and fractionated in iodixanol density gradients, and the radioactivity in the lipid raft fraction was analyzed as previously described. Some hydrolysis assays used 0.5–1.0 × 10^6 hepatocytes per well in 12-well culture plates with 0.75 ml of medium containing 0.1 mM taurocholate and 1-h incubations with or without 20 μg/ml CEL and HDL [^{3}H]CE as above.

RESULTS

The potential role of CEL in the selective uptake of lipids from HDL by hepatocytes was first explored using cultures of HepG2 cells. HDL labeled with \([^{3}H]\)cholesteryl ether was added to HepG2 cells in medium containing 2 mM taurocholate, and the cells were incubated with or without 60 μg/ml exogenous CEL for 4 h. After washing, cells were lysed, and the amount of cholesteryl ether that had been internalized during uptake was determined. As shown in Fig. 1, in the presence of exogenous CEL, HepG2 cells internalized ~4 times more cholesteryl ether than when CEL was not added (p < 0.05). This result is similar to that reported in a previous study (43).

To begin to understand the mechanism by which CEL might enhance selective uptake, experiments were performed to determine whether an association could be found between CEL and other components of the selective uptake pathway. In one experiment, HDL labeled with \([^{3}H]\)cholesteryl ether was added...
to HepG2 cells in the presence of $^{125}\text{I}$-labeled CEL protein and 2 mM taurocholate for 4 h. The cells were then homogenized and fractionated on a preformed 10–26% iodixanol density gradient. The results presented in Fig. 2 demonstrate that $^{125}\text{I}$-labeled CEL does cofractionate with selectively internalized [3H]cholesteryl ether, and both are consistently found in the greatest concentration in fraction 4. Western blot analysis was used to determine the distribution of SR BI in each fraction and is presented above the graph. These results suggest an association of CEL with HDL and SR BI in lipid rafts during selective uptake.

Although the distributions of CEL, SR BI, and HDL cholesteryl ether in iodixanol gradients were strikingly similar, consideration was given to the fact that this relationship was based on cofractionation after density centrifugation. Therefore, immunohistochemistry and confocal microscopy were utilized as a more direct approach to test for an association between CEL and SR BI. Fig. 3 illustrates that CEL does colocalize with SR BI in the plasma membrane of HepG2 cells. The yellow color represents the coincidence of red and green pixels, indicating colocalization of CEL and SR BI. The arrowheads in panels E and F indicate specific areas of apparent SR BI and CEL concentration and colocalization in plasma membranes. Images are representative of most slices seen in several experiments.

**FIG. 3.** Colocalization of CEL and SR BI in HepG2 cell plasma membrane by confocal microscopy. HepG2 cells grown on glass coverslips were incubated for 30 min with CEL (30 μg/ml) then rinsed with PBS, fixed, and permeabilized. CEL was immunostained with Texas Red, SR BI was immunostained with fluorescein isothiocyanate as described under “Experimental Procedures,” and cells were examined with a confocal microscope. Panels A and B show only fluorescein isothiocyanate-stained SR BI, whereas panels C and D show only Texas Red-stained CEL. Panels E and F show the merged fluorescein isothiocyanate and Texas Red images. The yellow color represents the coincidence of red and green pixels, indicating colocalization of CEL and SR BI. The arrowheads in panels E and F indicate specific areas of apparent SR BI and CEL concentration and colocalization in plasma membranes. Images are representative of most slices seen in several experiments.

**FIG. 4.** Cofractionation of mouse liver CEL and SR BI in iodixanol gradients. Mouse liver was homogenized and fractionated in an iodixanol density gradient as for HepG2 cells. Fractions were analyzed for the presence of bile salt-stimulated cholesterol esterase activity as described under “Experimental Procedures.” Only trihydroxy bile salt-stimulated activity (CEL-specific) is reported. The data represent the average of duplicate assays. The amount of SR BI in each fraction was determined by Western blot analysis and is presented above the graph.

**FIG. 5.** CEL and SR BI are present in the same endosome fraction from mouse liver. Mouse liver endosomes were prepared and separated into MVB, CURL, RRC, and CEF (lipid raft) fractions using sucrose density gradients as described under “Experimental Procedures.” These endosome fractions were analyzed for CEL activity and SR BI protein as described for Fig. 4. CEL activity data are presented as the amount of cholesteryl ester hydrolyzed/h/fraction and is the average of duplicate assays.

Experiments were next performed to determine whether the association between CEL and SR BI observed in HepG2 cells also exists in hepatocytes in vivo. The first experiment was to discover if endogenous CEL was present and had esterase activity in the lipid rafts of liver cells. Mouse liver was homogenized and fractionated in an iodixanol gradient in the same way as described for HepG2 cells, and the fractions were analyzed for the presence of bile salt-stimulated cholesterol esterase activity (CEL-specific) and SR BI protein. The data represent the average of duplicate assays. The amount of SR BI in each fraction was determined by Western blot analysis and is presented above the graph.
**CEL Enhances Hepatic Uptake and Hydrolysis of HDL CE**

**Fig. 6.** Effect of CEL and anti-SR B1-blocking antibody on the selective uptake of CE from HDL by primary WT and CEL KO hepatocytes. Primary hepatocytes were isolated from the livers of WT and CEL KO mice as described under “Experimental Procedures.” Some hepatocytes (+Ab) were pretreated with anti-SR B1 blocking antibody (1/100) for 1 h at 37 °C. Cells were then incubated for 1.5 h in medium containing 0.2 mM taurocholate and HDL [3H]CE with or without 30 μg/ml CEL. Cells were homogenized and fractionated in iodixanol density gradients as previously described, and the amount of [3H] in the lipid raft fraction was determined by scintillation spectrometry. Each bar represents the average ± S.D. of duplicate experiments with three wells of hepatocytes per group. Results are representative of three independent experiments. p < 0.05 for bars with different letters.

**Fig. 7.** Effect of heparin pretreatment on the selective uptake of CE from HDL by HepG2 cells. HDL [3H]CE uptake was determined by comparing the total cholesterol recovered by TLC from the lipid rafts of HepG2 cells as described under “Experimental Procedures.” Cells were pretreated with or without 100 μg/ml of heparin followed by incubation in 10% lipoprotein-deficient serum medium containing 0.2 mM taurocholate and [3H]CE-HDL with or without 30 μg/ml of CEL. Each bar represents the combined results from three dishes of cells per group. Results are representative of three independent experiments.

**Fig. 8.** Effect of CEL activity on the selective uptake and hydrolysis of HDL CE by HepG2 cells. HepG2 cells were incubated for 4 h at 37 °C with [3H]cholesteryl oleate-labeled HDL, and 60 μg/ml exogenous CEL in the presence of either 2 mM taurocholate or 0.5 mM taurodeoxycholate. Lipids were extracted from the cells and separated by thin layer chromatography to determine the amount of free and esterified cholesterol. Cholesteryl ester hydrolysis (open bars) is expressed as the percent of [3H] in free cholesterol from internalized lipids. The apparent HDL uptake (striped bars) represents HDL CE corrected for any whole particle uptake as determined by incorporation of label from 125I-labeled HDL. Results are the average ± S.D. of duplicate experiments with three dishes/group/experiment. * p < 0.01.

Hepatic CEL activity in lipid rafts was also measured using 10% lipoprotein-deficient serum medium containing 0.2 mM taurocholate and [3H]CE-HDL with or without 30 μg/ml of CEL. Each bar represents the combined results from three dishes of cells per group. Results are representative of three independent experiments.

To demonstrate that CEL enhances SR B1-mediated selective uptake directly, the effect of SR B1-blocking antibodies (54) on HDL [3H]CE uptake by primary hepatocytes from CEL−/− and control mice was investigated. Fig. 6 shows that the addition of CEL to knockout (KO) hepatocytes significantly increased the amount of HDL [3H]CE in lipid raft fractions to the same level seen in control (WT) hepatocytes (p < 0.05). Pretreatment with SR B1-blocking antibody decreased HDL [3H]CE in lipid rafts to the same degree in WT (36.5% less than WT without antibody) and CEL-treated KO (31.7% less than KO + CEL only) hepatocytes (p < 0.05 for both comparisons). Notably, the SR B1 antibody completely prevented CEL stimulation of HDL CE uptake by knockout cells. In control assays, nonspecific antibodies had no effect on uptake by either type of hepatocyte (data not shown).

Because CEL has a heparin binding site, the ability of the enzyme to enhance selective uptake may be influenced by an interaction between CEL and HSPGs on the cell surface instead of direct interaction with SR B1. This issue was addressed by pretreating HepG2 cells with 100 μg/ml heparin to specifically block any interaction between CEL and HSPGs without inhibiting binding of HDL to SR B1 (51). In Fig. 7 the uptake of [3H]CE from HDL in the presence or absence of CEL and heparin pretreatment was determined by looking at the total cholesterol recovered from the lipid rafts of HepG2 cells. As previously shown, the presence of CEL increased selective uptake of [3H]CE almost 2-fold as compared with adding HDL

enzized mouse liver and analyzed for CEL activity and for the presence of SR B1 as described under “Experimental Procedures.” In Fig. 5, the amount of cholesteryl ester hydrolyzed in vitro in 1 h by endogenous CEL is presented for each of the endosome fractions. Although minimal activity was found in the MVB, CURLs, and RRCs, the lipid raft fraction did exhibit a significant amount of bile salt-dependent esterase activity. The Western blot results shown in Fig. 5 suggest that the association between CEL and SR B1 seen in HepG2 cells is also present in vivo. Although the receptor is present in all four fractions, the greatest amount is found in the lipid raft fraction. Taken together, these cofractionation data are consistent with the hypothesis that an association between CEL and the selective uptake pathway exists in vivo as well as in cultured cells.

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**Fig. 9. Effect of taurocholate and CEL concentrations on the hydrolysis of HDL CE by HepG2 cells.** HDL $[^3H]$CE hydrolysis by HepG2 cells was measured as described above and is expressed as percent of $[^3H]$extracted as free cholesterol from lipid raft fractions. Each bar represents the average duplicate experiments with two or three dishes of cells per experiment. A, cells were treated with $30 \mu$g/ml CEL in the presence of the indicated concentrations of taurocholate (TC) or taurodeoxycholate (TDC) (*, p < 0.03 for comparison between each group (0.1 mM TC not done in duplicate)). B, cells were treated with the amounts of exogenous CEL and sodium taurocholate as indicated on the graph. All results were significantly greater than TDC controls (TDC bars of Fig. 8 and this figure, panel A).

**TABLE I**

| CEL genotype (n) | CEL hydrolyzed ± S.D. | Average time after injection (min) |
|------------------|-----------------------|-----------------------------------|
| $+/\text{TCD}$ (3) | 60.2 ± 12.6           | 90                                 |
| $+/\text{TC}$ (4) | 86.6 ± 9.5            | 45                                 |

$[^3H]$CE alone. However, this increase was not seen when heparin pretreatment blocked CEL interaction with HSPGs. This suggests the involvement of cell surface proteoglycans present in lipid rafts (55) in the ability of CEL to enhance the selective uptake of CE from HDL.

Experiments were next conducted to determine whether CEL contributes to the hydrolysis of HDL CE during or after selective uptake by hepatocytes. Hydrolysis of HDL labeled with $[^3H]$cholesteryl oleate instead of the unhydrolyzable cholesteryl ether was first measured in HepG2 cells. In this experiment HDL uptake was measured as described under “Experimental Procedures” in the presence of CEL and either 2 mM taurocholate or 0.5 mM taurodeoxycholate (TDC). Because CEL activity is stimulated only by trihydroxy bile salts, TDC, a dihydroxy bile salt, served as a negative control. The lower concentration of TDC was necessary because 2 mM is above its critical micelle concentration and causes cell disruption. Lipids were extracted from the lipid raft fractions of iodixanol gradients, and the amount of free and esterified radiolabeled cholesterol was determined by TLC. Fig. 8 shows the effect of CEL activity on both the uptake and hydrolysis of HDL cholesteryl ester. No significant difference in uptake was detected between the two bile salt treatments (striped bars). However, the extent of cholesteryl ester hydrolysis that occurred during or after internalization (open bars) was dramatically reduced (p < 0.002) by TDC treatment. In the presence of taurocholate, ~90% of the ester internalized during selective uptake had been hydrolyzed to free cholesterol, whereas only 50% was hydrolyzed when CEL was inhibited.

This initial hydrolysis experiment was performed under conditions used in previous studies (43) that were selected to maximize any CEL effects (2 mM bile salt, 60 $\mu$g/ml CEL). To better mimic physiologic conditions, the experiment was repeated using 10–20-fold less bile salt and 2–10-fold less exogenous enzyme. As shown in Fig. 9A, CEL significantly increased HDL CE hydrolysis in the presence of 0.2 mM taurocholate (85.8 ± 0.9% hydrolyzed) as well as with 2.0 mM taurocholate (97.1 ± 1.6% hydrolyzed) as compared with hydrolysis in the presence of 0.5 mM taurodeoxycholate (66.0 ± 4.5%; p < 0.03). A similar effect of CEL was also observed at 0.1 mM taurocholate (85.1% hydrolyzed). Hydrolysis at 0.2 mM taurocholate was less than that at 2.0 mM but remained significantly greater than taurodeoxycholate control values (p < 0.03). Reducing the amount of exogenous CEL as much as 10-fold did not diminish its effect on hydrolysis of HDL CE by HepG2 cells (Fig. 9B). The apparent difference seen in Fig. 9B is due to the lower concentration of taurocholate used in those samples.

The contribution of CEL to HDL cholesteryl ester hydrolysis in vivo was tested using knockout mice. For this experiment labeled HDL was injected into control or CEL–/– mice via either the jugular or saphenous vein. After 45 min (experiment 1) or 90 min (experiment 2), animals were sacrificed, and liver endosomes were fractionated by the sucrose gradient method described under “Experimental Procedures.” Lipids were extracted from the lipid raft fraction, and the amount of free and esterified cholesterol determined by TLC. Table I presents the results of two separate experiments. In both experiments significantly less of the radiolabeled cholesteryl ester was hydrolyzed in the knockout mice (p = 0.03). In the first experiment, less hydrolysis was detected in both control and CEL–/– mice than in the second experiment, although the difference between groups was similar in the two experiments. This may have resulted from the shorter incubation time used in the first experiment but might also be affected by differences in HDL preparation (CETP versus potato starch labeling) and/or the site of injection (saphenous versus jugular vein).

To demonstrate that these in vivo results were a direct effect of CEL and that they paralleled results obtained from HepG2 cell culture experiments, primary hepatocytes were prepared from CEL–/– mice and tested for the extent to which they hydrolyzed HDL CE in the presence versus absence of exogenous enzyme. The experiments were performed as described for
the HepG2 cells except that taurocholate was present at 0.1 mM only, and CEL, when added, was present at 20 μg/ml. For each experiment, the % of HDL CE hydrolyzed by KO hepatocytes with or without added CEL was normalized to the % hydrolyzed by WT hepatocytes isolated and assayed at the same time. The combined results of three separate experiments (three different KO and WT hepatocyte preparations) were that 63.4 ± 5.3 (S.D.) % of internalized HDL \[^{3}H]\]CE was hydrolyzed by KO hepatocytes as compared with 87.8 ± 11.2 (S.D.) % hydrolyzed by KO hepatocytes supplemented with CEL (p < 0.03). These results are strikingly similar to those obtained both in vivo (Table I) and with HepG2 cells (Fig. 8), supporting the hypothesis that the effects of CEL seen in vitro reflect the physiological role of this enzyme in HDL metabolism.

DISCUSSION

The goal of this study was to determine whether CEL contributes to hepatic uptake and hydrolysis of HDL CE via the SR BI-mediated-selective uptake pathway. A role for CEL in HDL metabolism has been suggested by previous studies. The enzyme is expressed and secreted by hepatocytes both in culture and in vivo (30, 38, 43) and is found in intracellular vesicles as well (44). Li et al. (43) show that selective uptake of HDL cholesteryl ether by HepG2 cells was increased by CEL and that this effect was blocked by antibodies to the enzyme. A role for hepatic CEL in HDL metabolism is also supported by a recent report (56) that both the CEL and SR BI genes are regulated by LRH-1, liver receptor homologue 1, a nuclear receptor that is important for the regulation of cholesterol homeostasis and expression of bile acid synthesis genes (57).

The experiments presented in the current report build on this previous work. SR BI has been shown to be the HDL receptor that facilitates selective uptake (11–21). It was of interest, then, to determine whether the CEL effects are also part of this pathway. The cofractionation of \[^{125}I\]labeled CEL, HDL \[^{3}H\]cholesteryl ether, and SR BI in iodixanol gradients of HepG2 cell homogenates suggests that this is so (Fig. 2). Confocal microscopy was used as a second approach in an attempt to show colocalization of CEL and SR BI (Fig. 3). Results from both experiments are consistent with the presence of CEL in lipid rafts that also contain SR BI. CEL and SR BI were also found to cofractionate from homogenates of mouse liver by two different fractionation methods (Figs. 4 and 5). Using the sucrose gradient method, CEL was found primarily in the lipid raft fraction (also called CEF). These results differ from a previous study (44) that reported CEL in retosomes. However, the method used in that study separated endosomes somewhat differently and may not have separated the RRC and CEF. The current results support the co-localization of CEL and SR BI in vivo and are consistent with it being internalized as part of the selective uptake process. In addition to co-localization, the experiments with SR BI-blocking antibodies (Fig. 6) strongly suggest that CEL enhances HDL uptake directly through the SR BI pathway.

Because CEL binds to heparin sulfate proteoglycans on cell surfaces, a physical association between the enzyme and SR BI does not need to be invoked. Instead, the involvement of HSPGs in the ability of CEL to enhance the selective uptake of cholesteryl esters from HDL is strongly supported by the results of the HepG2 experiment, in which heparin was used to block this interaction (Fig. 7). Treating the cells with heparin effectively blocked proteoglycans and inhibited the increased uptake of cholesteryl ester induced by CEL. Proteoglycan involvement is also supported by attempts at co-immunoprecipitation of CEL with antibodies directed against SR BI, which suggests their association involves a membrane component and not direct protein interactions (data not shown).

The bile salt dependence of CEL is not a limitation to its potential function outside of the gastrointestinal tract. Circulating bile salt concentration is ~10 μM and may increase 3-fold postprandially (42). Concentrations in portal blood are typically 6 times higher than in peripheral circulation. The K_M for bile salt binding to CEL is ~20 μM, and it is enzymatically active at approximately this same concentration (37, 42). The high bile salt concentrations (~10 mM) present in intestine and typically used with CEL in vitro are important for substrate solubility, not enzyme activity. This point is demonstrated by the current results showing an effect of CEL on HDL CE hydrolysis by both HepG2 cells and primary hepatocytes at concentrations of only 100 μM taurocholate, a level approximating physiological conditions.

At low bile salt concentrations CEL is not able to hydrolyze HDL CE in solution (Ref. 42 and data not shown), but CEL activity markedly increased hydrolysis of HDL CE in vitro in HepG2 cells and in primary hepatocytes even at low bile salt concentrations. This result combined with the presence of CEL in lipid rafts suggests that this enzyme is responsible at least in part for hydrolyzing HDL CE during or soon after its uptake by SR BI. This model would be consistent with the results of Silver et al. (20, 21), who show that HDL is internalized, the lipids are removed, and the remainder of the HDL particle is resorbed by a retromer recycling mechanism. The presence of CEL in these vesicles could facilitate hydrolysis of the cholesteryl esters before delivery to the bile canaliculus (17).

In CEL/−/− mice, primary hepatocytes, and HepG2 cells, the absence of CEL activity reduced HDL CE hydrolysis to a very similar degree. In the absence of CEL, only ~60% of the cholesteryl ester was hydrolyzed, whereas ~90% was hydrolyzed in the presence of SR BI activity. It may be that a second enzyme is induced to compensate for the lack of CEL. Another possibility is that, in the absence of CEL, the cholesteryl esters of HDL are alternatively processed by hepatocytes and are hydrolyzed by a cholesterol esterase associated with a different pathway.

The exact mechanism by which CEL enhances hepatic selective uptake remains unclear. The data presented show that CEL plays an important role in hydrolysis of HDL CE but do not identify whether this occurs during SR BI-mediated internalization or within an endosomal vesicle compartment. In addition, the data suggest that CEL has a positive effect that is independent of its enzyme activity, raising the possibility that CEL enhances or stabilizes the binding of HDL to SR BI. Interestingly, previous work has shown that CEL can exhibit cholesterol transfer activity in vitro (13). In vivo data presented here suggest that CEL/−/− mice may manifest differences in HDL metabolism. Recent results indicate elevated plasma cholesterol in these mice, and studies are in progress to determine which lipoproteins are involved and to determine whether hepatic metabolism of HDL cholesterol is altered.

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Carboxyl Ester Lipase Cofractionates with Scavenger Receptor BI in Hepatocyte Lipid Rafts and Enhances Selective Uptake and Hydrolysis of Cholesteryl Esters from HDL₃

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