Hepatic SR-BI-mediated cholesteryl ester selective uptake occurs with unaltered efficiency in the absence of cellular energy

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Abstract **Scavenger receptor class B type I (SR-BI) plays a critical role in the delivery of HDL cholesterol and cholesteryl esters (CEs) to liver and steroidogenic tissues by a selective process that does not result in significant degradation of HDL protein. Recently, SR-BI-mediated endocytosis and recycling of HDL have been demonstrated. However, it remains unclear whether efficient SR-BI-mediated selective uptake occurs strictly at the plasma membrane or at additional sites along its endocytic itinerary. To examine the requirement for SR-BI endocytosis in HDL selective uptake, we determined the effects of energy depletion on the levels of cell-associated HDL protein and CE in primary mouse hepatocytes. Compared with CHO cells, we observed a much larger energy-dependent effect on CE uptake in primary mouse hepatocytes. Although varying the levels of caveolin-1 and carboxyl ester lipase altered the efficiency of selective uptake, neither was able to account for the energy-dependent component of HDL-CE uptake. Finally, we demonstrate that the hepatocyte-specific, energy-dependent effects on HDL-apolipoprotein A-I and -CE uptake are independent of SR-BI and are not required to achieve efficient SR-BI-mediated selective uptake of CE. Together, these data support the conclusion that neither the intracellular trafficking of HDL nor any energy-dependent cellular process affects the ability of the cell to maximally acquire CE through SR-BI-mediated selective uptake from HDL.—** Harder, C. J., G. Vassiliou, H. M. McBride, and R. McPherson. **Hepatic SR-BI-mediated cholesteryl ester selective uptake occurs with unaltered efficiency in the absence of cellular energy. *J. Lipid Res.* 2006.** 47: **492–503.**

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HDL has a functional role in the protection against atherosclerosis, and its plasma concentration is inversely correlated with the risk of cardiovascular disease. One of the protective actions of HDL involves cholesterol removal from peripheral cells for transport to the liver for biliary secretion (reviewed in 1). This process, termed "reverse cholesterol transport," is mediated, in part, by the well-established HDL receptor, scavenger receptor class B type I (SR-BI) (2). In contrast to the holoparticle uptake of the LDL pathway (3), SR-BI mediates cholesterol, cholesteryl ester (CE), and phospholipid uptake via a pathway that does not involve significant degradation of the HDL particle, a process known as "selective uptake." This process was originally described to consist of two phases. First, the lipoprotein binds to SR-BI on the cell surface, and second, the lipids in the lipoprotein are transferred to a membrane (reviewed in 1).

Despite intense interest in SR-BI, the cellular mechanism by which SR-BI contributes to the selective uptake and intracellular trafficking of HDL-derived CE is still not fully understood. Early kinetic studies demonstrated that the rate of CE transfer is proportional to the amount of CE in HDL, suggesting that SR-BI forms a hydrophobic channel that facilitates the movement of sterol down a concentration gradient into the plasma membrane (4). Experiments involving reconstitution of SR-BI into liposomes showed that SR-BI mediates CE selective uptake from HDL into multilamellar vesicles independent of cellular cofactors (5). These data are consistent with a model in which HDL binding to SR-BI is sufficient to mediate the diffusional transfer of CE into the plasma membrane.

However, these studies did not address the potential role of other cellular processes in accelerating CE transfer. Accordingly, defects in HDL internalization have been suggested to decrease the selective uptake of CE (6). HDL recycling, analogous to the internalization and recycling of transferrin and its receptor, was speculated to play a role in efficient selective uptake (7). Subsequent studies indicated that SR-BI mediated HDL endocytosis and recycling,

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Abbreviations: apoA-I, apolipoprotein A-I; Cav-1, caveolin-1; CE, cholesteryl ester; CEL, carboxyl ester lipase; CFP, cyan fluorescent protein; COE, cholesteryl oleoyl ether; RFP, red fluorescent protein; SR-BI, scavenger receptor class B type I; YFP, yellow fluorescent protein.

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with concurrent depletion of HDL lipids (8). However, other data supported the conclusion that efficient selective uptake of CE does not require endocytosis (9–11). To address these discrepancies, we examined the requirement for endocytosis for selective uptake of CE in both steroidogenic cells and hepatocytes. We confirm that in CHO cells, a block in endocytosis has no effect on the efficiency of CE selective uptake. Furthermore, we determine that in primary mouse hepatocytes, efficient CE selective uptake also occurs despite blocking HDL endocytosis and demonstrate that in hepatocytes, SR-BI mediates a very small fraction of total HDL endocytosis.

MATERIALS AND METHODS

Materials

Cycloheximide, 2-deoxyglucose, monensin, NaN_3, and HBSS were obtained from Sigma-Aldrich. BODIPY-CE (cholesteryl 4, 4-difluoror-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate) was obtained from Molecular Probes. Lipofectamine Plus and Lipofectamine reagents were obtained from Invitrogen. Cy3 and Cy5 protein labeling kits, $Na^{125}I$, [^{3}H]CE, and cholesteryl oleoyl ether (COE) were purchased from Amersham Biosciences. Anti-Fluorescent Protein (Aequorea victoria monoclonal antibody JL-8) was purchased from BD Biosciences Clontech, and anti-mSR-BI antibody (NB 400-104) was purchased from Novus Biologicals.

Cloning

Human SR-BI (CD-36 LIMPII Analogous-1)-cyan fluorescent protein (CFP) and -yellow fluorescent protein (YFP) were generated by PCR amplification of the cDNA using Pfu thermal polymerase and ligated into the pECFP-C3 and pEYFP-C3 vectors, respectively (Clontech). The sequences were consistent with published GenBank sequence gi:33620766 as determined by direct sequencing. Caveolin-1 (Cav-1)-YFP was kindly provided by R. Parton (University of Queensland, Brisbane, Australia). The Cav-1 red fluorescent protein (RFP) was generated from Cav-1 YFP by subcloning the coding sequence and has been characterized (12).

Cell lines and culture

CHO cells were grown in F-12 medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Transient transfections were performed using Lipofectamine and Lipofectamine Plus according to the manufacturer's protocol (Invitrogen, Burlington, Canada) using 0.4 μg of DNA, 2–3 μl of Lipofectamine Plus, and 0.7 μl of Lipofectamine per well of a 24-well plate. SR-BI-CFP stable cells were generated in ldlA7 cells (M. Krieger, Massachusetts Institute of Technology, Boston, MA) selected using 1.4 mM G418 and maintained with 0.5 mM G418. ldlA7[mSR-BI] cells were kindly provided by M. Krieger.

Primary mouse hepatocyte isolation

Retired breeders of the C57BL6 (wild-type) mouse strain were purchased from Charles River (Wilmington, MA). SR-BI$^{-/-}$ mice (strain B6; 129S2-*Scarb1*^{tm1Kri}/J) and Cav-1$^{-/-}$ mice (strain STOCK-*Cav1*^{tm1Mls}/J) were purchased from the Jackson Laboratory (Bar Harbor, ME). SR-BI$^{-/-}$ mice were generated from male homozygous knockout mice crossed with heterozygous females. The Cav-1$^{-/-}$ mice were crossed with C57BL6/J mice to obtain matching heterozygous and wild-type controls (Cav-1$^{+/-}$ and Cav-1$^{+/+}$, respectively). The mice were maintained on a 12 h light/12 h dark schedule on a normal chow diet. Primary hepatocytes were prepared from these mice according to established protocols (13, 14). Briefly, mice were sedated and their livers were perfused with collagenase solution. The cells were seeded on fibronectin-coated (4 μg/well) 24-well plates at an initial density of 1.9×10^5 cells per well in William's medium containing penicillin (100 U/ml), streptomycin sulfate (100 U/ml), Fungizone (250 ng/ml; Invitrogen), and 10% fetal bovine serum (Sigma). Six hours after the initial plating, the cells were washed in William's medium (without FBS) and fresh medium was added (as above). The cells were used 12–36 h after the initial wash.

HDL purification and protein labeling

All HDL was purified by density gradient ultracentrifugation using plasma from a healthy female normolipidemic donor (15). The HDL contained 0.98 mg of phospholipid, 0.087 mg of free cholesterol, 0.22 mg of CE, and 0.088 mg of triglycerides per milligram of HDL protein. Samples were dialyzed against 4 liters of nitrogen-sparged PBS, pH 7.4, with 2 g of Chelex for a minimum of 4 h. The purified HDL was labeled with [^{3}H]CE or COE (16). A total of 400 μg of human apolipoprotein A-I (apoA-I; kindly provided by D. L. Sparks, University of Ottawa Heart Institute, Ottawa, Canada) was iodinated in two batches in Iodotubes (Pierce) with 350 μCi of $Na^{125}I$, yielding an apoA-I specific activity of ~900,000 cpm/μg (16). The ^{125}I-apoA-I was exchanged onto HDL overnight, followed by a 16 h 1.21 g/ml density spin and isolation of the top fraction. The HDL was then dialyzed as described above. The specific activities of the 5 mg of ^{125}I-apoA-I-labeled HDL ranged between 20,000 and 50,000 cpm/μg. Transferrin was first purified by HPLC on a Sephacryl S-200 column (BD Biosciences), and 200 μg was labeled with 100 μCi of $Na^{125}I$, yielding a specific activity of ~400,000 cpm/μg, as described for apoA-I above. HDL was also labeled using a Cy5 protein labeling kit. One to 3.5 mg of protein was labeled using the manufacturer's protocol and separated from free label on a 30 cm P10 column. The cross-linking buffer 0.2 M $NaCO_3$ was used at pH 8.3 to prevent excessive labeling. The protein moiety of HDL was labeled with Cy5 at an average of two to five dye molecules per particle. When the cross-linking was performed at pH 8.3, the Cy5-HDL was completely competed off the cell surface with 50-fold unlabeled HDL. BODIPY-CE HDL was made as described previously (17). BODIPY-CE labeling of HDL was considered sufficient if >30% of the label was quenched in the core of the HDL particle (measured in a fluorimeter as an increase in BODIPY fluorescence upon addition of SDS).

Selective uptake assays

Confluent ldlA[mSR-BI] cells (see Figs. 1, 2A below), ldlA7 cells (see Fig. 2B–H below), or primary mouse hepatocytes (see Figs. 4–8 below) were plated on 24-well plates, washed twice at 37°C with 2 ml of ligand buffer (HBSS, 20 mM HEPES, and 5 mg/ml BSA, pH 7.4), and then pretreated in ligand buffer for 30 min alone (control), with monensin (20 μM), or with 50 mM 2-deoxyglucose and 5 mM NaN_3 (energy depletion). Cells were then incubated at 37°C with the specified ligand for the specified time. At the end of the incubation, the ligand buffer was removed and cells were washed six times on ice with 4°C HBSS. In time-based assays, the 0 min time point consisted of 2 h at 4°C incubation with the ligands. After the final washes, the remaining buffer was removed and the cells were solubilized with 500 μl of

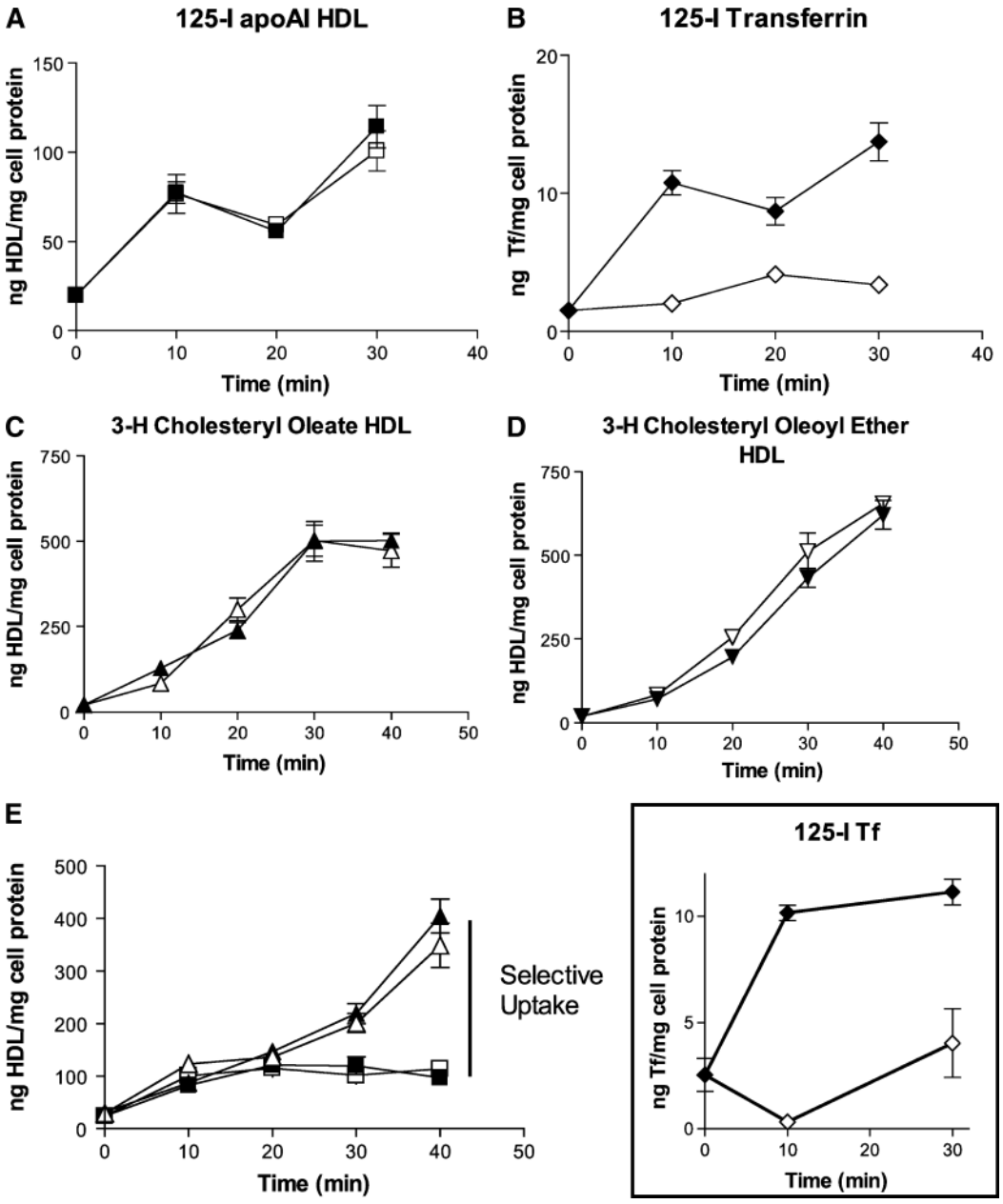

Fig. 1. Cellular energy is not required for the efficient selective uptake of cholesteryl ester (CE) in ldlA[mSR-BI] (for scavenger receptor class B type I) cells. Cells were incubated with HBSS/HEPES/BSA (closed symbols) or HBSS/HEPES/BSA/50 mM 2-deoxyglucose/5 mM NaN_3 (energy depletion; open symbols) for 30 min at 37°C before the addition of the ligands. A total of 300 μl of ^{125}I-apolipoprotein A-I (apoA-I)-HDL (30 μg/ml; squares) (A), ^{125}I-transferrin (Tf) (2 μg/ml; diamonds) (B), [^{3}H]CE-HDL (30 μg/ml; triangles) (C), or [^{3}H]cholesteryl oleoyl ether (COE) (30 μg/ml; inverted triangles) (D) was simultaneously added to the appropriate wells immediately after removing HBSS with a 24-well pump. Cells were washed, solubilized with NaOH, and processed. E: The same experiment was repeated with the addition of both ^{125}I-apoA-I-HDL (30 μg/ml; squares), [^{3}H]CE-HDL (30 μg/ml; triangles) and adjacent transferrin control. n ≥ 4; error bars indicate SEM.

0.2 N NaOH overnight at room temperature with gentle shaking. The protein content of 40 μl from each well was measured using a BSA standard and the bicinchoninic acid protein assay reagent according to the manufacturer's instructions (Pierce). The cell-associated ^{3}H or ^{125}I radioactivity in 400 μl of each cell lysate was measured by liquid scintillation counting using Ecolite (ICN, Costa Mesa, CA) or by γ counting, respectively. The cell association or degradation of radioactivity was measured in units of the amount of label in 1 ng (protein content) of HDL and corrected for levels of total cell protein.

Live cell fluorescence microscopy

For analysis of the subcellular distribution of SR-BI-CFP and labeled HDL, cells were seeded on glass coverslips (Fisher Scientific) and transiently transfected/cotransfected with fluorescent

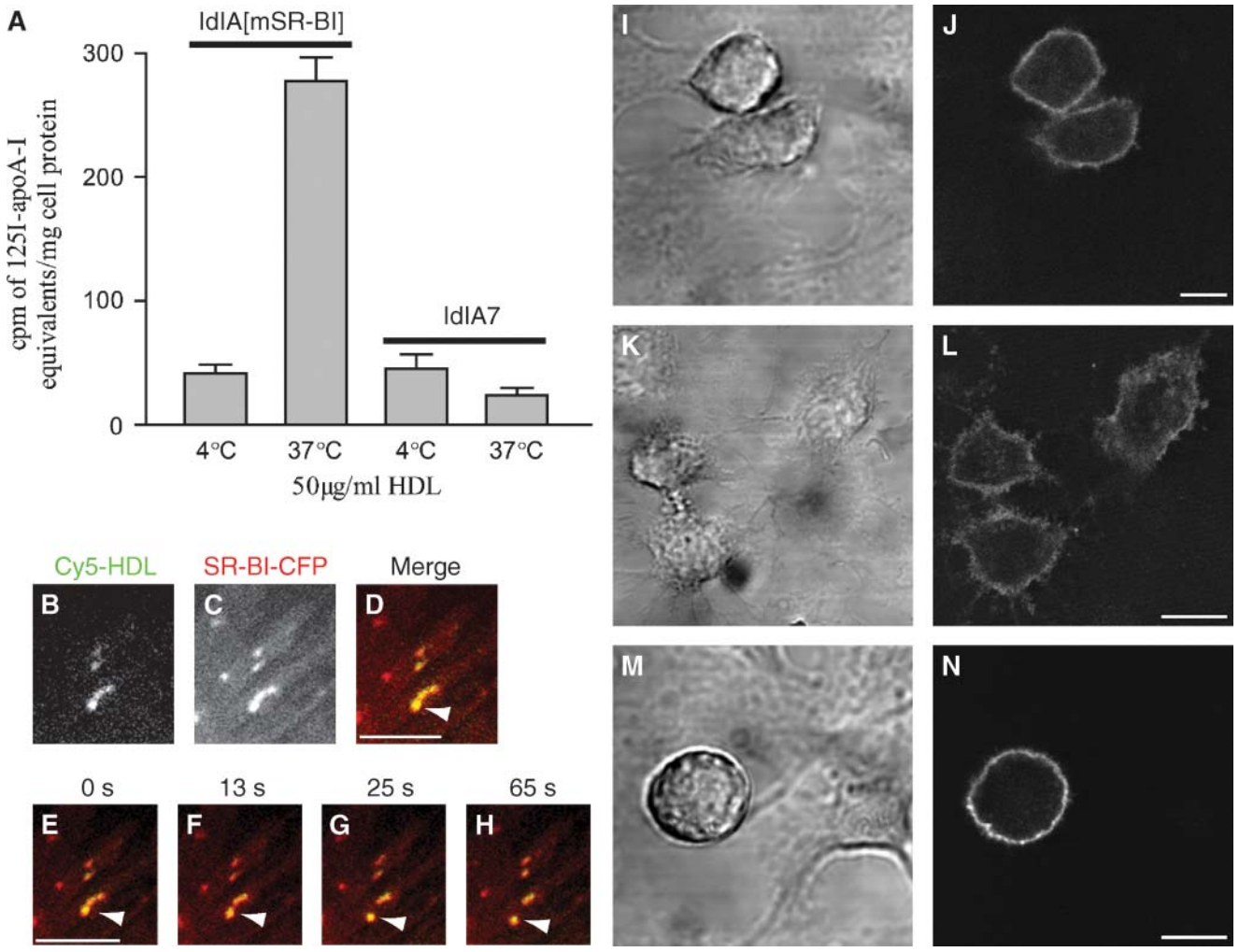

Fig. 2. SR-BI is required for and participates in HDL endocytosis in ldlA7 cells, but this does not occur at 4°C or with energy depletion. A: Cell surface trypsin cleavage assay described in Materials and Methods. Error bars represent SEM, n = 4. B–H: ldlA7 cells were transiently transfected with SR-BI-cyan fluorescent protein (CFP), incubated with Cy5-HDL for 10 min at 37°C, washed with PBS, and immediately mounted in a live cell chamber buffered with 20 mM HEPES. Subsequently, HDL and SR-BI endocytic events were captured at various time points (where 0 s represents the beginning of the observed event; a representative example is shown). I–N: ldlA7 cells transiently transfected with SR-BI-CFP were treated with energy depletion as described for Fig. 1A except with Cy5-HDL instead of ^{125}I-apoA-I-HDL. Cells were fixed with 3.3% Paraformaldehyde (PFA) and visualized using a laser scanning confocal microscope. Bars = 5 μm.

proteins. The coverslips were then mounted in live cell chambers in regular growth medium supplemented with 20 mM HEPES (pH 7.4) and visualized with an Olympus 100× oil-immersion objective, numerical aperture 1.35, in an enclosed climate-controlled (37°C) Olympus 1 × 70 inverted microscope operated by TILLvisION software version 4.0 (TILL Photonics, Heidelberg, Germany). The protocol for recording images documenting CFP localization with respect to Cy5-HDL consisted of exciting the fluorophores with a Polychrome IV monochronometer (CFP at 434 nm and Cy5 at 647 nm) with exposure times of 300–1,000 ms, and analysis through a CFP/YFP/Cy5 or FITC/Cy3/Cy5 triple-pass filter (Chroma). All images were merged as RGB images using the TILLvisION software. All images shown demonstrate cells that are representative of moderate transfection efficiencies and that have been verified by at least two independent experiments.

HDL endocytosis assays

ldlA7 and ldlA[mSR-BI] cells seeded on six-well plates were incubated with ligand buffer on ice for 20 min before labeling to ensure that the temperature of the cells was 4°C. Cells were then preincubated on ice with 50 μg/ml ^{125}I-apoA-I-HDL. After 2 h, the medium was removed, the cells were transferred to a 37°C incubator (where specified), and fresh (37°C) ^{125}I-apoA-I-HDL was added. After a 10 min incubation, the cells were washed on ice sequentially once with cold PBS, twice with cold trypsin [0.05% (w/v), 15 min], and again three times with PBS. Cells were solubilized, counted, and analyzed as described for the selective uptake assay above.

Microscopy with energy depletion

SR-BI-CFP cells were preincubated with energy depletion medium (50 mM 2-deoxyglucose and 5 mM NaN_3) at 37°C for 20 min to ensure complete inhibition of endocytosis. Cells were then incubated in energy depletion medium with 30 μg/ml Cy5-HDL for 30 min, washed with ligand buffer, mounted, and immediately examined for localization of Cy5-HDL. Images were taken with a laser scanning confocal microscope.

Laser scanning confocal microscopy

Fluorescent confocal images were obtained using a Bio-Rad MRC 1024 confocal microscope. The 522 nm line of an argon

ion laser and the 630 nm line (Cy5-HDL) or the 580 nm line (RFP) of a HeNe laser was used with a 60×, 1.4 numerical aperture objective.

RESULTS

To address the requirement of HDL endocytosis for selective uptake, we blocked all endocytic processes by depletion of cellular energy with 50 mM 2-deoxyglucose and 5 mM NaN_3 (18) in two different cell models: CHO cells lacking the LDL receptor (ldlA[mSR-BI]) (see Figs. 1, 3 below) and primary mouse hepatocytes (see Figs. 4–8 below). This technique permits direct comparison of the extent of HDL endocytosis, the cell association of HDL-CE, and ultimately, the levels of selective uptake in the absence of endocytosis.

We incubated both ldlA[mSR-BI] cells and primary mouse hepatocytes with ^{125}I-transferrin as a positive control to ensure that endocytosis was efficiently blocked with energy depletion (**Fig. 1B**). As anticipated from previous studies (11, 19, 20), ^{125}I-apoA-I-HDL binding to the cell surface was not significantly changed in energy-depleted conditions, indicating that HDL remains mostly bound to the cell surface in CHO cells (Figs. 1A; see Fig. 2I–N below). Also consistent with recent work (11), we demonstrate that blocking endocytosis does not change the cell association of CE (Fig. 1C). To verify that the rate of cell association of CE is not affected by the diffusion of free cholesterol resulting from CE hydrolysis, we also examined the uptake of the nonhydrolyzable CE analog, COE. We found that, like CE, energy depletion had no effect on COE cell association (Fig. 1D), confirming that CE hydrolysis cannot account for these results (18). Even at the short time points used in these experiments, uptake of CE occurs in a molar excess of HDL protein, indicating that this CE cell association is selective uptake (Fig. 1E).

Because energy depletion had an insignificant effect on the level of ^{125}I-apoA-I-HDL cell association in ldlA[mSR-BI] cells (Fig. 1A), we next determined whether HDL internalization occurs in these cells and whether this endocytosis is dependent on SR-BI. Using trypsin cleavage to remove cell surface HDL (shown to be ≥98% efficient; data not shown), we demonstrate that the presence of SR-BI at 37°C is required for internalization of HDL (**Fig. 2A**). Although the percentage of total surface-bound HDL that undergoes internalization may be relatively low (Fig. 1A), these data indicate that HDL is nevertheless a significant endocytic cargo (Fig. 2A).

To follow SR-BI-mediated HDL endocytosis, we tagged human SR-BI with YFP at the N terminus. Western blot analysis indicated that the tagged form is glycosylated normally (2), as indicated by a 109 kDa form of the protein in addition to the unglycosylated form at 82 kDa (data not shown). We also observed an ~218 kDa band, suggesting the presence of glycosylated/dimeric SR-BI-YFP (21). In addition, no degradation fragments of the YFP SR-BI were observed, confirming the stability of the receptor. Importantly, SR-BI-CFP facilitated HDL binding to the cell

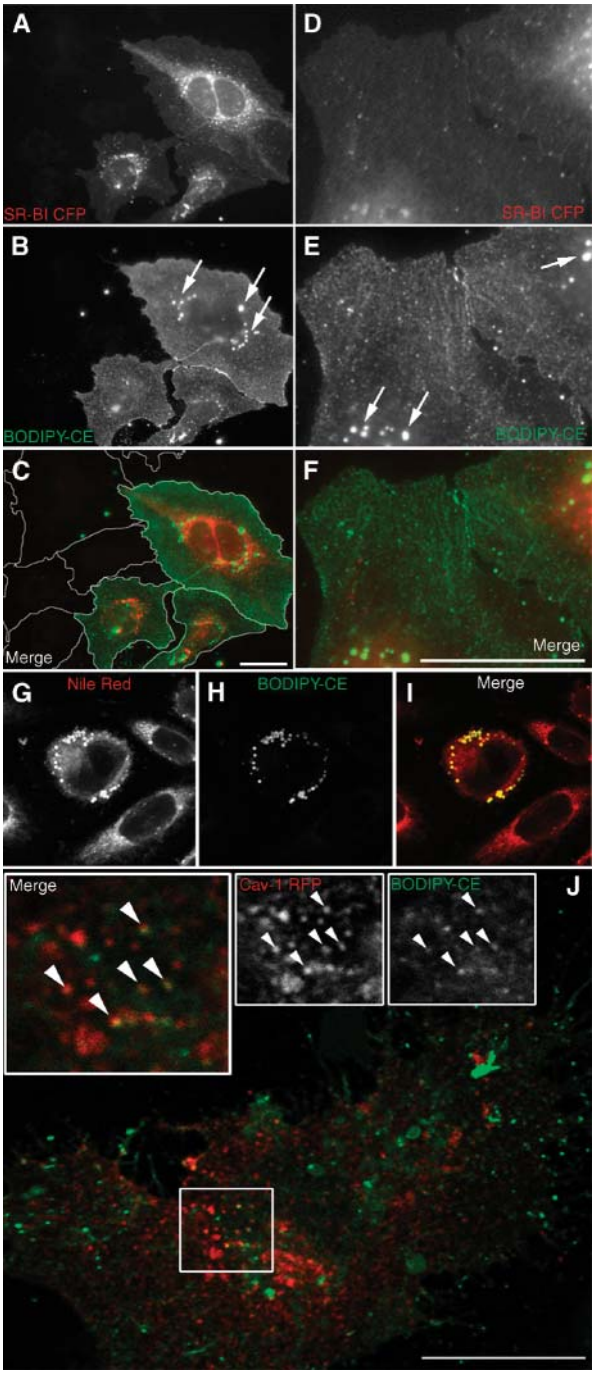

Fig. 3. BODIPY-CE derived from SR-BI-mediated HDL selective uptake in the absence of energy accumulates in cell surface caveolae and in intracellular lipid droplets. A–F: ldlA7 cells expressing SR-BI-CFP were treated with energy depletion as described for Fig. 1A except with 30 μg/ml BODIPY-CE-HDL instead of [^{3}H]CE-HDL. G–I: ldlA7 cells expressing SR-BI-CFP were incubated with 30 μg/ml BODIPY-CE-HDL for 30 min at 37°C, fixed with 3.3% Paraformaldehyde (PFA), and incubated with 0.01% Nile Red. J: ldlA7 cells expressing SR-BI-CFP and caveolin-1-red fluorescent protein (Cav-1-RFP) were incubated with 30 μg/ml BODIPY-CE-HDL for 30 min at 37°C, treated with energy depletion as described for Fig. 1A, fixed with 3.3% PFA, and imaged using laser scanning confocal microscopy. Arrowheads highlight areas of BODIPY-CE and Cav-1-RFP colocalization (inset). Bars = 5 μm.

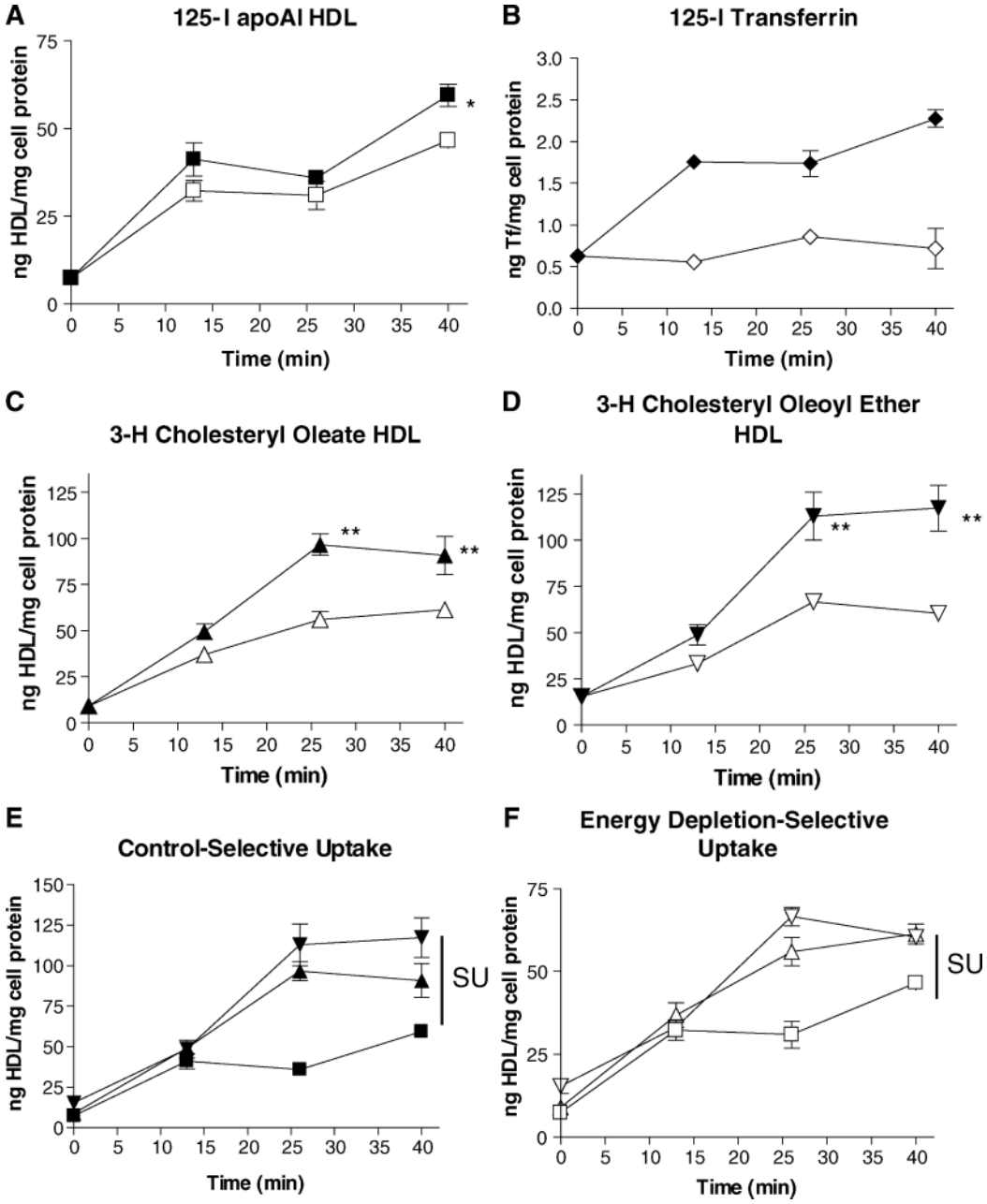

Fig. 4. Cellular energy is required for the efficient uptake of CE in primary mouse hepatocytes. Cells were incubated with HBSS/HEPES/BSA (closed symbols) or HBSS/HEPES/BSA/50 mM 2-deoxyglucose/5 mM NaN_3 (energy depletion; open symbols) for 30 min at 37°C before the addition of the ligands ^{125}I-apoA-I-HDL (30 μg/ml; squares) (A, E and F), ^{125}I-transferrin (Tf) (2 μg/ml; diamonds) (B), [^{3}H]CE-HDL (30 μg/ml; triangles) (C, E and F), and [^{3}H]COE (30 μg/ml; inverted triangles) (D, E and F). Cells were subsequently processed and analyzed as described in Materials and Methods. The selective uptake (SU) is displayed for both CE and COE in control and energy depletion conditions (E and F respectively). n ≥ 4; error bars indicate SEM.

surface in an SR-BI-dependent manner, and we observed subsequent streaming of HDL-positive vesicles into the cell (Fig. 2B–H), consistent with the previously reported endocytic uptake of HDL (7).

Having established that HDL endocytosis is not required for selective uptake in ldlA[mSR-BI] cells, we examined the distribution of CE in these cells using a nonhydrolyzable fluorescent CE analog (BODIPY-CE) (22). We found that despite the absence of cellular energy, notable levels of BODIPY-CE still accumulated in perinuclear puncta (**Fig. 3A–F**, arrows). Using laser scanning confocal microscopy and costaining with Nile Red, we confirmed that these perinuclear puncta are indeed lipid droplets (Fig. 3G–I). Interestingly, the BODIPY-CE also accumulated in cell surface puncta that aligned along cytoskeletal elements in a pattern reminiscent of cell surface caveolae (Fig. 3D–F). These puncta did not colocalize with SR-BI-CFP, indicating that the BODIPY-CE had been

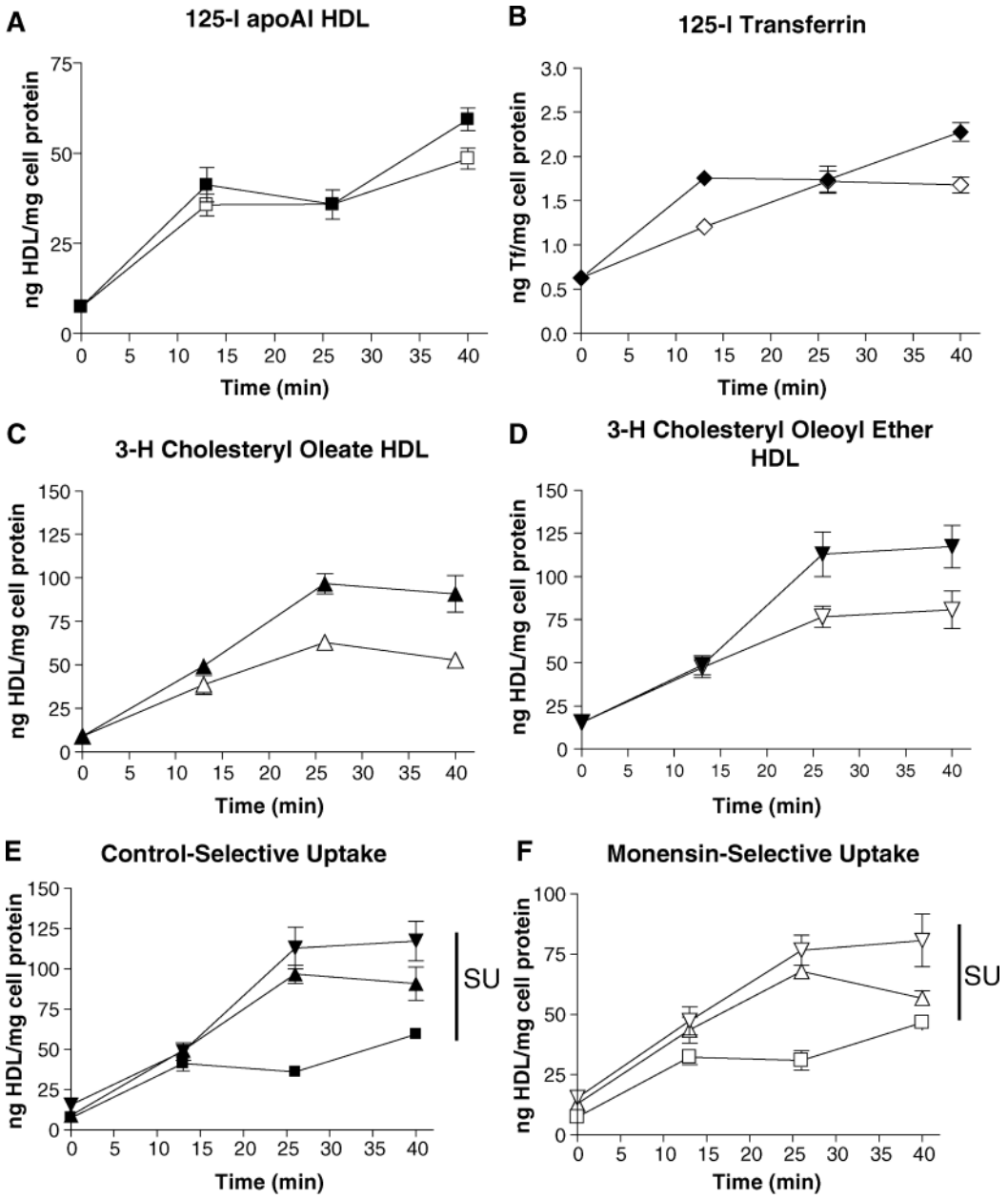

Fig. 5. Endocytosis is required for energy-dependent CE uptake in primary mouse hepatocytes. Cells were incubated with HBSS/HEPES/BSA (closed symbols) or HBSS/HEPES/BSA/20 μM monensin (open symbols) for 30 min at 37°C before the addition of the ligands ^{125}I-apoA-I-HDL (30 μg/ml; squares) (A, E and F), ^{125}I-transferrin (Tf) (2 μg/ml; diamonds) (B), [^{3}H]CE-HDL (30 μg/ml; triangles) (C, E and F), and [^{3}H]COE (30 μg/ml; inverted triangles) (D, E and F). Cells were subsequently processed and analyzed as described in Materials and Methods. The selective uptake (SU) is displayed for both CE and COE in control and energy depletion conditions (E and F respectively). n ≥ 4; error bars indicate SEM.

separated from HDL/SR-BI complexes by selective uptake. To test this caveolar localization, we transiently transfected low levels of Cav-1 in ldlA7 cells stably expressing SR-BI-CFP, depleted the cells of cellular energy, and loaded them with BODIPY-CE derived from HDL by selective uptake. Although we did not observe colocalization of SR-BI-CFP with Cav-1 RFP (data not shown), we did see extensive colocalization of BODIPY-CE and Cav-1 RFP (Fig. 3J).

In contrast to ldlA[mSR-BI] cells, cultured primary mouse hepatocytes exhibited much lower levels of HDL cell association and selective uptake (Fig. 1 vs. **Fig. 4:** two times less HDL cell association, five times less CE cell association, and eight times less selective uptake). In addition, energy depletion significantly decreased ($P = 0.02$) the levels of cell-associated ^{125}I-apoA-I-HDL at 40 min, indicating that much higher amounts of HDL holoparticle uptake occur in this cell model (Fig. 4A). Consistent with decreased HDL endocytosis, we also observed significant ($P \leq 0.03$) decreases in CE and COE cell association with energy depletion in these cells (Fig. 4C, D). Again, there were no significant dif-

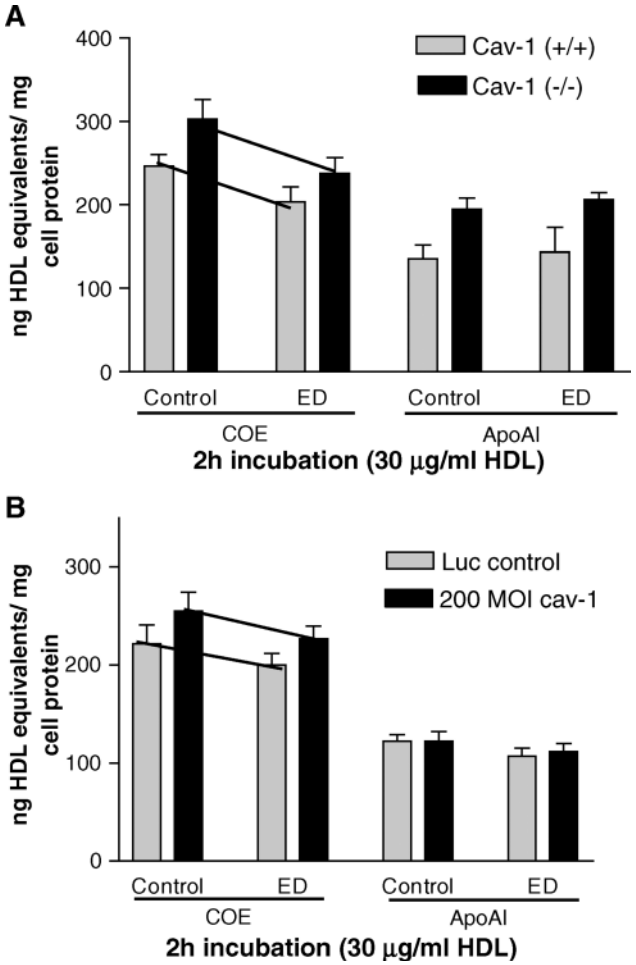

Fig. 6. Expression of Cav-1 affects the efficiency of COE selective uptake from HDL but does so in an energy-independent manner. A: Primary mouse hepatocytes from Cav-$1^{-/-}$ mice (black bars) and Cav-$1^{+/+}$ mice (gray bars) were incubated with HBSS/HEPES/BSA (control) or 50 mM 2-deoxyglucose/5 mM NaN_3 [energy depletion (ED)] for 30 min at 37°C before the addition of the ligands. B: Alternatively, primary mouse hepatocytes from C57BL/6J mice were isolated and infected with either multiplicity of infection (MOI) 200 of luciferase (Luc) adenovirus (gray bars) or 200 MOI of Cav-1 adenovirus (black bars) for 24 h. A total of 300 μl of ^{125}I-apoA-I-HDL (30 μg/ml) or [^{3}H]COE-HDL (30 μg/ml) was simultaneously added to the appropriate wells. Cells were then incubated for 2 h, washed, solubilized, and processed as described above. n = 6; error bars indicate SEM.

ferences between the levels of CE and COE cell association, indicating that hydrolysis of CE is not a factor in determining the levels of cell association during energy depletion. Given that the decreases in CE and COE cell association with energy depletion could be attributable to either a block in HDL endocytosis or to any number of cellular cofactors that require energy, we blocked endocytosis with monensin (**Fig. 5**). We found that 20 μM monensin and energy depletion had almost identical effects on CE and COE cell association and selective uptake, indicating that a block in endocytosis specifically decreases total cellular accumulation of CE and COE in hepatocytes but not in ldlA[mSR-BI] cells (Figs. 4, 5C–F).

The differences observed between cell models with energy depletion may result from differences in the tissue-specific regulation of cofactors that affect the efficiency of selective uptake. Cav-1 is one protein that has been reported to affect the efficiency of selective uptake (23). Because Cav-1 is able to transport CE in cytosol, its lower expression levels in hepatocytes could result in poor maintenance of CE concentration gradients from HDL to the plasma membrane (24, 25); consequently, this may account for the lower levels of CE uptake. For this reason, we isolated primary hepatocytes from Cav-$1^{+/+}$ and Cav-$1^{-/-}$ mice and examined the effect of Cav-1 on the selective uptake efficiency of COE (to avoid the complication of CE hydrolysis) at 2 h. Although the absence of Cav-1 had no effect, overexpression of Cav-1 slightly increased the efficiency of COE selective uptake (up 19.25% compared with control) (**Fig. 6A, B**). These experiments confirm that Cav-1 is not required for selective uptake, but they do suggest that overexpression of Cav-1 may have an effect on the efficiency of selective uptake. However, despite any changes in COE uptake in the presence or absence of Cav-1, the magnitude of the decreases with energy depletion always remained the same. Because Cav-1 expression had no effect on the energy-dependent cell association of COE, Cav-1 overexpression slightly affects the efficiency of selective uptake in an energy-independent manner not involving endocytosis.

Another cellular cofactor that has been shown to increase the efficiency of CE selective uptake is carboxyl ester lipase (CEL) (26). CEL or bile salt-dependent lipase is expressed mainly in pancreas but is also expressed in hepatocytes (27). It increases the efficiency of the SR-BI-mediated selective uptake of CE or COE by hydrolyzing

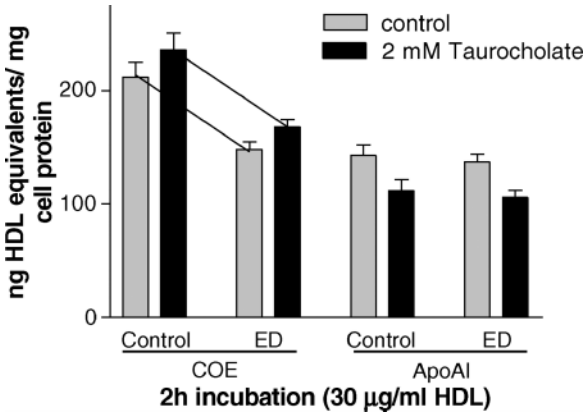

Fig. 7. Induction of carboxyl ester lipase (CEL) with taurocholate increases the efficiency of COE selective uptake in an energy-independent manner. Primary mouse hepatocytes from C57BL/6J mice were isolated and cultured for 48 h to increase the levels of secreted CEL. The medium from these cells was then collected and pooled. Cells were preincubated with HBSS/HEPES/BSA (control) or 50 mM 2-deoxyglucose/5 mM NaN_3 [energy depletion (ED)] for 30 min at 37°C before the addition of the ligands. A total of 300 μl of the conditioned medium was supplemented with either ^{125}I-apoA-I-HDL (30 μg/ml) or [^{3}H]COE-HDL (30 μg/ml). Cells were then incubated for 2 h, washed, solubilized, and processed as described above. n = 6; error bars indicate SEM.

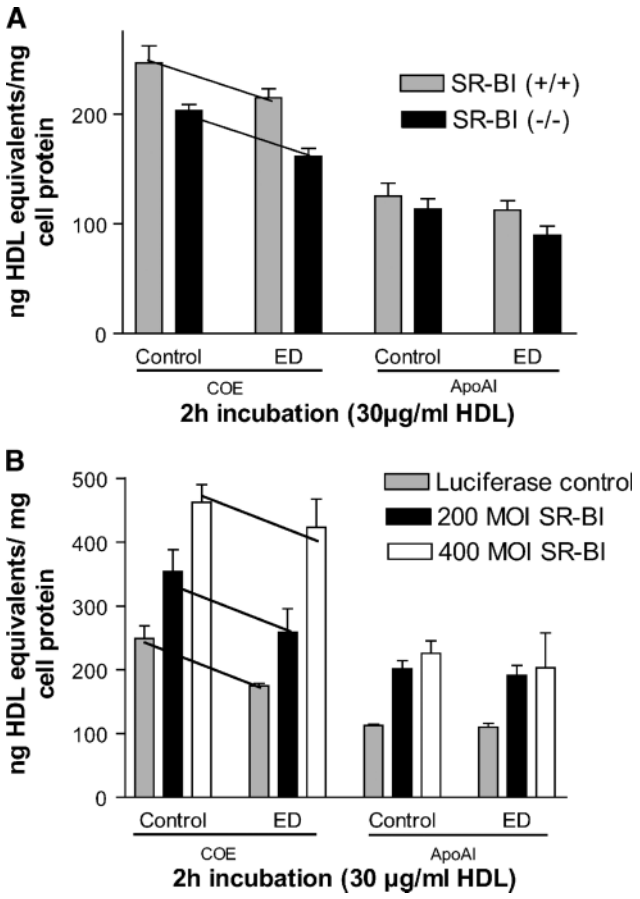

Fig. 8. Expression of SR-BI affects the efficiency of COE selective uptake from HDL but does so in an energy-independent manner. A: Primary mouse hepatocytes from SR-BI$^{-/-}$ mice (black bars) and SR-BI$^{+/+}$ mice (gray bars) were incubated with HBSS/HEPES/BSA (control) or 50 mM 2-deoxyglucose/5 mM NaN_3 [energy depletion (ED)] for 30 min at 37°C before the addition of the ligands. B: Alternatively, primary mouse hepatocytes from C57BL/6J mice were isolated and infected with either multiplicity of infection (MOI) 400 of luciferase adenovirus (light gray bars) or 200 MOI (black bars) or 400 MOI (white bars) of SR-BI adenovirus for 24 h. ^{125}I-apoA-I-HDL (30 µg/ml) or [^{3}H]COE-HDL (30 µg/ml) was added to the appropriate wells. Cells were then incubated for 2 h, washed, solubilized, and processed as described above. n = 6; error bars indicate SEM.

raft-associated CE and presumably maintaining a high CE concentration gradient (28). To examine the effect of CEL on HDL COE cell association, we plated hepatocytes and allowed the media to be enriched in CEL for 48 h. The media were then pooled, supplemented with radiolabeled ligands ([^{3}H]COE or ^{125}I-apoA-I-HDL), and added to the cells for 2 h with or without 2 mM taurocholate [a stimulator of CEL hydrolytic activity (26)] (**Fig. 7**). Stimulation of CEL with taurocholate increased the efficiency of COE selective uptake from HDL (82.3% compared with control), and interestingly, this increase was also independent of cellular energy.

Given that the energy-dependent decrease in COE cell association was not mediated by CEL and appeared to require endocytosis, we postulated that it was independent of SR-BI. To test this possibility, we isolated primary hepatocytes from SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice and examined the effect of SR-BI on COE cell association after 2 h (**Fig. 8A**). We found that knockdown of SR-BI significantly decreased the amount of cell-associated COE. Likewise, overexpression of SR-BI by adenovirus resulted in significant increases in COE cell association (Fig. 8B). We determined from these experiments that changing the expression levels of SR-BI affects the selective uptake of COE (decreased by 29.4% with SR-BI knockdown and increased by 8.88% for multiplicity of infection 200 and by 69.6% for multiplicity of infection 400 compared with controls) but has no effect on the energy-dependent endocytosis of HDL (Fig. 8B, black lines).

DISCUSSION

These studies clearly demonstrate that neither endocytosis nor recycling of HDL is required for efficient SR-BI-mediated CE selective uptake in hepatocytes. We determined that CE derived from HDL selective uptake localizes to caveolae in CHO cells and that Cav-1 increases the efficiency of CE selective uptake in hepatocytes. These findings highlight the importance of nonvesicular CE trafficking in SR-BI-mediated selective uptake and establish that the vast majority of HDL endocytosis in hepatocytes is SR-BI-independent holoparticle uptake. Given that previous experiments suggesting that HDL recycling played a functional role in CE selective uptake were conducted in hepatic cell models (6, 8), we originally hypothesized that this process might be cell type-specific. Accordingly, we conducted experiments in primary hepatocytes to determine whether blocking endocytosis affected the efficiency of selective uptake. We used COE to avoid the complication of free cholesterol efflux attributable to CE hydrolysis and used established "energy poisons" to inhibit all energy-dependent cellular processes, including endocytosis. However, the only significant difference in HDL metabolism was a decrease in both COE and apoA-I cell association in hepatocytes in response to energy depletion but not in ldlA[mSR-BI] CHO cells (compare Figs. 1C, D and 4C, D). Using monensin, we established that this energy-dependent decrease was attributable to a decrease in HDL endocytosis in hepatocytes (Fig. 5) and, importantly, that this endocytosis was unaffected by Cav-1 (Fig. 6), CEL (Fig. 7), or SR-BI (Fig. 8) expression. These results indicate that HDL endocytosis in primary mouse hepatocytes occurs independently of SR-BI and does not affect the efficiency of selective uptake.

These results do not exclude the possibility that CE selective uptake also occurs while small amounts of HDL are undergoing endocytosis for degradation, transcytosis, or recycling. However, they do argue that the majority of HDL endocytosis in hepatocytes is SR-BI-independent, making the contribution of SR-BI-mediated CE selective uptake during recycling insignificant. Importantly, these results do not exclude a role for SR-BII in HDL recycling, nor do they imply that the intracellular trafficking or the distribution of SR-BI lacks importance in hepatocytes;

rather, they suggest that under steady-state conditions, SR-BI trafficking does not contribute to the efficiency of SR-BI-mediated CE selective uptake. In vivo, factors that affect the cell surface distribution of SR-BI will certainly affect the efficiency of selective uptake. For example, the liver contains a PSD-95/Discs Large/ZO-1 (PDZ) domain binding protein (PDZK1) that interacts with the PDZ domain on the C-terminal tail of SR-BI and regulates its cell surface localization (reviewed in 29, 30). If PDZK1 binding to SR-BI stimulates rapid cell surface localization of intracellular SR-BI, then PDZK1 may represent one mechanism for the intracellular trafficking of SR-BI in hepatocytes. However, unless the regulation of SR-BI by PDZK1 is very rapid, its presence in hepatocytes is unlikely to have affected our results given the short time course of our experiments. It is also important to note that our results indicating the energy independence of selective uptake might alternatively be interpreted as indicating the recycling of HDL and transcytosis of cholesterol. However, transcytosis has only been reported for free cholesterol and not CE, and given that our experiments were performed with nonhydrolyzable COE, the subsequent transcytosis and efflux of free cholesterol can be excluded.

Hydrolysis of CE by CEL is one proposed method of maintaining the CE concentration gradient required for selective uptake in hepatocytes and is supported by cofractionation of SR-BI and CEL in hepatic cells (28). We found that, as expected, CEL stimulation with taurocholate increased the efficiency of COE selective uptake, but this increase was independent of cellular energy. Our data are consistent with an earlier report indicating that CEL hydrolyzes raft-associated CE, thereby increasing the CE concentration gradient and facilitating increased SR-BI-mediated COE transfer (28). Although CEL has been shown to colocalize with SR-BI intracellularly (28), our results suggest that this localization is not causative in CE selective uptake and that CEL acts efficiently in the absence of HDL or CEL internalization.

Additional insights can also be gained from these studies on the contribution of Cav-1 to selective uptake. SR-BI has been postulated to function as a hydrophobic channel permitting CE to be transferred down a concentration gradient into the plasma membrane (4). Therefore, the CE concentration in the membrane may affect the efficiency of CE transfer. The clearance of CE from the plasma membrane may involve a cytosolic carrier and/or endocytosis of membranes enriched in CE. Because in our experiments, energy depletion and monensin treatment precluded the endocytosis of membranes enriched in CE, it is possible that the cytosolic transfer of CE to internal membranes maintains the CE concentration gradient required for efficient selective uptake. Surprisingly, we found that despite the depletion of energy in ldlA[mSR-BI] cells, BODIPY-CE was able to traffic to perinuclear lipid droplets. Because of structural differences between BODIPY-CE and CE, it is possible that BODIPY-CE has increased solubility and thus artificially desorbs from the plasma membrane and adsorbs onto these lipid droplets. However, this is unlikely because if BODIPY-CE had enough solubility to diffuse unassisted between the plasma membrane and the lipid droplet, it would also be expected to diffuse between cells. Yet, only cells that expressed the SR-BI-CFP accumulated the BODIPY-CE (Fig. 5A–C), indicating that the BODIPY modification has not significantly altered the solubility of CE. The observation that BODIPY-CE is localized to lipid droplets clearly indicates that there is cytosolic transport of CE in these cells. Accordingly, complexes of Cav-1, annexin II, and CE have been shown to regulate the intracellular accumulation of CE in CHO cells (24). In fact, acylation of residue 133 of Cav-1 was shown to almost eliminate the cytosolic transport of CE to internal membranes (presumably lipid droplets) (24). We also observed a similar time line for the internalization of CE. At 14 min, both HDL protein and CE or COE were present in 1:1 ratios (Fig. 4E, F). It was only after 26 min that CE selective uptake was evident, indicating that it takes between 14 and 26 min for an HDL particle to be depleted of CE and dissociate from SR-BI. This nonvesicular cytosolic transfer of CE was first proposed in light of previous studies that localized both SR-BI and CE derived from HDL selective uptake to caveolae. Original experiments suggested that SR-BI and CE (derived from HDL selective uptake) were localized to caveolae (31, 32). However, SR-BI was subsequently shown to be in a detergent-resistant membrane that was independent of Cav-1 (8, 33) and localized to a microdomain that was independent of caveolae (19, 34). Because of the initial mislocalization of SR-BI to caveolae (reviewed in 1), few studies have explored the relationship between Cav-1 and CE internalization. Intriguingly, because we have shown that cell-associated BODIPY-CE appears in SR-BI-CFP-void/Cav-1-positive puncta on the cell surface, Cav-1 may play an important role in the subsequent internalization of CE in CHO cells.

Although the effects of modulating Cav-1 expression are complicated, our biochemical data overexpressing Cav-1 (Fig. 6) support a model in which Cav-1/annexin II complexes (24) facilitate the energy-independent internalization of CE. Importantly, we are not suggesting that caveolae or rafts are required for selective uptake (35), as Cav-1$^{-/-}$ hepatocytes did not have decreased selective uptake. We are, however, highlighting the fact that plasma membrane CE clearance may be important for efficient selective uptake and speculate that in hepatocytes, Cav-1/annexin II complexes facilitate this process. It must also be noted that selective uptake has been documented in FRT cells that lack Cav-1 (36). Interestingly, in that report, increased HDL degradation was observed only in cells that expressed SR-BI, and not in cells that coexpressed SR-BI and Cav-1. These results would be consistent with a requirement for CE to be depleted from the plasma membrane (to maintain the CE concentration gradient for selective uptake) by either cytosolic transport of CE or endocytosis of CE-rich membranes. It is possible that the SR-BI-dependent degradation of HDL may be coincident with the internalization of CE-enriched membranes. This observation may also explain why some reports document a chloroquine-sensitive component to HDL-CE selective uptake (37, 38).

Our data also provide support for earlier work demonstrating that CE derived from HDL selective uptake localizes to caveolae (24, 32). Although we did not specifically distinguish between BODIPY-CE in HDL and BODIPY-CE that was separated from HDL by selective uptake, we conclude that the puncta observed are not BODIPY-CE in HDL because they do not colocalize with SR-BI. Because we have demonstrated in the ldlA7 cell model that SR-BI is required for HDL binding, we believe that localization of BODIPY-CE without SR-BI-CFP is indicative of BODIPY-CE separation from HDL. This colocalization may represent one site of CE exit from the plasma membrane for Cav-1/annexin II-dependent trafficking.

In conclusion, we demonstrate that in primary mouse hepatocytes, efficient SR-BI-dependent CE selective uptake does not require endocytosis or recycling of HDL and that the detectable HDL endocytosis is SR-BI-independent holoparticle uptake mediated by one of several possible HDL receptors (16, 39). Although Cav-1, CEL, and SR-BI affect the efficiency of COE selective uptake, they accomplish this in an energy- and endocytosis-independent manner.

This work is dedicated to the memory of our friend, Dr. Gerard Vassiliou, whose passion, encouragement, inspiration, and friendship were invaluable. The authors thank Andrew Meng for adenoviral development, Vivian Franklin for help with hepatocyte isolation, and Zdena Harder, Ross Milne, and Yves Marcel for critical review of the manuscript. This work was supported by grants from the Canadian Institutes of Health Research (Grant 44360 to R.M. and Grant 43935 to H.M.M.) and by an Ontario Graduate Scholarship in Science and Technology (to C.J.H.).

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