# Quantitative analysis of SR-BI-dependent HDL retroendocytosis in hepatocytes and fibroblasts

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**Abstract Previous studies have suggested that HDL retroendocytosis may play a role in scavenger receptor class B type I (SR-BI)-dependent selective lipid uptake in a cell-specific manner. To investigate this possibility, we developed methods to quantitatively measure HDL uptake and resecretion in fibroblast (COS-7) and hepatocyte (HepG2) cells expressing exogenous SR-BI. Approximately 17% and 24% of HDL associated in an SR-BI-dependent manner with COS-7 and HepG2 cells, respectively, accumulates intracellularly after a 10 min incubation. To determine whether this intracellular HDL undergoes retroendocytosis, we developed a pulse-chase assay whereby internalized biotinylated $^{125}$I-HDL$_3$ secreted from cells is quantitatively precipitated from cell supernatants using immobilized streptavidin. Our results show a rapid secretion of a portion of intracellular HDL from both cell types (representing 4–7% of the total cell-associated HDL) that is almost complete within 30 min (half-life ~ 10 min). In COS-7 cells, the calculated rate of HDL secretion (~0.5 ng HDL/mg/min) was >30-fold slower than the rate of SR-BI-dependent selective cholesteryl ester (CE) uptake (~17 ng HDL/mg/min), whereas the rate of release of HDL from the cell surface (~19 ng HDL/mg/min) was similar to the rate of selective CE uptake. Notably, the rate of SR-BI-dependent HDL resecretion in COS-7 and HepG2 cells was similar. BLT1, a compound that inhibits selective CE uptake, does not alter the amount of SR-BI-mediated HDL retroendocytosis in COS-7 cells. From these data, we conclude that HDL retroendocytosis in COS-7 and HepG2 cells is similar and that the vast majority of SR-BI-dependent selective uptake occurs at the cell surface in both cell types.**—Sun, B., E. R. M. Eckhardt, S. Shetty, D. R. van der Westhuyzen, and N. R. Webb. **Quantitative analysis of SR-BI-dependent HDL retroendocytosis in hepatocytes and fibroblasts.** ***J. Lipid Res.*** **2006.** 47: **1700–1713.**

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Abundant evidence has established that scavenger receptor class B type I (SR-BI) plays a primary role in HDL metabolism by mediating selective cholesteryl ester (CE) uptake, a process in which HDL CE is taken up without intracellular apolipoprotein degradation (1). In addition to mediating the selective uptake of HDL CE, SR-BI also plays a role in facilitating the bidirectional flux of unesterified cholesterol between cells and extracellular acceptors (2–4). SR-BI has a large extracellular loop (~400 residues), two transmembrane domains, and two short cytoplasmic tails at its N and C termini (5). SR-BI is highly expressed in liver and steroidogenic tissues, which are known to be major sites for selective lipid uptake. SR-BI is also expressed in other cells such as macrophages (6, 7), intestinal cells (8–10), and endothelial cells (11–13). Although SR-BI is best known as a physiological HDL receptor, it also binds other lipoprotein ligands, such as LDL, oxidized LDL, and acetylated LDL (1, 14), as well as several other nonlipoprotein ligands (15, 16). Studies have shown that SR-BI mediates selective CE uptake from LDL (2, 17, 18). In mouse models of atherosclerosis, hepatic overexpression of SR-BI has been shown to be protective against lesion formation (19–23), whereas SR-BI deficiency in hyperlipidemic strains of mouse accelerates atherosclerosis (24–26). The protective effect of hepatic SR-BI is most likely attributable to its contribution to reverse cholesterol transport, whereby excess cholesterol is transported from peripheral tissues to the liver for storage, catabolism, or biliary excretion.

Despite the importance of selective CE uptake in the liver, the precise mechanism for this process is unknown. One model suggests that selective lipid uptake occurs at the plasma membrane, which is supported by the finding that reconstituted liposomes containing only SR-BI are able to selectively absorb lipid from HDL (27, 28). Treatments that block HDL internalization, such as hyperosmotic sucrose or depletion of $K^+$ or ATP, have no effect on selective lipid uptake in SR-BI-expressing cells (28, 29). Electron microscopic analysis of cultured steroidogenic tis-

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Abbreviations: CE, cholesteryl ester; MFI, mean fluorescence intensity; MOI, multiplicity of infection; SR-BI, scavenger receptor class B type I.

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sues and murine adrenal cells showed little or no intracellular HDL, consistent with CE uptake taking place at the surface of these cells (30). Based on estimates of the activation energy for selective CE uptake, Rodrigueza et al. (31) proposed that SR-BI creates a hydrophobic channel at the cell surface through which CE from the core of the HDL particle is transported down a concentration gradient to the plasma membrane.

Several studies have shown that HDL is internalized by a number of different cell types in an SR-BI-dependent manner (29, 32–36). This has led to an alternative model for selective CE uptake, in which HDL particles undergo lipid transfer during SR-BI-dependent internalization, sorting, and resecretion (32, 33). However, evidence suggests that SR-BI internalization of HDL is not an absolute requirement for selective CE uptake, as shown by the fact that inhibiting endocytosis in SR-BI-expressing cells does not block selective CE uptake (28, 29). Nevertheless, it is possible that HDL internalization and selective lipid uptake are not mutually exclusive processes and that lipid transfer can occur during HDL recycling. Based on available data, it is also possible that the contribution of SR-BI-mediated HDL recycling to selective lipid uptake may be different in different cell types.

By quantifying the internalization and resecretion of biotinylated $^{125}$I-HDL in SR-BI-expressing COS-7 and HepG2 cells, we have determined for the first time the rate of SR-BI-mediated HDL uptake and resecretion. Our results indicate that 4–7% of HDL associated with COS-7 and HepG2 cells in an SR-BI-dependent manner is in a retroendocytic pool and that the rate of SR-BI-dependent HDL resecretion in the two cell types is similar. Based on a comparison of the rates of SR-BI-dependent HDL retroendocytosis and selective lipid uptake, we conclude that the bulk of lipid transfer mediated by SR-BI occurs at the plasma membrane in both cell types.

## EXPERIMENTAL PROCEDURES

### Cells

COS-7 and HepG2 cells were originally obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM (Gibco) or MEM (Gibco), respectively, supplemented with 2 mM L-glutamine, 50 U/l penicillin G, 50 μg/l streptomycin (all from Invitrogen), and 10% heat-inactivated fetal calf serum (Gibco). SR-BI was expressed in COS-7 and HepG2 cells using an adenoviral vector, AdSR-BI, that encodes mouse SR-BI (18, 37). For biochemical assays, cells were seeded in 10 cm dishes and, when ~80% confluent, inoculated with AdSR-BI at a multiplicity of infection (MOI) of 5,000 particles/cell, unless indicated otherwise. Twenty-four hours after inoculation, cells were reseeded in 12-well clusters and incubated at 37°C for an additional 24 h before the uptake assays. To confirm SR-BI expression, Western blot analysis of representative cell lysates was performed as described previously (38).

### Isolation and labeling of lipoproteins

Human $HDL_3$ (d = 1.13–1.18 g/ml) was isolated from fresh human plasma by density gradient ultracentrifugation as described previously (39). The $HDL_3$ fraction was dialyzed against 150 mM NaCl and 2.5 mM EDTA, sterile-filtered, and stored under $N_2$ gas at 4°C. $HDL_3$ apolipoproteins were iodinated in the presence of $^{125}$I (Amersham Biosciences) by the iodine monochloride method (40). Specific activity of $^{125}$I-HDL was 100–300 cpm/ng protein. HDL-associated CE was traced with nonhydrolyzable [1,2(*n*)-$^3$H]cholesteryl oleoyl ether (Amersham Biosciences) according to the method of Gwynne and Mahaffee (41), with the following modifications: [1,2(*n*)-$^3$H]cholesteryl oleoyl ether was dried in a 12 × 75 mm borosilicate glass tube (20 μCi/mg HDL protein), after which $HDL_3$ and partially purified CE transfer protein were added. After 16 h of incubation at 37°C, HDL was reisolated by ultracentrifugation at a density of 1.21 g/ml. The specific activity of the [$^3$H]HDL ranged from 10 to 30 dpm/ng protein. For preparation of fluorescent HDL, $HDL_3$ protein was covalently modified with Alexa 488 (Molecular Probes) according to the manufacturer's directions and stored under $N_2$ gas at 4°C.

### Association and selective lipid uptake assays

Cell association and selective lipid uptake assays were performed as described previously (38), except that media containing radiolabeled lipoproteins were supplemented with 0.5% BSA instead of serum. Approximately 90% confluent cells expressing SR-BI by adenoviral vector or control cells were incubated with 10 μg/ml $^3$H,$^{125}$I-HDL or biotinylated $^3$H,$^{125}$I-HDL in complete medium supplemented with 0.5% BSA. For some experiments, COS-7 cells were preincubated with 10 μM BLT1 (an inhibitor of selective lipid uptake; kindly provided by Dr. G. H. Rothblat, University of Pennsylvania) at 37°C for 1 h before the addition of HDL ligands. After incubation at 37°C, the medium was removed and the cells were washed three times with ice-cold washing buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4, and 2 mg/ml fatty acid-free BSA) followed by three washes in the same buffer without BSA. The cells were then solubilized in 0.1 N NaOH for 60 min at room temperature, and cellular protein and radioactivity content were measured in the lysate. $^{125}$I present in the lysate corresponded to cell-associated HDL protein, and cell-associated $^3$H corresponded to the uptake of cholesteryl ether tracer. To allow for a direct comparison of different radiolabeled tracers, $^3$H uptake was expressed as apparent uptake of HDL protein, assuming whole particle uptake. Selective uptake was defined as the difference between cell-associated $^3$H-derived values and $^{125}$I-derived values and represents the uptake of CE that cannot be accounted for by the uptake of intact particles. The trichloroacetic acid-soluble degraded material in the cell medium was assayed as described (42). In all experiments, SR-BI-specific values were calculated as the difference between values from AdSR-BI-treated COS-7 or HepG2 cells and control cells.

### Preparation of biotinylated $HDL_3$

Biotinylation of $^{125}$I-$HDL_3$ or $^3$H,$^{125}$I-$HDL_3$ was carried out using EZ-link™ Sulfo-NHS-LC-Biotin according to the manufacturer's recommendations (Pierce). Briefly, radiolabeled $HDL_3$ (1.5 mg/ml) was mixed with EZ-link™ reagent at selected molar ratios in a total volume of 300 μl and then incubated at room temperature for 1 h. The mixture was subsequently dialyzed overnight against 154 mM NaCl and 1 mM EDTA (three changes of 4 liters of dialysis buffer) to remove unincorporated biotin. To assess the efficacy of biotinylation, 25 ng of biotinylated $^{125}$I-HDL was mixed with 50 μl of MagPrep® streptavidin beads (Novagen) in a total volume of 300 μl of PBS and incubated at room temperature while rocking for 30 min. The streptavidin beads were collected with a Magnetic Particle Concentrator™ (Dynal AS, Oslo, Norway), and the amount of $^{125}$I-HDL recovered in the

supernatant and pellet was quantified. Biotinylated $^{125}$I-HDL preparations were not used in biotin assays unless >90% of $^{125}$I-HDL could be recovered by streptavidin precipitation.

### Biotin assay for the measurement of intracellular and secreted HDL

Control cells or cells overexpressing SR-BI were incubated with 10 μg/ml biotinylated $^{125}$I-HDL at 4°C for 60 min or at 37°C for the indicated times, then washed three times with cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-CM) to remove unbound ligand. For some experiments, cells were preincubated with 10 μM BLT1 at 37°C for 1 h before the addition of HDL ligands and was present at the same concentration during the pulse-chase protocol. To "mask" cell surface biotinylated $^{125}$I-HDL, cells were incubated at 4°C for 15 min with 0.15 mg/ml NeutrAvidin (Pierce). Cells were washed three times with cold PBS-CM to remove unbound NeutrAvidin and then lysed in 300 μl of 25 mM MES, 150 mM NaCl, 1% Triton X-100, and 60 mM octyglucopyranoside, pH 6.7. Clarified cell lysates were incubated with 20 μg of MagPrep® streptavidin beads at room temperature for 30 min. The streptavidin beads were collected with a Magnetic Particle Concentrator™ (Dynal AS), and the amount of $^{125}$I-HDL in the supernatant (representing cell surface, masked HDL) and pellet (representing intracellular HDL) was quantified with a γ-counter. To quantify the secretion of intracellular HDL, cells were incubated with biotinylated $^{125}$I-HDL at 37°C for the indicated times, then surface-bound HDL was masked with NeutrAvidin as described above. The NeutrAvidin-treated cells were incubated in prewarmed complete medium at 37°C for 5 min to 8 h as indicated, and the amount of $^{125}$I-HDL precipitated from the medium by streptavidin beads was determined. This represents the secreted HDL fraction. The addition of a 20-fold molar excess of unlabeled HDL during the chase period had no effect on the amount of biotinylated $^{125}$I-HDL recovered from the medium by streptavidin precipitation, demonstrating that the rate of secretion of intracellular HDL is independent of extracellular HDL.

### Measurement of HDL released from the cell surface

Cells were incubated with prewarmed complete medium supplemented with 0.5% fatty acid-free BSA at 37°C with or without 10 μM BLT1 for 1 h and cooled to 4°C by washing two times with ice-cold washing buffer, with the last wash on ice for 5 min. Thereafter, cells were incubated in the presence or absence of BLT1 in ice-cold medium containing 10 μg/ml $^{125}$I-HDL. After 1 h of incubation at 4°C, the medium was removed and cells were washed three times with ice-cold washing buffer followed by three washes with washing buffer without BSA to remove unbound ligand. All washes were performed at 4°C with prechilled solutions. Cells were either lysed to determine the total cell-associated $^{125}$I-HDL at 4°C or incubated at 37°C for selected intervals with prewarmed complete medium with or without 10 μM BLT1. The medium was collected after the "chase" period to determine the amount of $^{125}$I-HDL dissociated from the cell surface. HDL dissociation is expressed as the percentage of surface-bound $^{125}$I-HDL at the end of the 1 h, 4°C pulse. In separate experiments, cells were processed similarly, except that the 37°C chase period followed a 10 min incubation with 10 μg/ml $^{125}$I-HDL at 37°C. In all experiments, SR-BI-specific values were calculated as the difference between values from SR-BI-expressing COS-7 cells and control COS-7 cells.

### Fluorescence energy transfer assay for the measurement of surface HDL

COS-7 cells expressing SR-BI by adenoviral vector or control cells were incubated with 10 μg/ml Alexa 488-HDL. After incubation at 4°C or 37°C as indicated, cells were collected from the dish by gentle rinsing and incubated with or without Trypan Blue (0.4% solution in PBS; Cellgro, Richmond, VA) at 4°C for 15 min. Cells were then washed once in PBS before analysis by flow cytometry. Trypan Blue is a non-cell-permeant molecule whose absorption spectra overlaps the emission spectra of Alexa 488 and quenches fluorescence by Forster resonance energy transfer with a calculated $R_o$ value (the distance at which energy transfer efficiency is 50%) of 3.8–4.2 nm (43). This approach has been used previously to investigate phagocytosis and endocytosis of fluorescent molecules (44, 45). For time course studies, fluorescent ligand was added at staggered intervals so that cells were harvested at the same time. Visual inspection of cells under light microscopy indicated that the vast majority (>97%) of cells excluded Trypan Blue. Cells were analyzed by flow cytometry, and cell-surface HDL was estimated by calculating the difference in mean fluorescent intensity (MFI) in cells with or without Trypan Blue. The percentage of total cell-associated HDL that was intracellular was calculated by dividing the MFI of cells in the presence of Trypan Blue by the MFI of cells in the absence of Trypan Blue. In all experiments, SR-BI-specific MFIs were calculated as the difference between values from SR-BI-expressing COS-7 cells and control COS-7 cells.

### Confocal microscopy

COS-7 cells were seeded in 10 cm dishes, and when ~80% confluent, they were inoculated with AdSR-BI (MOI = 5,000 particles/cell). Twenty-four hours after inoculation, cells were reseeded on glass coverslips in 12 well cluster dishes and incubated at 37°C for an additional 24 h. SR-BI-expressing COS-7 cells and control COS-7 cells were incubated with 10 μg/ml Alexa 488-HDL at 37°C for 1 h and washed three times with PBS-CM to remove unbound ligands. Cells were fixed with 4% paraformaldehyde (pH 7.2) at room temperature for 15 min and washed two times with PBS. Cells were mounted on slides using fluorescence-protecting medium (Vectashield; Vector Laboratories, Burlingame, CA). Confocal microscopy was performed at the University of Kentucky Imaging Facility using a Leica laser scanning confocal microscope with argon (488 nm) lasers.

### Data and statistical analysis

Calculations of kinetic parameters were performed using Prism® software (GraphPad Software, San Diego, CA). Data are expressed as means ± SEM. Results were analyzed by Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

## RESULTS

### HDL internalization by COS-7 cells expressing SR-BI

In this study, we set out to measure SR-BI-mediated HDL internalization and resecretion in COS-7 cells, a cell line commonly used to investigate selective lipid uptake by exogenously expressed SR-BI (46–48). Because COS-7 cells do not express detectable endogenous SR-BI, SR-BI-dependent activity can be assessed in these cells with negligible background receptor activity. We manipulated SR-BI expression in COS-7 cells by varying the MOI of an adenoviral vector expressing mouse SR-BI (37). For the range of MOIs used in this experiment, selective lipid uptake increased linearly with increasing MOI and corresponded to receptor expression (**Fig. 1A**).

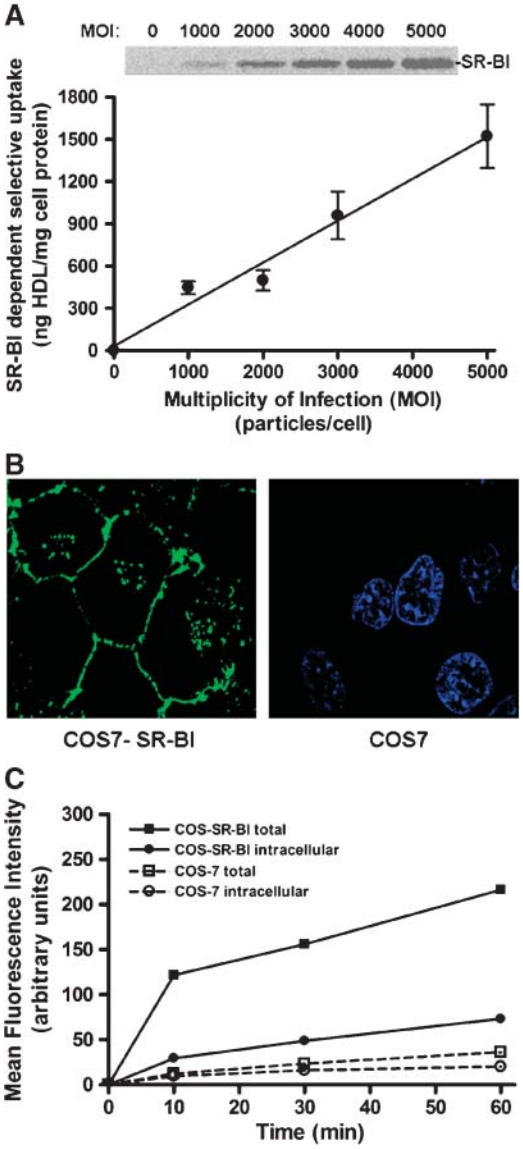


**Fig. 1.** COS-7 cells as a model system for investigating scavenger receptor class B type I (SR-BI)-dependent selective lipid uptake. A: Relationship between exogenous SR-BI expression and selective lipid uptake in COS-7 cells. SR-BI was expressed in COS-7 cells using adenovirus vector at selected multiplicities of infection (MOI) as indicated. After a 2 h incubation with 10 μg/ml $^{3}$H,$^{125}$I-HDL at 37°C, selective lipid uptake was quantified as described in Experimental Procedures. SR-BI-specific values are shown, which are defined as the difference between SR-BI-expressing and nonexpressing COS-7 cells. Values represent means ± SEM of results from triplicate determinations. The inset shows immunoblot analysis of representative total cell lysates (5 μg) using anti-SR-BI antibody. B: COS-7 cells expressing SR-BI by adenovirus vector (COS-SR-BI) and control COS-7 cells were incubated for 1 h at 37°C with 10 μg/ml HDL labeled on the apolipoprotein moiety with Alexa 488. To aid in the visualization of control COS-7 cells, the cells were incubated with 4′,6-diamino-phenylindole for 5 min at room temperature before confocal microscopy. Representative confocal cross-sections from the central region of cells are shown. C: Cells were incubated with 10 μg/ml Alexa 488-HDL at 37°C for the indicated times. Intracellular HDL was quantified by fluorescence energy transfer assay, as described in Experimental Procedures. The results shown represent the mean fluorescence intensity of control COS-7 cells (open symbols) and COS-SR-BI cells (closed symbols) in the absence (squares) or presence (circles) of Trypan Blue. Values are representative of three independent experiments.

Because HDL internalization by SR-BI in COS-7 cells has not been specifically addressed, we first qualitatively assessed SR-BI-mediated HDL internalization in these cells by confocal microscopy. COS-7 cells expressing exogenous SR-BI were incubated for 1 h at 37°C with HDL labeled on the apolipoprotein moiety with Alexa 488. Confocal microscopy revealed that a portion of HDL was intracellular and distributed in a punctate pattern throughout the cytoplasm. However, the majority of cell-associated HDL appeared to be on or close to the plasma membrane. Control COS-7 cells had no detectable cell-associated Alexa 488-HDL (Fig. 1B).

To quantify HDL internalization by SR-BI-expressing COS-7 cells, we developed a fluorescence-based assay that takes advantage of the ability of Trypan Blue to quench fluorescence at certain wavelengths (43). Because Trypan Blue does not enter living cells, this reagent may be used to distinguish surface-bound Alexa 488-HDL (i.e., quenchable by Trypan Blue) and HDL that has accumulated intracellularly. To quantify SR-BI-mediated HDL internalization by this assay, SR-BI-expressing COS-7 (COS-SR-BI) cells and control COS-7 cells were incubated at 37°C with Alexa 488-HDL for selected intervals, followed by incubation with or without 0.4% Trypan Blue at 4°C for 15 min. For each time point, the MFI of cells in the absence and presence of Trypan Blue was quantified by flow cytometry. This analysis showed a time-dependent increase in the fluorescence of COS-SR-BI cells that was not quenchable by Trypan Blue (Fig. 1C). After 1 h of incubation at 37°C, 27.0 ± 3.3% of total cell-associated Alexa 488-HDL accumulated in a nonquenchable, presumably intracellular pool (Fig. 1C). This contrasts with COS-SR-BI cells incubated with Alexa 488-HDL for 1 h at 4°C, in which <8% of total fluorescence was not eliminated in the presence of Trypan Blue (data not shown). After 60 min of incubation with Alexa 488-HDL, the MFI of COS-SR-BI cells in the absence and presence of Trypan Blue was ~6- and 3.6-fold higher than that of control COS-7 cells, respectively (Fig. 1C). Although incomplete quenching likely results in a slight overestimation of intracellular HDL, our analysis nevertheless indicates SR-BI-dependent HDL endocytosis in COS-7 cells.

### Biotin assay for quantifying intracellular and surface-bound HDL

We developed another approach to quantify the SR-BI-mediated internalization of HDL using biotinylated $^{125}$I-HDL. In this assay, internalized biotinylated HDL can be quantitatively recovered from cell lysates through its high-affinity interaction with streptavidin beads, whereas surface-bound biotinylated HDL can be prevented from binding to streptavidin beads by incubating cells before lysis with soluble NeutrAvidin. To investigate the feasibility of this approach, we performed a series of experiments to verify that HDL particles can undergo covalent modification with biotin without altering their interaction with SR-BI.

First, the optimal conditions for HDL biotinylation were determined. To this end, $^{125}$I-HDL was incubated with EZ-

Link™ Sulfo-NHS-LC-Biotin (Pierce) at different molar ratios. Aliquots of the biotinylated $^{125}$I-HDL were then tested for their ability to be precipitated by streptavidin beads. As shown in **Fig. 2A**, virtually none of the non-biotinylated $^{125}$I-HDL was precipitable with streptavidin beads. As expected, biotinylated $^{125}$I-HDL was precipitated by streptavidin, with maximal recovery (92 ± 1.7%) of HDL modified using a biotin/HDL protein molar ratio of 80:1 (Fig. 2A). Accordingly, all subsequent preparations of biotinylated HDL incorporated this molar ratio. To verify that cell-associated biotinylated HDL is also efficiently precipitated by streptavidin, COS-SR-BI cells were incubated at 4°C with 10 μg/ml biotinylated $^{125}$I-HDL, followed by incubation of cell lysates with streptavidin beads. Our results showed that >93% of the $^{125}$I-HDL biotinylated in the presence of an 80-fold molar excess of biotin was precipitated from cell lysates by streptavidin (Fig. 2B). Similar recovery of biotinylated $^{125}$I-HDL was observed in the case of COS-SR-BI cells incubated with this ligand at 37°C for up to 3 h (data not shown). This compares with nonbiotinylated $^{125}$I-HDL, in which only 2.6 ± 1.0% of total cell-associated ligand was precipitated from cell lysates by streptavidin beads (Fig. 2B).

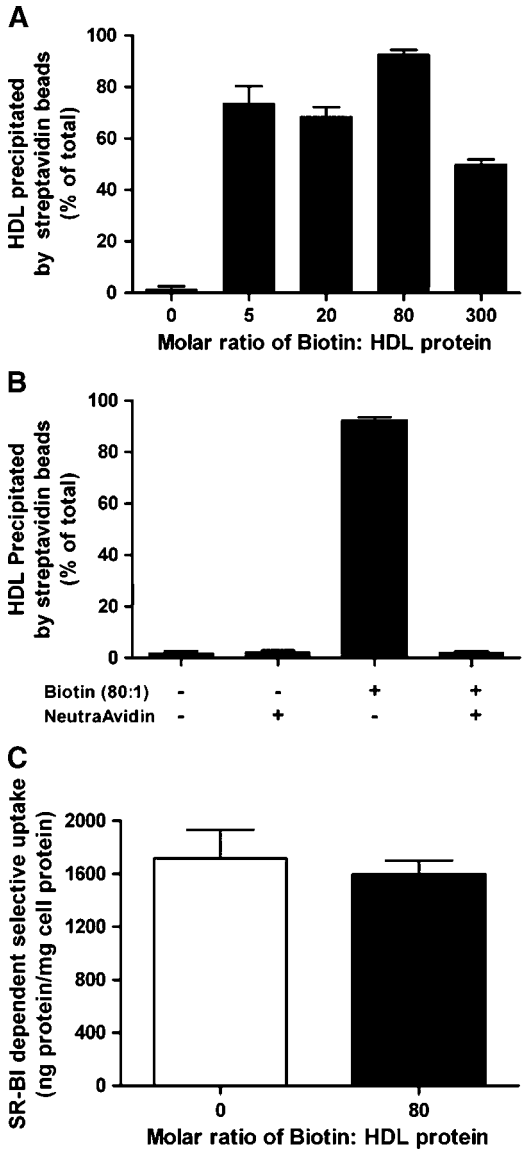


**Fig. 2.** Biotin assay for quantifying intracellular and surface-bound HDL. A: $^{125}$I-HDL biotinylated in the presence of the indicated biotin. HDL molar ratios were mixed with streptavidin beads, and the amounts of $^{125}$I-HDL recovered in the pellet and supernatant were determined. B: COS-SR-BI cells were incubated at 4°C with 10 μg/ml unmodified $^{125}$I-HDL or $^{125}$I-HDL biotinylated at a molar ratio of 80:1. Cells were washed to remove excess ligand and incubated with or without 0.15 mg/ml NeutrAvidin at 4°C for 15 min, and the recovery of biotinylated $^{125}$I-HDL by streptavidin precipitation was determined as described in Experimental Procedures. Values represent means ± SEM of results from triplicate determinations. C: After a 2 h incubation with 10 μg/ml $^{3}$H,$^{125}$I-HDL at 37°C, selective lipid uptake was quantified in control cells and COS-SR-BI cells as described in Experimental Procedures. Shown are SR-BI-dependent values, which are defined as the difference between COS-SR-BI and control cells and which represent means ± SEM of results from triplicate determinations.

Next, we assessed whether biotinylation of HDL alters the interaction between the lipoprotein and SR-BI. Control COS-7 cells and COS-SR-BI cells were incubated for 2 h at 37°C with 10 μg/ml biotinylated or nonbiotinylated $^{3}$H,$^{125}$I-HDL. As shown in Fig. 2C, there was no difference in SR-BI-dependent selective lipid uptake between nonbiotinylated and biotinylated HDL. Moreover, SR-BI-dependent HDL cell association and HDL degradation were similar for the two HDL preparations (data not shown).

We next determined whether NeutrAvidin incubations would block streptavidin precipitation of cell surface-bound biotinylated $^{125}$I-HDL. COS-SR-BI cells were incubated at 4°C with 10 μg/ml unmodified or biotinylated $^{125}$I-HDL. Cells were washed to remove excess ligand and incubated with or without 0.15 mg/ml NeutrAvidin at 4°C for 15 min, and the recovery of biotinylated $^{125}$I-HDL was determined after streptavidin precipitation. As shown in Fig. 2B, ~95% of the $^{125}$I-HDL precipitated by streptavidin could be blocked by preincubating cells with NeutrAvidin. Because NeutrAvidin is not taken up by cells at 4°C, any biotinylated $^{125}$I-HDL that is localized intracellularly would be expected to be precipitable by streptavidin. To verify that this is the case, we performed a time course study wherein control COS-7 cells and COS-SR-BI cells were incubated with 10 μg/ml biotinylated $^{125}$I-HDL at 37°C. At selected intervals, excess ligand was removed, cells were incubated for 15 min at 4°C with 0.15 mg/ml NeutrAvidin, and intracellularly accumulated HDL was quantified by measuring the amount of $^{125}$I-HDL precipitated by streptavidin beads. The results from the biotin assay indicated a time-dependent increase in streptavidin-precipitable HDL in COS-SR-BI cells but not in control COS-7 cells (**Fig. 3A**). After 1 h of incubation at 37°C, 22.7 ± 1.3% of total cell-associated HDL in COS-SR-BI cells was intracellular. Importantly, results from the biotin assay were in close agreement with results obtained with the fluorescence energy transfer assay (Fig. 3B). Based on these data, we estimate that the rate of SR-BI-dependent HDL internalization is ~3 ng HDL/mg cell protein/min.

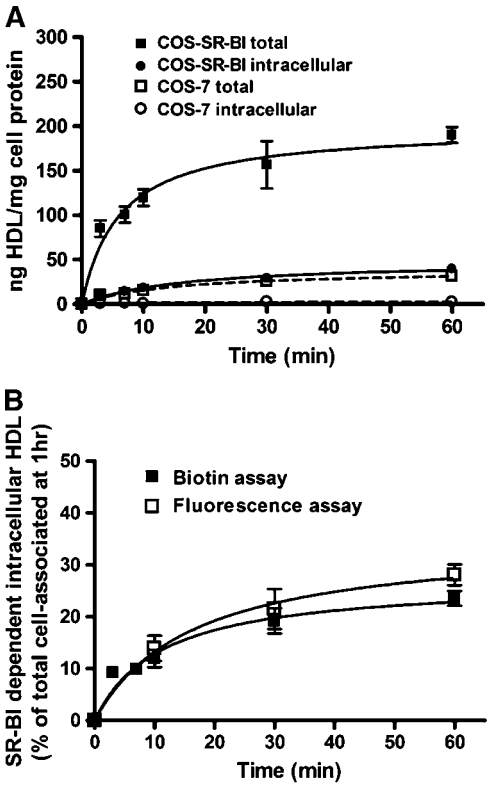


**Fig. 3.** Quantitative determination of intracellular HDL accumulation in COS-SR-BI cells by two biochemical assays. A: Control COS-7 cells (open symbols) and COS-SR-BI cells (closed symbols) were incubated at 37°C with biotinylated $^{125}$I-HDL for the indicated times, and then total cell-associated (squares) and intracellular (circles) HDL was quantified as described in Experimental Procedures. Values represent means ± SEM of results from triplicate determinations. B: Cells were incubated with 10 μg/ml Alexa 488-HDL (open squares) or biotinylated $^{125}$I-HDL (closed squares) at 37°C for the indicated times. Intracellular HDL was quantified as described in Experimental Procedures. The values shown are amounts of intracellular HDL at each time interval, expressed as a percentage of the total cell-associated HDL after the 60 min incubation. SR-BI-specific values are shown, which are defined as the difference between COS-SR-BI and control COS-7 cells. Values represent means ± SEM of results from triplicate determinations.

### Quantification of SR-BI-dependent HDL retroendocytosis

The biotin assay also enabled us to examine retroendocytosis quantitatively and to measure the rate of secretion of intracellular HDL. COS-SR-BI and control cells were incubated for 10 min with biotinylated $^{125}$I-HDL, at which time ~17% of the total cell-associated HDL was found within the cell (**Table 1**). After this pulse, cell surface-bound biotinylated $^{125}$I-HDL was masked by NeutrAvidin and the resecretion of intracellular HDL into the medium during a subsequent chase was quantified by streptavidin precipitation. Results from this analysis showed rapid secretion of a portion of the intracellular HDL that appeared to be almost complete within 30 min (**Fig. 4A**). Approximately 44% of the HDL that accumulated intracellularly during the 10 min incubation, representing ~7% of the total cell-associated HDL, was secreted into the medium during the 2 h chase period. We estimate that the size of the secreted pool for cells pulsed for 10 min was 7.6 ± 0.44 ng HDL/mg cell protein, and the calculated rate of HDL secretion was 0.53 ± 0.11 ng HDL/mg cell protein/min (Table 1). Thus, the rate of HDL secretion is markedly slower than the rate of SR-BI-dependent selective CE uptake (16.9 ± 2.2 ng HDL/mg cell protein/min) determined in these cells (Table 1).

The finding that not all internalized HDL was recovered in the medium after the 2 h chase suggests the presence of at least two intracellular pools of HDL: a rapidly turning-over retroendocytic pool and a more slowly filled pool that remains intracellular. To investigate the fate of the latter, intracellular pool, we performed additional pulse-chase experiments to measure HDL secretion and degradation during a longer (8 h) chase period. As indicated by the previous experiment, the recovery of secreted HDL after a 10 min pulse was complete by 60 min and represented ~40% of the original intracellular pool (Fig. 4B). After the 8 h chase period, virtually no biotinylated $^{125}$I-HDL was collected from cell lysates by streptavidin precipitation, suggesting that this pool of HDL had undergone at least partial degradation. This conclusion is supported by the finding that a large portion (60%) of the nonsecreted intracellular pool was present as trichloroacetic acid-soluble degraded material in the cell medium after the 8 h chase (Fig. 4B, diamonds).

### Release of SR-BI-bound HDL at the cell surface

We have demonstrated that the rate of SR-BI-mediated HDL retroendocytosis cannot account for selective lipid uptake and have shown that the bulk of HDL associated with COS-SR-BI cells is bound to the cell surface (Table 1). Thus, it was of interest to determine whether the rate of exchange of this surface-bound HDL was sufficiently rapid to mediate selective lipid uptake. For this analysis, cells were incubated with 10 μg/ml $^{125}$I-HDL at 4°C for 1 h and

TABLE 1. Rate of SR-BI-dependent HDL secretion and selective uptake in COS-7 cells

| Total Cell-Associated HDL[a] | Intracellular HDL[a] | Intracellular/ Total Ratio | Secreted Pool[a] | Secretion Rate $k$ | Secretion Rate | Release Rate $k$ | Release Rate | Selective CE Uptake[b] |
|---|---|---|---|---|---|---|---|---|
| *ng HDL/mg cell protein* | | | *ng HDL/mg cell protein* | *min*$^{-1}$ | *ng/mg/min* | *min*$^{-1}$ | *ng/mg/min* | |
| 104.4 | 17.3 | 0.17 | 7.6 ± 0.44 | 0.07 ± 0.01 | 0.53 ± 0.11 | 0.19 ± 0.02 | 19.3 ± 2.1 | 16.9 ± 2.2 |

CE, cholesteryl ester; SR-BI, scavenger receptor class B type I.
[a] After a 10 min, 37°C pulse.
[b] Calculated from the measurement of selective CE uptake at 37°C for 1 h.

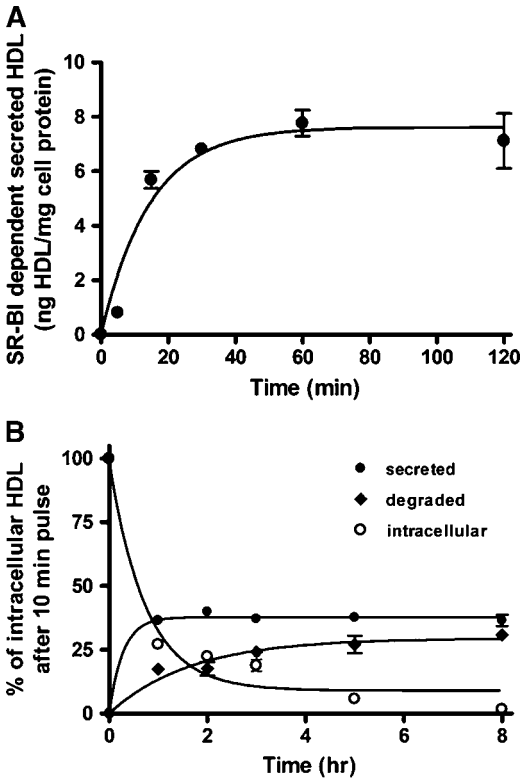


**Fig. 4.** SR-BI-dependent HDL resecretion and degradation. A: Control COS-7 cells and COS-SR-BI cells were incubated at 37°C with 10 μg/ml biotinylated $^{125}$I-HDL for 10 min and then washed and incubated with 0.15 mg/ml NeutrAvidin for 15 min at 4°C. Cells were then chased in complete medium at 37°C for the indicated times. Secreted HDL is defined as the $^{125}$I-HDL present in the medium that is precipitable by streptavidin beads. SR-BI-specific values are shown, which are defined as the difference between COS-SR-BI and control COS-7 cells. Values represent means ± SEM of results from triplicate determinations. B: Cells were incubated at 37°C with 10 μg/ml biotinylated $^{125}$I-HDL for 10 min and then washed and incubated with 0.15 mg/ml NeutrAvidin for 15 min at 4°C. Cells were then chased in complete medium at 37°C for the indicated times, and intracellular, secreted, and degraded HDL was quantified as described in Experimental Procedures. SR-BI-dependent values, which represent means ± SEM of results from triplicate determinations, are expressed as percentages of the intracellular biotinylated $^{125}$I-HDL at the end of the 10 min pulse.

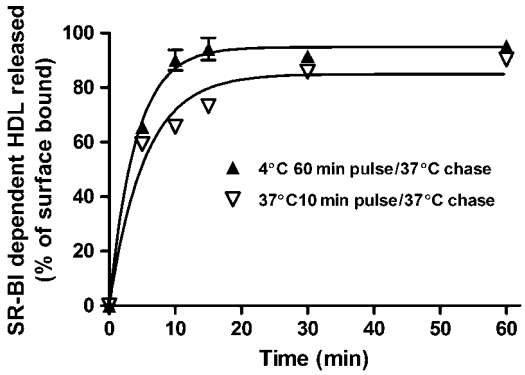


**Fig. 5.** Dissociation of surface bound HDL. Control COS-7 cells and COS-SR-BI cells were cooled to 4°C and, after washing with ice-cold PBS, incubated with ice-cold medium containing 10 μg/ml $^{125}$I-HDL for 1 h. Alternatively, cells were incubated with 10 μg/ml $^{125}$I-HDL at 37°C for 10 min. After incubation, the medium was removed and cells were washed to remove unbound ligand. Cells were either lysed to determine the total cell-associated $^{125}$I-HDL at 4°C and 37°C or incubated at 37°C with prewarmed complete medium containing a 60-fold excess of unlabeled HDL. At the indicated times, medium was collected to determine the amount of $^{125}$I-HDL dissociated from the cell surface. Values, which represent means ± SEM of results from triplicate determinations, are expressed as percentages of surface-bound $^{125}$I-HDL at the end of the 1 h, 4°C pulse or the 10 min, 37°C pulse.

then washed to remove unbound ligand. The release of $^{125}$I-HDL into medium containing a 60-fold excess of unlabeled HDL was quantified as described in Experimental Procedures. During the first 5 min of the chase period (when only a small amount of HDL had undergone internalization and resecretion), ~60% of cell-associated HDL was released into the medium (**Fig. 5**, closed triangles). Maximal release was reached by ~15 min and corresponded to 90–95% of cell-associated HDL. Our data indicate a relatively rapid release of HDL from SR-BI (half-life = 2.4–3.4 min). We also investigated the kinetics of release of HDL associated with COS-SR-BI cells at 37°C. Cells were pulsed with biotinylated $^{125}$I-HDL for 10 min, and total cell-associated $^{125}$I-HDL and release of cell-associated HDL were measured. The rate of appearance of HDL in the medium was somewhat slower for HDL incubated at 37°C (half-life = 3.4–4.8 min) compared with 4°C. We attribute this difference to the small amount of HDL that is internalized during the 10 min, 37°C pulse and subsequently resecreted. Our data suggest that the rate of exchange of HDL bound to SR-BI is similar to the rate of selective CE uptake (Table 1).

### Effect of BLT1, an inhibitor of selective lipid uptake, on SR-BI-mediated HDL internalization, secretion, and surface release

BLT1 has been shown previously to block SR-BI-mediated selective lipid uptake from HDL (49). To test whether BLT1 blocks selective lipid uptake by altering HDL internalization and/or resecretion, we measured these processes in COS-SR-BI cells in the presence and absence of BLT1. In confirmation of previous reports (28, 49), we showed that BLT1 promoted SR-BI-mediated HDL cell association (**Fig. 6B**) but decreased selective lipid uptake (Fig. 6A). BLT1 did not alter SR-BI-mediated HDL degradation (data not shown). Interestingly, our results using the biotin assay demonstrated that this decrease in SR-BI-dependent selective lipid uptake is not accompanied by a decrease in intracellular HDL (Fig. 6B), a finding that was confirmed using the fluorescence energy transfer assay (data not shown). Furthermore, using the biotin assay, we determined that the amount of intracellular biotinylated $^{125}$I-HDL resecreted into the medium was not altered in cells incubated with BLT1 (Fig. 6C). However, the rate of release of surface-bound HDL was reduced significantly by BLT1 (Fig. 6D), consistent with an earlier report (49). Although we cannot exclude the possibility that BLT1 alters SR-BI

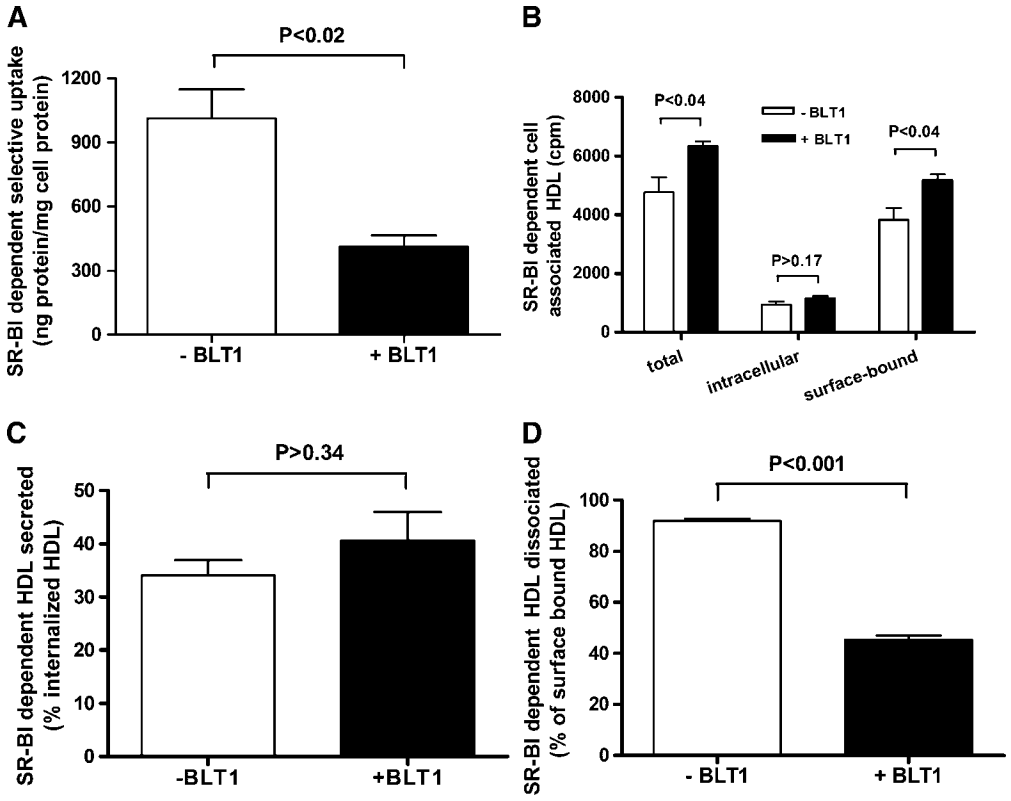


**Fig. 6.** Effect of BLT1 on SR-BI-mediated intracellular HDL accumulation and HDL resecretion. Control COS-7 and COS-SR-BI cells were preincubated with or without 10 μM BLT1 at 37°C for 1 h before the addition of HDL ligands. A: Selective uptake from $^{3}H$,$^{125}I$-HDL at 37°C for 1 h in the presence or absence of BLT1 was quantified as described in Experimental Procedures. The results shown are SR-BI-dependent values and represent means ± SEM of triplicate determinations. B: Cells were incubated with 10 μg/ml biotinylated $^{125}I$-HDL in the presence or absence of BLT1 at 37°C for 1 h. Cells were washed and incubated with 0.15 mg/ml NeutrAvidin for 15 min at 4°C and then processed to quantify cell-associated, intracellular, and surface-bound biotinylated $^{125}I$-HDL. The results shown are SR-BI-dependent values and represent means ± SEM of triplicate determinations. C: Cells were incubated with 10 μg/ml biotinylated $^{125}I$-HDL in the presence or absence of BLT1 at 37°C for 10 min. Cells were washed and incubated with 0.15 mg/ml NeutrAvidin for 15 min at 4°C and then either processed to quantify intracellular biotinylated $^{125}I$-HDL or chased in complete medium at 37°C for 15 min. Secreted HDL is defined as the $^{125}I$-HDL present in the medium that is precipitable by streptavidin beads. Values, which represent means ± SEM of triplicate determinations, are expressed as percentages of intracellular biotinylated $^{125}I$-HDL at the end of the 10 min incubation with HDL. D: Cells were preincubated with or without 10 μM BLT1 at 37°C for 1 h and then cooled to 4°C, and after washing with ice-cold PBS, they were incubated with ice-cold medium containing 10 μg/ml $^{125}I$-HDL with or without 10 μM BLT1. After a 1 h incubation at 4°C, medium was removed and cells were washed to remove unbound ligand. Cells were either lysed to determine the total cell-associated $^{125}I$-HDL at 4°C or incubated at 37°C with prewarmed complete medium with or without 10 μM BLT1 for 15 min, and the amount of $^{125}I$-HDL present in the medium was determined. Values, which represent means ± SEM of triplicate determinations, are expressed as percentages of surface-bound $^{125}I$-HDL at the end of the 1 h, 4°C pulse.

and/or HDL trafficking, our data strongly suggest that the marked inhibitory effect of BLT1 on selective uptake is largely related to its ability to alter the interaction of HDL particles with SR-BI to decrease the rate of ligand/receptor dissociation.

### SR-BI-dependent HDL retroendocytosis in HepG2 cells

To study SR-BI activity in HepG2 cells, we quantified HDL uptake and resecretion in these cells with and without exogenous SR-BI overexpression mediated by adenoviral vector gene transfer. Based on the biotin assay, cells expressing exogenous SR-BI (HepG2-SR-BI cells) had an ~3.3-fold increase in the total amount of cell-associated HDL, and a 4.7-fold increase in intracellular HDL, compared with control HepG2 cells after a 10 min incubation with biotinylated $^{125}I$-HDL (**Fig. 7A**, **Table 2**). This increase in HDL association was accompanied by a 3.8-fold increase in the rate of selective lipid uptake (Table 2). Notably, the exogenous SR-BI-dependent increase in HDL cell association (63.2 ng/mg cell protein; defined as the difference in cell association for HepG2-SR-BI cells and HepG2 cells) was ~60% of the SR-BI-dependent cell association in COS-SR-BI cells (104.4 ng/mg cell protein; Table 1), suggesting that exogenous expression of the

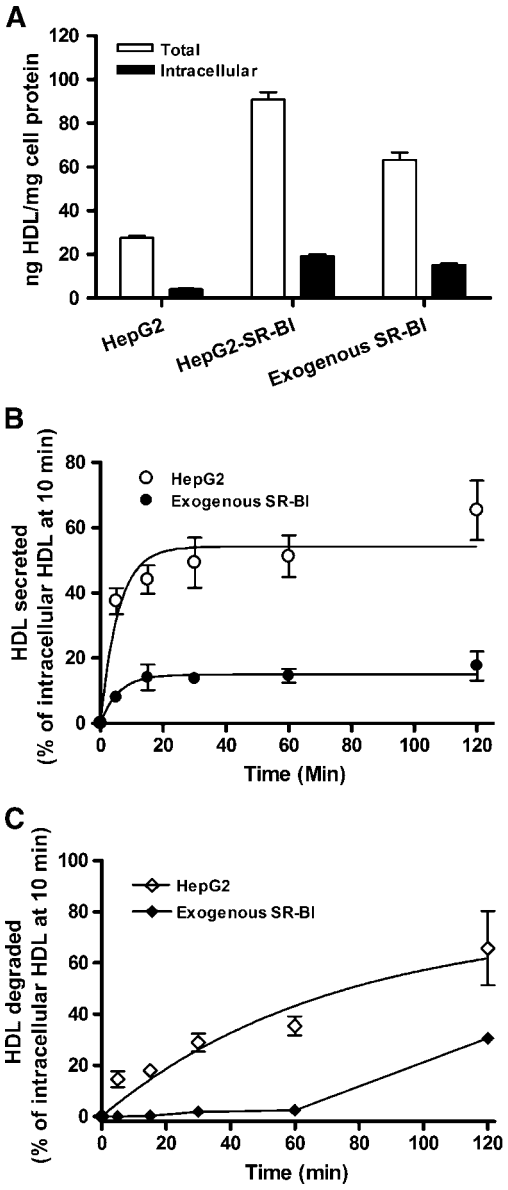


**Fig. 7.** SR-BI-dependent HDL uptake, resecretion, and degradation in HepG2 cells. A: Control HepG2 cells or HepG2 cells overexpressing SR-BI by adenovirus vector (HepG2-SR-BI) were incubated at 37°C with 10 μg/ml biotinylated $^{125}$I-HDL for 10 min, and total cell-associated and intracellular HDL was quantified as described in Experimental Procedures. Results shown represent means ± SEM of triplicate determinations. Exogenous SR-BI values are defined as the differences in values for HepG2-SR-BI and HepG2 cells. B: Control HepG2 and HepG2-SR-BI cells were incubated at 37°C with 10 μg/ml biotinylated $^{125}$I-HDL for 10 min and then washed and incubated with 0.15 mg/ml NeutrAvidin for 15 min at 4°C. Cells were then chased in complete medium at 37°C for the indicated times, and secreted HDL was quantified as described in Experimental Procedures. Shown are values derived from HepG2 cells and exogenous SR-BI-dependent values, which are differences in secretion from HepG2-SR-BI cells and control HepG2 cells. Data are expressed as percentages of the intracellular biotinylated $^{125}$I-HDL at the end of the 10 min pulse and represent means ± SEM of triplicate determinations. C: Medium from the experiment described in B was assayed to quantify $^{125}$I degradation products. Values represent means ± SEM of results from triplicate determinations.

receptor was comparable in the two cell types. Interestingly, despite similar amounts of HDL cell association and internalization, the rate of exogenous SR-BI-dependent selective lipid uptake was >4.4-fold slower in HepG2-SR-BI cells compared with COS-SR-BI cells (Tables 1, 2). Although the reason for this discrepancy is unknown, the low rate of selective uptake we observed is consistent with previous reports that selective lipid uptake in HepG2 cells is relatively inefficient (50). It is notable that the rate of exogenous SR-BI-dependent HDL release from the surface of HepG2 cells was the same as the rate measured in COS-7 cells ($k \sim 0.19$ min$^{-1}$) and is sufficiently fast to account for selective lipid uptake (Table 2).

The resecretion of intracellular biotinylated $^{125}$I-HDL that had accumulated in HepG2 and HepG2-SR-BI cells after a 10 min incubation was measured using the biotin assay (Fig. 7B). Similar to results in COS-SR-BI cells, we identified an exogenous SR-BI-dependent intracellular HDL pool in HepG2-SR-BI cells that was rapidly secreted. The rates of exogenous SR-BI-dependent HDL secretion in HepG2 cells ($k = 0.15 \pm 0.06$ min$^{-1}$) and COS-7 cells ($k = 0.07 \pm 0.01$ min$^{-1}$) were similar. Interestingly, the rate of exogenous SR-BI-dependent HDL secretion from HepG2 cells (0.35 ± 0.13 ng/mg/min) appeared to be ~10-fold slower than the rate of exogenous SR-BI-dependent selective uptake (3.8 ± 0.6 ng/mg/min), similar to results in COS-7 cells (Table 2). A rapidly recycling HDL pool was also detected in HepG2 cells without SR-BI overexpression (Fig. 7B). Compared with the exogenous SR-BI-dependent components in HepG2-SR-BI cells, the size of the secreted HDL pool and the rate of HDL secretion in control HepG2 cells appeared to be similar. Again, the rate of HDL secretion in HepG2 cells (0.4 ± 0.16 ng/mg/min) was too slow to totally account for selective uptake (1.4 ± 0.2 ng/mg/min) (Table 2). After the 2 h chase, ~85% and 47% of the exogenous SR-BI-dependent and -independent intracellular HDL pools, respectively, in HepG2-SR-BI cells was not resecreted (Fig. 7B, Table 2). Although these intracellular HDL pools appear to be eventually turned over through lysosomal degradation (Fig. 7C), the kinetics of $^{125}$I-HDL degradation in the two cell types were clearly different. Whereas degradation of the intracellular pool in control HepG2 cells appeared to gradually decline with time, very little exogenous SR-BI-dependent $^{125}$I degradation product was detected during the first 60 min of chase, before degradation increased markedly during the second hour (Fig. 7C).

## DISCUSSION

SR-BI-dependent HDL internalization has been described previously in a number of studies (29, 32–36), leading to speculation that SR-BI mediates selective CE uptake at least partially through a process that involves HDL internalization, selective sorting, and resecretion (32, 33, 35). This model for selective uptake is currently limited by the fact that rates of SR-BI-dependent HDL selective uptake and retroendocytosis have not been com-

TABLE 2. Rate of HDL secretion and selective uptake in HepG2 cells

| Cell | Total Cell-Associated HDL[a] | Intracellular HDL[a] | Intracellular/Total Ratio | Secreted Pool[a] | Secretion Rate $k$ | Secretion Rate | Release Rate $k$ | Release Rate | Selective CE Uptake[b] |
|---|---|---|---|---|---|---|---|---|---|
| | *ng HDL/mg cell protein* | | | *ng HDL/mg cell protein* | $min^{-1}$ | *ng/mg/min* | $min^{-1}$ | *ng/mg/min* | |
| HepG2 | 27.7 | 4.1 | 0.15 | 2.2 ± 0.15 | 0.18 ± 0.07 | 0.40 ± 0.16 | 0.10 ± 0.01 | 1.8 ± 0.2 | 1.4 ± 0.17 |
| HepG2 + SR-BI | 90.9 | 19.3 | 0.21 | 4.3 ± 0.22 | 0.24 ± 0.07 | 1.03 ± 0.30 | – | – | 5.3 ± 0.55 |
| SR-BI-dependent | 63.2 | 15.2 | 0.24 | 2.3 ± 0.19 | 0.15 ± 0.06 | 0.35 ± 0.13 | 0.19 ± 0.03 | 9.9 ± 1.6 | 3.8 ± 0.59 |

[a] After a 10 min, 37°C pulse.
[b] Calculated from the measurement of selective CE uptake at 37°C for 2 h.

pared directly. In this study, we set out to carefully quantify SR-BI-dependent HDL retroendocytosis in COS-7 and HepG2 cells, in vitro model systems widely used to study selective CE uptake (17, 35, 36, 46–48, 50, 51). Our investigations demonstrate that HDL trafficking by SR-BI exogenously expressed in COS-7 and HepG2 cells shares several features in common. *1*) SR-BI mediates HDL internalization in both COS-7 and HepG2 cells, representing 17% and 24%, respectively, of the total SR-BI-dependent HDL cell association after a 10 min incubation. *2*) There appear to be at least two intracellular SR-BI-dependent HDL pools in both cell types, a rapidly turning-over retroendocytic pool ($k \sim 0.1$ $min^{-1}$) that in the steady state represents ~5% of the total HDL associated with cells and a more slowly filled pool that is not resecreted during a 2 h chase and is eventually turned over by lysosomal degradation. *3*) For both cell types, the rate of HDL secretion from the retroendocytic pool is too slow to account for the majority of selective CE uptake.

For most of our analyses, we chose to express SR-BI in COS-7 cells by adenoviral vector-mediated gene transfer. These cells exhibit robust selective lipid uptake that, within the dose range of adenoviral vector used in our experiments, was directly proportional to the amount of receptor expressed. In the absence of exogenous SR-BI, these cells exhibited virtually no HDL association, internalization, or selective CE uptake; in all cases, SR-BI-dependent values were ~10-fold higher compared with values from control cells. This low background facilitated accurate measurements of relatively small amounts of SR-BI-specific uptake and resecretion. The quantification of SR-BI-mediated HDL uptake required a method that distinguished intracellularly accumulated particles from particles bound to SR-BI at the cell surface. A commonly used approach for this is to "displace" extracellular $^{125}$I-HDL by incubating cells for an extended period with a 100-fold excess of unlabeled HDL at 0–4°C (52). In the case of rat hepatoma cells, ~88% of surface-bound HDL can be displaced under these conditions (52). Using a similar approach, Pagler et al. (36) reported that 80% of surface-bound HDL could be displaced from SR-BI-expressing CHO cells. To determine whether this method could be used in our studies, we investigated whether $^{125}$I-HDL associated with SR-BI-expressing COS cells at 4°C (to inhibit cellular HDL uptake) could be effectively displaced by a subsequent incubation with excess cold HDL at 4°C. Unexpectedly, we found that <70% of HDL bound at 4°C in an SR-BI-dependent manner was removed under these conditions (data not shown). The difference in results from our experiments and published reports is likely attributable to differences in the cell types under study. For example, it is possible that a large portion of "displaceable" HDL binding sites present on rat hepatocytes is not SR-BI. Experiments to investigate other methods for "stripping" ligands from the cell surface, including trypsin digestion (29, 35, 52–55), low pH (56, 57), and polyanions and heparin (35, 58–60), also failed to effectively remove $^{125}$I-HDL bound to SR-BI at 4°C (data not shown). Our newly developed method, in which >95% of biotinylated $^{125}$I-HDL bound extracellularly to SR-BI-expressing cells can be blocked by NeutrAvidin, provides sufficient sensitivity to quantify SR-BI-dependent HDL internalization and resecretion. It should be noted that our estimates of intracellular and resecreted HDL do not take into account the fact that <5% of surface-bound biotinylated $^{125}$I-HDL cannot be masked by NeutrAvidin, which leads to slight overestimates of SR-BI-dependent HDL retroendocytosis by our assay. An advantage of our protocol is that to be detected in the streptavidin-precipitable fraction, "intracellular" and "secreted" HDL must contain both $^{125}$I and biotin labels and thus presumably represents largely intact apolipoprotein on HDL.

Using the biotin assay, our kinetics studies identified at least two intracellular pools in SR-BI-expressing COS-7 cells, which together represent ~23% of the HDL that is associated with the cells after a 1 h incubation (17% after a 10 min incubation). This estimate of intracellular HDL was verified using an alternative biochemical method, which quantified intracellularly accumulated fluorescent HDL. One intracellular pool is distinguished by the fact that it undergoes rapid retroendocytosis (half-life ~ 10 min). This pool appears to fill rather rapidly (within 10 min) and represents only ~5% of total HDL associated with SR-BI-expressing COS-7 cells (40% of the intracellular HDL). Importantly, we have been able to quantify the rate of secretion of this retroendocytic pool (~0.5 ng HDL/mg protein/min) and have determined that it is too slow to account for the SR-BI-dependent selective lipid uptake (~17 ng/mg/min) that occurs in these cells. From these data, we conclude that HDL retroendocytosis contributes little, if any, to selective uptake in COS-7 cells. An additional, more slowly filling intracellular HDL pool can be detected in SR-BI-expressing COS-7 cells, which is not secreted

during a 2 h chase. This intracellular pool appears to be eventually turned over by lysosomal degradation, because ~60% of the nonsecreted, intracellular HDL is degraded after an 8 h chase. Our results in COS-7 cells may be compared with the findings of Pagler et al. (36), who quantified HDL uptake and resecretion in SR-BI-transfected CHO cells. They estimated that ~64% of HDL associated with cells in an SR-BI-dependent manner was intracellular (1 h of incubation) and that 43% of this pool of HDL was recovered in the medium after a 30 min chase period (i.e., 28% of total cell-associated HDL). Compared with our results in COS-7 cells, these findings suggest that the extent of SR-BI-dependent HDL internalization and resecretion may be greater in CHO cells (64% and 28% of total cell-associated HDL, respectively) compared with COS-7 cells (23% and 5%, respectively). It seems likely, however, that some of the discrepancy from the two studies is attributable to differences in methodology. For measurements of SR-BI-dependent HDL internalization and resecretion in CHO cells, a displacement procedure was used that was only 80% effective at removing surface-bound HDL (36). Because estimates of intracellular and resecreted HDL were not corrected for this amount of "background" HDL, and because it is likely that the nondisplaced surface pool of HDL would be released during a 37°C chase, we interpret these findings to suggest that ~45% and 8% of total cell-associated HDL is internalized and resecreted, respectively, in SR-BI-expressing CHO cells. These values are in closer agreement with our assay results in COS-7 cells.

We also investigated HDL recycling in HepG2 cells. Based on results from the biotin assay, we estimate that ~15% of the total cell-associated HDL in HepG2 cells accumulates intracellularly, and ~54% of this intracellular HDL pool (8% of total cell-associated HDL) is resecreted (half-life = 3.8 min). During a 2 h chase, virtually all of the nonsecreted intracellular HDL in HepG2 cells is degraded. Our findings appear to be in close agreement with results reported by Wustner et al. (61), who used quantitative fluorescence to investigate intracellular trafficking of HDL in polarized and nonpolarized HepG2 cells. Using pulse-chase protocols, they estimated that in polarized cells >50% of intracellular Alexa 488-HDL was rapidly recycled to the basolateral membrane and released from cells (half-life ~ 6.9 min). Mathematical modeling of the kinetic data derived from this study predicted a transport pathway whereby internalized HDL is targeted from the basolateral membrane to sorting endosomes from which rapid recycling occurs. Some HDL escaping this recycling pathway is transcytosed to bile canaliculi via the subapical compartment/apical recycling compartment or, alternatively, delivered to the late endosome/lysosome for degradation (62). In another recently published study, it was estimated that ~40% of $^{125}$I-HDL$_3$ associated with HepG2 cells was resecreted during a 2 h, 37°C chase (35). The CE/protein ratio of this "resecreted" pool of HDL was reduced compared with the starting HDL, suggesting that this pool had undergone selective lipid uptake. However, this interpretation is complicated by the fact that in this pulse-chase study, the chase period followed a 1 h, 4°C heparin treatment that in control experiments detached only ~54% of $^{125}$I-HDL bound to HepG2 cells at 4°C. Thus, the resecreted HDL likely contained a significant amount of HDL that was readily released from the cell surface during the 37°C chase.

Approximately 45–65% of HDL taken up by HepG2 cells can be inhibited by antibodies to SR-BI (61, 63), demonstrating that these cells express HDL binding sites in addition to SR-BI. To assess "SR-BI-specific" HDL trafficking, our approach was to compare uptake and resecretion in control HepG2 cells and HepG2 cells overexpressing SR-BI by adenoviral vector. In our experiments, the size of the secreted HDL pool and the rate of HDL secretion mediated by "exogenous" SR-BI were comparable to those mediated by control HepG2 cells. The similar rates of HDL secretion for exogenous SR-BI and for HepG2 cells are in agreement with the fact that a large portion of HDL taken up by HepG2 cells has been attributed to SR-BI and suggests that exogenous and endogenous SR-BI metabolize HDL similarly. Interestingly, the rates of exogenous SR-BI-dependent HDL resecretion in HepG2 cells (0.35 ± 0.13 ng/mg/min) and COS-7 cells (0.53 ± 0.11 ng/mg/min) were similar. By comparing rates of exogenous SR-BI-dependent selective lipid uptake and HDL resecretion, we estimate that HDL retroendocytosis contributes <3% and 10% of selective lipid uptake in COS-7 and HepG2 cells, respectively, assuming that these processes are not mutually exclusive. In addition to a rapidly recycling HDL pool, there appears to be a pool of HDL internalized by exogenously expressed SR-BI in HepG2 cells that escapes resecretion. Similar to what occurs in COS-7 cells, this pool is eventually targeted to lysosomes and degraded.

Our conclusion that HDL internalization and resecretion do not account for selective lipid uptake is consistent with recently published work showing that treatments that block endocytosis, such as hyperosmotic sucrose, $K^+$ depletion, or 2-deoxyglucose/$NaN_3$ treatment, do not prevent selective lipid uptake (28, 29). We confirm an earlier finding that BLT1 does not interfere with selective lipid uptake by reducing the amount of HDL internalized (28), and we further show that BLT1 does not alter the amount of SR-BI-dependent retroendocytosis. Interestingly, BLT1 appears to reduce selective lipid uptake by altering the cell surface interaction of HDL with SR-BI, such that the rate of HDL dissociation from the receptor is decreased (Fig. 6D) (49). Thus, selective lipid uptake may be inhibited in the presence of BLT1, because the number of HDL particles processed by SR-BI in a given period may be reduced significantly as a result of a decreased rate of ligand dissociation from the receptor. It is also possible that BLT1 directly inhibits SR-BI-mediated lipid transfer through an unknown mechanism and that HDL dissociation is secondarily affected, because the HDL particle is normally more readily released from SR-BI after it is lipid-depleted, possibly as a result of conformational changes in apolipoprotein A-I (64).

We have previously described an alternatively spliced form of SR-BI (designated SR-BII) that is identical to SR-BI except for the presence of an entirely different C-terminal

cytoplasmic tail (37, 38). Using a cell surface biotinylation approach, we recently reported that the vast majority (80–90%) of SR-BII in transfected CHO cells is intracellular (34). This contrasts with SR-BI, in which ~70% of the receptor is present on the cell surface in transfected CHO cells (34). We also demonstrated by confocal microscopy that SR-BII, and not SR-BI, undergoes rapid endocytosis and mediates the intracellular accumulation of HDL in the endosomal recycling compartment. By fluorescence energy transfer assay, we confirmed the finding that significantly more Alexa 488-HDL associated with SR-BII-expressing cells is intracellular (~85%; data not shown) compared with SR-BI-expressing cells (<25%; Fig. 3B). We investigated SR-BII-dependent HDL uptake by our newly developed biotin assay, as described in this study for SR-BI. Unexpectedly, after a 1 h incubation, only 50% of biotinylated $^{125}$I-HDL associated with SR-BII-expressing cells was recovered from cell lysates by streptavidin precipitation in the absence of NeutrAvidin block, whereas >93% of HDL associated with SR-BI-expressing cells could be recovered by streptavidin precipitation. We interpret these findings to suggest that HDL trafficking by SR-BI and SR-BII is distinct, because it appears that HDL taken up by SR-BII-expressing cells moves to intracellular compartment(s) where it is subject to at least partial degradation.

In summary, we have quantified SR-BI-dependent HDL uptake and resecretion in COS-7 and HepG2 cells. Our results suggest that SR-BI-mediated intracellular trafficking of HDL in COS-7 cells may share features in common with HDL transport in hepatocytes. After internalization, a portion of intracellular HDL undergoes rapid retroendocytosis, with a half-life typical of recycling via the endocytic recycling compartment (62, 65). Although the functional significance of HDL recycling by SR-BI is not clear, recent studies have suggested that this pathway may play a role in cellular cholesterol efflux (36). In both HepG2 and COS-7 cells, a second pool of internalized HDL that escapes resecretion is eventually targeted to lysosomes and degraded. We have determined that the amount of HDL trafficking through the retroendocytic pool is too small to support selective lipid uptake.

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