Expression of Scavenger Receptor BI in COS-7 Cells Alters Cholesterol Content and Distribution†

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ABSTRACT: Previous studies have shown that scavenger receptor BI (SR-BI) stimulates the bidirectional flux of free cholesterol (FC) between HDL and SR-BI-expressing cells. A major component of the enhanced FC flux appears to occur independently of HDL binding to SR-BI and may be due to changes in membrane lipid domains resulting from SR-BI expression (*1*). In the present study, the impact of SR-BI on cellular cholesterol metabolism was determined by examining SR-BI-mediated changes in cellular cholesterol mass, the esterification of HDL-derived FC, and changes in membrane lipid pools. Growth of SR-BI-expressing cells in medium containing HDL led to increased cellular cholesterol mass, most of which accumulated as ester. The esterification of HDL-derived FC was enhanced by SR-BI-expression to a far greater extent than the SR-BI mediated increase in FC uptake, suggesting an SR-BI-mediated effect on cholesterol utilization in the cell. This observation was tested by comparing FC esterification rates in SR-BI positive and negative cells when equivalent amounts of extracellular FC were taken up via cyclodextrins or apolipoprotein AI/phospholipid disks, neither of which contained cholesteryl ester. Under these conditions, SR-BI did not preferentially stimulate cholesterol esterification. These results indicate that the enhanced esterification of HDL-derived FC in SR-BI-expressing cells is due to the expanded pool of cellular FC and not to a specific effect of SR-BI on cholesterol utilization. Two approaches were used to test the effects of SR-BI expression on membrane lipid organization. In the first, the sensitivity of cellular FC to exogenous cholesterol oxidase was tested under conditions in which there is a preferential oxidation of caveolar cholesterol. SR-BI-expression was found to greatly increase the fraction of cellular cholesterol available to the oxidase as compared to either vector-transfected cells or cells expressing the related class B scavenger receptor CD36. These results suggest that SR-BI expression alters the distribution of membrane-free cholesterol to a caveolar fraction or alters the accessibility of this membrane fraction to exogenous cholesterol oxidase. In the second approach, the efflux of cellular FC to high concentrations of cyclodextrins was monitored under conditions where desorption of FC from the plasma membrane is rate limiting for efflux. SR-BI-expressing cells showed a shift in the distribution of FC between two kinetic pools with more FC in the fast pool and less in the slow pool. These data support a model in which SR-BI expression leads to a redistribution of cholesterol to membrane domains that serve to facilitate the flux of FC between cells and lipoproteins.

There has been increasing interest in the role of scavenger receptor BI (SR-BI) in cholesterol metabolism since its identification as an HDL receptor (*2*). SR-BI has been shown to be abundant in liver and adrenals as well as other cells such as macrophages within atherosclerotic plaques (*3*−*5*). This receptor recognizes a broad range of ligands including negatively charged phospholipid liposomes (*6*, *7*), LDL (*8*), VLDL (*8*), and HDL (*2*). Because of its ability to bind HDL with high affinity, it has been proposed that SR-BI plays a role in the metabolism of HDL cholesterol (*9*−*15*). In contrast to other lipoprotein receptors such as the apo B/E and scavenger receptor class A, which bind and internalize lipoproteins, SR-BI binds ligands such as HDL but does not internalize the lipoprotein. Rather, the binding of HDL to SR-BI results in selective incorporation of cholesteryl ester (*9*, *16*−*18*).

Studies from this laboratory have demonstrated that SR-BI, in addition to promoting selective uptake of cholesteryl ester, stimulates the bidirectional flux of unesterified cholesterol between cells and HDL (*1*, *5*, *19*). We have also shown that the rate of release of cellular cholesterol from different cell types to phospholipid containing extracellular acceptors generally correlates with the expression level of SR-BI protein (*5*, *19*). Although it was first assumed that the ability of SR-BI to bind HDL particles with high affinity was the reason for this enhanced cholesterol flux, recent

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studies from our laboratory revealed that a major component of the stimulation of cholesterol flux was not directly linked to the binding of HDL to the receptor (*1*). This conclusion was supported by the observation that the expression of SR-BI stimulated efflux of cell cholesterol to neutral SUV, even though such vesicles do not bind to the receptor (*1*). In addition, it was demonstrated that although HDL bound with high affinity to CD36, a receptor structurally related to SR-BI (*17*, *18*, *20*) CD36 promotes only a modest increase in the amount of cholesterol efflux (*1*). These results suggest that transient expression of SR-BI in COS-7 cells produced changes in cholesterol flux that were not related to lipoprotein binding. Furthermore, it was observed that cholesterol oxidase treatment of SR-BI-expressing COS-7 cells resulted in greater cholesterol oxidation than was observed in vector transfected cells, suggesting that the presence of SR-BI in the plasma membrane produced a change in the organization of the membrane. This re-organization may be responsible for enhanced cholesterol flux between plasma membrane and HDL (*1*).

Because SR-BI may influence a number of steps in cellular cholesterol metabolism, we initiated the current studies to examine various aspects of cholesterol metabolism in COS-7 cells transiently transfected with SR-BI or CD36. The results demonstrate that aspects of cholesterol metabolism were significantly modified by the expression of SR-BI when compared to vector transfected cells.

EXPERIMENTAL PROCEDURES

Materials. Fetal bovine serum (FBS), bovine serum albumin (BSA, essentially fatty acid free), gentamicin, unesterified cholesterol (FC), cholesteryl methyl ether (CME), glutaraldehyde, and cholesterol oxidase from streptomyces were purchased from Sigma Chemicals (St. Louis, MO). Organic solvents were products of Fisher Scientific (Pittsburgh, PA). Tissue culture flasks and plates were obtained through Corning (Corning, NY) or Falcon (Lincoln, NJ). [1,2-^{3}H]Cholesterol and [1-^{14}C]oleate were purchased from New England Nuclear (Boston, MA). Tissue culture media were obtained from Gibco (Grand Island, NY). Human HDL ($1.125 < d < 1.21$ g/mL) was fractionated by sequential ultracentrifugation (*21*). Cholesterol oxidase from *Nocardia erythropolis* was purchased from Boehringer-Manheim (Indianapolis, IN). 1-Palmitoyl-2-oleoyl phosphatidylcholine (PC) was purchased from Avanti Polar Lipids. FC and PC dispersions were made by the method of Arbogast et al. (*22*) with the addition of 1.5 μCi [^{3}H]cholesterol/mg unlabeled cholesterol. Pfizer CP-113,818 was a gift from Pfizer Pharmaceuticals. 2-Hydroxypropyl-β-cyclodextrin (2OHCD) and methyl-β-cyclodextrin (MCD) were gifts from Cerestar USA.

Cell Culture and Transient Transfection. COS-7 cells were routinely grown in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, and antibiotics in T75 flasks and subcultured once a week using a 1:20 split ratio. For transfection, cells (1.5×10^6) were seeded in 100 mm plates in DMEM supplemented with 10% FBS and incubated for 18 h at 37 °C in a humidified 5% CO_2 incubator. Cells were transfected with a mixture of 10 μg of the desired plasmid, diluted in serum-free DMEM, and Fugene-6 (Boehringer-Manheim) prepared in a sterile polystyrene tube (Falcon #2058). This mixture was added dropwise to the plated cells. Murine SR-BI, rat CD36, or vector (*17*) were prepared using endotoxin-free Qiagen Maxiprep kits. After transfection, cells were maintained on normal growth media and some incubations contained 1 μCi/mL [^{3}H]cholesterol.

Labeling and Cholesterol Loading of HDL$_3$. Labeling of control and cholesterol-enriched HDL with [^{3}H]cholesterol was accomplished by adding [^{3}H]cholesterol (25−100 μCi in ethyl ether) to a fiberglass filter in a 7 mL glass scintillation vial and drying the solvent under N_2. HDL_3 (6−10 μg protein) was added to the vial in addition to 2.6 mL MEM-HEPES and incubated overnight at 4 °C on a rotating platform. The HDL was removed after 24 h and the filter washed 2× with MEM-HEPES. The combined original HDL and washes were diluted to a final concentration of 200 mg HDL_3 protein/mL with MEM. To enrich HDL_3 with excess free cholesterol, the lipoprotein was incubated with cholesterol/phospholipid dispersions (2:1 mole:mole) as detailed in earlier studies (*23*). This procedure yielded HDL in which the free cholesterol content was increased 4-fold, without major changes in other lipoprotein components. Cholesterol-enriched HDL was labeled with [^{3}H]cholesterol as described above.

Uptake and Esterification of [^{3}H]Cholesterol from HDL. Cells were initially depleted of cholesterol by growing them on delipidized serum protein (5 mg protein/mL) overnight (DLP) (*24*). HDL_3 (200 μg protein/mL) was added to cells for 4, 8, and 24 h. At each time point, the monolayers were washed 2× with MEM-HEPES. The incorporation of [^{3}H]-cholesterol from HDL_3 and mass of cholesterol and cholesteryl ester were determined as described below.

Cholesterol Mass Quantification. At the end of each experiment, lipids were extracted with 2-propanol, containing CME as an internal standard. The amount of [^{3}H]cholesterol incorporated into cells was determined by liquid scintillation counting. The distribution of the labeled cholesterol between free and esterified cholesterol was determined by LSC after thin-layer chromatography (*25*, *26*). Total and free cholesterol mass analysis was done by gas−liquid chromatography (GLC) as previously described (*27*).

Protein Determination. After lipid extraction, cell monolayers were solubilized in 10% SDS and protein was determined by a Lowry assay, as modified by Markwell et al. (*28*).

Uptake and Esterification of [^{3}H]Cholesterol from Methyl-β-cyclodextrin (MCD). After transfection with SR-BI or vector, the cells were incubated overnight with DLP to initially deplete the cells of lipid. The media containing DLP was then removed and the cells were washed twice with MEM-HEPES. The cells were then incubated for 4 h (15 °C) in DMEM containing 250 μg [^{3}H]cholesterol/phospholipid dispersions and MCD (0.1 mM) This media was then removed and the cells were incubated in serum-free DMEM (37 °C) for 24 h. At the end of the experimental period, the cells were washed twice with PBS and lipids were extracted with 2-propanol. The percent of cellular free and esterified cholesterol was determined by thin-layer chromatography as described above.

Uptake and Esterification of [^{3}H]Cholesterol from Apo-AI/phospholipid Disks. Apo-AI/phospholipid disks were prepared using the cholate dispersion/Bio-Bead method as previously described (*29*). The final composition of the disks was determined after concentration (Centiprep 30, Amicon). The complexes were purified and characterized as described previously (*30*). After transfection with SR-BI or vector, the cells were incubated overnight with DLP to initially deplete the cells of lipid. The media containing DLP was then

removed, and the cells were washed twice with MEM-HEPES. The cells were then incubated for 24 h (37 °C) in DMEM media containing 10, 25, 50, 80, or 250 μg apo-AI/mL. The cells were then washed twice with PBS, and lipids were extracted with 2-propanol. The amount of [^{3}H]FC incorporated was determined and the percent of this labeled cholesterol converted to EC was established after thin-layer chromatography separation of FC from EC. The mass of cholesterol incorporated and esterified was calculated from the specific activity of the FC in the reconstituted particles.

Cholesterol Oxidase. After transfection and during the growth period, COS-7 cells were co-incubated with [^{3}H]-cholesterol (1 μCi/mL) in growth medium. Cholesterol oxidase assay was performed using one of two protocols, one involves fixing the cells (*31*) and the second does not (*32*).

Fixed Protocol. [^{3}H]Cholesterol-labeled cells were chilled on ice, washed twice with cold PBS, and fixed for 10 min with 1% glutaraldehyde. After fixing, the cells were washed again with cold PBS. Cholesterol oxidase from streptomyces was added at a concentration of 1 U/mL for 30 min at 37 °C. After this time, the cells were chilled and washed 2× with ice cold PBS.

Nonfixed Protocol. [^{3}H]Cholesterol-labeled cells were chilled on ice, washed twice with cold PBS. Cholesterol oxidase from *Nocardia erythropolis* was added to the cells for 1 h to 4 h at 37 °C; after this time, the cells were chilled and washed 2× with ice cold PBS. In both protocols, lipids were extracted with 2-propanol and separated by TLC (mobile phase: 96 mL hexane, 15 mL methanol, 8 mL ethyl ether). Quantification was accomplished using LSC.

Efflux of FC to Cyclodextrins. Transfected COS-7 cells were incubated overnight in growth medium supplemented with 3 μCi/mL [^{3}H]cholesterol and the ACAT inhibitor CP-113,818 (2 μg/mL). Monolayers were washed 2× with MEM-HEPES and exposed to trypsin (900 BAEE units/mL) for 5 min at 37 °C. The cells were then harvested by low speed centrifugation (1000 rpm for 5 min) at room temperature. The cell pellet was washed 2× by alternating pelleting and resuspension in MEM-HEPES. The cells were brought to a final concentration of 4×10^6 cell/mL and 600 μL of the cell suspension was added to 3 mL of efflux medium. The efflux medium consisted of MEM-HEPES containing 100 mM 2-hydroxypropyl β cyclodextrin (2OH-βCD), which was 50% saturated with FC (*33*). The cells in suspension were kept at a constant temperature (37 °C) in a shaking water bath. To measure efflux of cellular cholesterol, an aliquot of the cell suspension was removed at desired times and filtered (Multiscreen filtration system, Millipore Corp., Bedford, MA), and the radioactivity was determined in each aliquot. An aliquot of the cell suspension was used to determine total radioactivity in the experimental solution. Cholesterol efflux was determined by dividing the total ^{3}H counts in the medium after efflux by the amount that was originally in the experimental solution. The GraphPad Prism software (version 2.0, GraphPad Software Inc., CA) was used to analyze the efflux kinetics. The data were fitted to a biexponential equation by nonlinear regression as described previously (*34*). The equation used describes efflux from two pools; $Y = Ae^{-k_1 - t} + Be^{-k_2 - t}$ assuming all of the cholesterol is available for efflux. *A* and *B* are the fractional sizes of the independent pools, 1 and 2, respectively. The apparent rate constants for fractional efflux from pools 1 and 2 are k_1 and k_2, respectively. The apparent half-times were calculated as $t_{1/2} = \ln 2\ k$.

ACAT Activity. Transfected COS-7 cells were plated in 100 mm dishes three days prior to the experiments (8.8×10^5 cells per dish). On the day of the experiment, the dishes were rinsed 3× with PBS and the monolayers were scraped into 1 mL PBS. The cells were lysed by nitrogen cavitation (30 min, 250 psi). The lysed cells were centrifuged at 25000*g* for 20 min to sediment nuclei, mitochondria and lysosomes. The resulting supernatant was centrifuged at 105000*g* for 60 min to collect the microsomes. Microsomal protein content was determined as described above. The ACAT assay was carried out as described by Tavani et al. (*35*). Briefly, the standard assay medium contained 100 μg microsomal protein, 1 mg fatty acid free BSA, 0.1 M potassium phosphate buffer (pH 7.4), 2 mM glutathione, 200 μg/mL cholesterol presented in Triton WR-1339 (at a Triton WR-1339 to cholesterol ratio of 30:1) and 0.4 μCi [1-^{14}C]oleoyl-CoA in a total volume of 1.6 mL. The reactions were initiated by the addition of labeled oleoyl-CoA after the other ingredients had been preincubated at 37 °C for 30 min. Reactions were run for 10 min at 37 °C and were terminated by the addition of 4 mL methanol, 4 mL chloroform, and 2 mL H_2O; 10 μg cholesterol oleate and 0.25 μCi [^{3}H]-cholesterol were added to serve as an internal standard and recovery markers. After separation of the phases, the chloroform layer was collected and dried under nitrogen. Cholesteryl esters were isolated by thin-layer chromatography, using a mobile phase of petroleum ether/ethyl ether/acetic acid (90:10:1). Lipids were visualized by exposure to iodine and quantitated by liquid scintillation counting.

Statistical Analysis. Values are expressed as mean ± SD. Unpaired Student's *t* test was used to determine statistical differences between groups (GraphPad Prism version 2.01, GraphPad Software). The criterion for significance was set at $p \leq 0.05$.

RESULTS

Effect of SR-BI on Cholesterol Content and Esterification. Since SR-BI has been shown to mediate the uptake of cholesteryl ester from HDL (*2*, *16*) and to stimulate the bidirectional flux of free cholesterol between cells and HDL (*1*, *5*, *19*), we first determined whether SR-BI would alter the cholesterol mass of transfected COS-7 cells. Cells were routinely grown on 10% FBS for two days following transfection; the data illustrated in Figure 1A are values from a representative determination. Although expression of either CD36 or SR-BI had no consistent effect on free cholesterol (FC) values, the expression of SR-BI resulted in cells having a significant level of esterified cholesterol (EC), whereas vector transfected and CD36-expressing COS-7 had essentially none. Thus, SR-BI expression results in a greater amount of total cellular cholesterol mass compared to vector transfected cells and cells expressing CD36.

The EC that accumulated in SR-BI positive cells could have originated from a number of extracellular sources including either the FC or EC pools in the array of lipoproteins present in the serum. To determine if lipoprotein FC was a source of the cellular EC, [^{3}H]cholesterol was added to the FBS-containing growth medium and the total ^{3}H incorporated and fractional esterification of the labeled sterol was determined. Figure 1A indicates that the cells expressing SR-BI when routinely grown in culture contain significantly more EC mass than vector transfected and

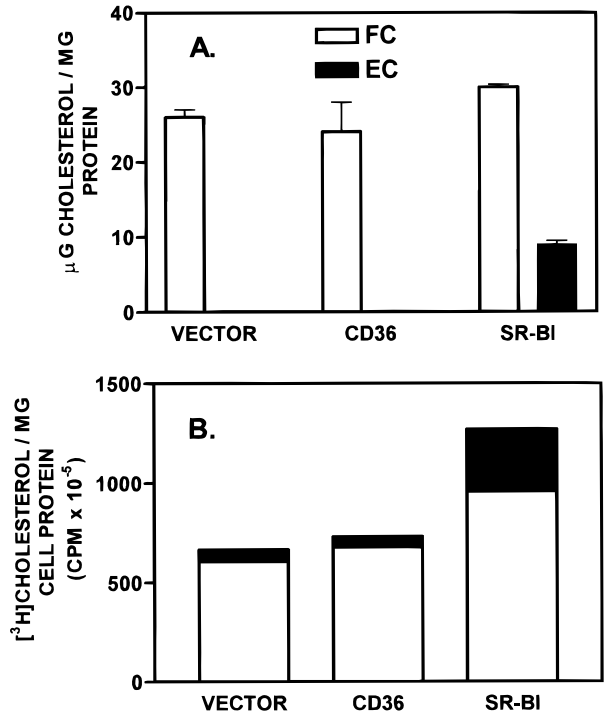


FIGURE 1: (A) Cholesterol content of COS cells transfected with SR-BI and CD36. COS-7 cells were transiently transfected with SR-BI, CD36 or vector and maintained in culture as described in "Methods". Lipids were extracted from the monolayers and cholesterol mass was determined by gas chromatography and normalized to cell protein. (B) COS-7 cells were treated as described above, and [^{3}H]cholesterol was added to the growth medium after transfection. Cells were incubated in this medium for 24 h. Total counts incorporated into cells were measured and normalized to cell protein. Lipids were extracted from monolayers, separated by thin-layer chromatography. ^{3}H-label in each lipid class was quantitated by liquid scintillation counting.

CD36-expressing cells. There was no consistent effect on FC mass in CD36-expressing, SR-BI-expressing, and vector-transfected cells. As shown in Figure 1B, SR-BI expression resulted in greater cellular [^{3}H]FC. Additionally, SR-BI expression resulted in an increase in the amount of the incorporated [^{3}H]FC esterified (Figure 1B, 8.0 ± 0.6%, vector transfected, 6.0 ± 0.5%, CD36-expressing, 24.0 ± 2% SR-BI-expressing). These results suggest that SR-BI may facilitate the esterification of FC entering the cell from medium lipoproteins.

To further examine the uptake and esterification of cholesterol by SR-BI-expressing and vector transfected cells, transfected monolayers of COS-7 cells were incubated with human HDL_3 that contained [^{3}H]cholesterol. Since previous studies from this laboratory have demonstrated that the flux of free cholesterol between cells and HDL is markedly influenced by the composition of HDL (*23*, *36*−*40*), we compared two preparations of HDL, native HDL with a normal FC content and HDL that had been approximately 4-fold enriched with FC (see "Methods"). We measured both [^{3}H]FC influx and the conversion of [^{3}H]FC to [^{3}H]EC. The results in Table 1 demonstrate that cells expressing SR-BI, when exposed to control or FC-enriched HDL, incorporate more HDL FC than vector transfected or CD36-expressing cells. FC uptake was increased 4-fold by enrichment of HDL, but the relative increase was the same for all cell types. Table 1 also illustrates that the relative esterification of HDL-derived FC was markedly stimulated by expression of SR-BI when compared to vector or CD36 transfected cells. Thus,

Table 1: Uptake and Esterification by Transfected COS-7 Cells of Free Cholesterol from Control and Free Cholesterol-Enriched HDL[a]

	FC influx	% of vector	% of control HDL	ester	% of vector
		control HDL			
vector	167 ± 3	100		2.4 ±0.2	100
CD36	172 ± 10	103		2.0 ± 0.1	100
SR-BI	234 ± 29[b]	140		13.1 ± 0.7[b]	650
		enriched HDL			
vector	720 ± 3	100	431	21.6 ±3.1	100
CD36	674 ± 57	94	392	20.8 ± 1.7	105
SR-BI	1000 ± 52[b]	139	427	98.8 ± 8.3[b]	450

[a] (ng HDL free cholesterol/mg cell protein/h); rates of HDL [^{3}H]-free cholesterol influx and esterification calculated from a linear regression fit of a 12 h incubation of cells with the radiolabeled HDL. [b] Significantly different than vector ($p \leq 0.05$, $n = 3$).

although SR-BI expression produced only a 1.4-fold increase in [^{3}H]FC uptake, the percent esterification of the newly incorporated [^{3}H]FC was increased 6.5-fold or 4.5-fold with the two HDL preparations. Esterification of HDL-derived FC was a result of acyl-CoA cholesterol acyltransferase (ACAT) activity since the inhibition of this enzyme with the ACAT inhibitor CP113818 completely blocked the esterification of labeled cholesterol incorporated from FBS or human HDL (data not shown). In addition, the stimulated esterification was not a reflection of an increase in ACAT activity, since there was no difference in the incorporation of ^{14}C-oleate into EC by cholesterol-enriched microsomal preparations (*35*) from vector transfected and SR-BI positive COS-7 cells (data not shown). Thus, these data suggest that SR-BI somehow facilitated the utilization of [^{3}H]FC for ACAT mediated esterification.

Figure 2 illustrates the cholesterol mass of COS-7 cells transfected with vector CD36 or SR-BI and then exposed to native or FC-enriched HDL_3 for up to 12 h. In this series of experiments, the transfected monolayers were grown on medium supplemented with delipidized serum protein (DLP) for 18 h prior to the addition of the HDL preparations. Since the expression of SR-BI produced an increase in cell cholesterol content when cells were grown on FBS (see Figure 1), this DLP treatment was used to normalize cholesterol content in all cells before the addition of the HDL. However, even after the monolayers were maintained in the absence of exogenous lipids for 18 h, SR-BI transfected cells had a significant level of EC, whereas vector transfected and CD36-expressing cells contained no detectable EC. Vector transfected cells exposed to native HDL exhibited a small, but significant increase in FC after 12 h incubation, whereas incubation with FC-enriched HDL produced a significant FC accumulation after 4 h, which continued to increase thereafter (Figure 2A). Cells expressing-CD36 had changes in FC mass similar to that of vector transfected cells with both control and enriched HDL (Figure 2B). The changes in FC content in the SR-BI transfected cells were much more dramatic (Figure 2C). Incubation of SR-BI-expressing cells with native HDL resulted in an increase in FC at 12 h, similar to vector transfected cells. However, in SR-BI cells exposed to FC-enriched HDL, there was a markedly higher accumulation of FC mass throughout the incubation period compared to vector transfected and CD36-expressing cells (Figure 2A−C). Vector transfected cells or CD36-expressing cells contained no detectable EC before or after the HDL incubations (data not shown), whereas the SR-BI transfected COS-7 cells increased their ester content when exposed to FC-enriched

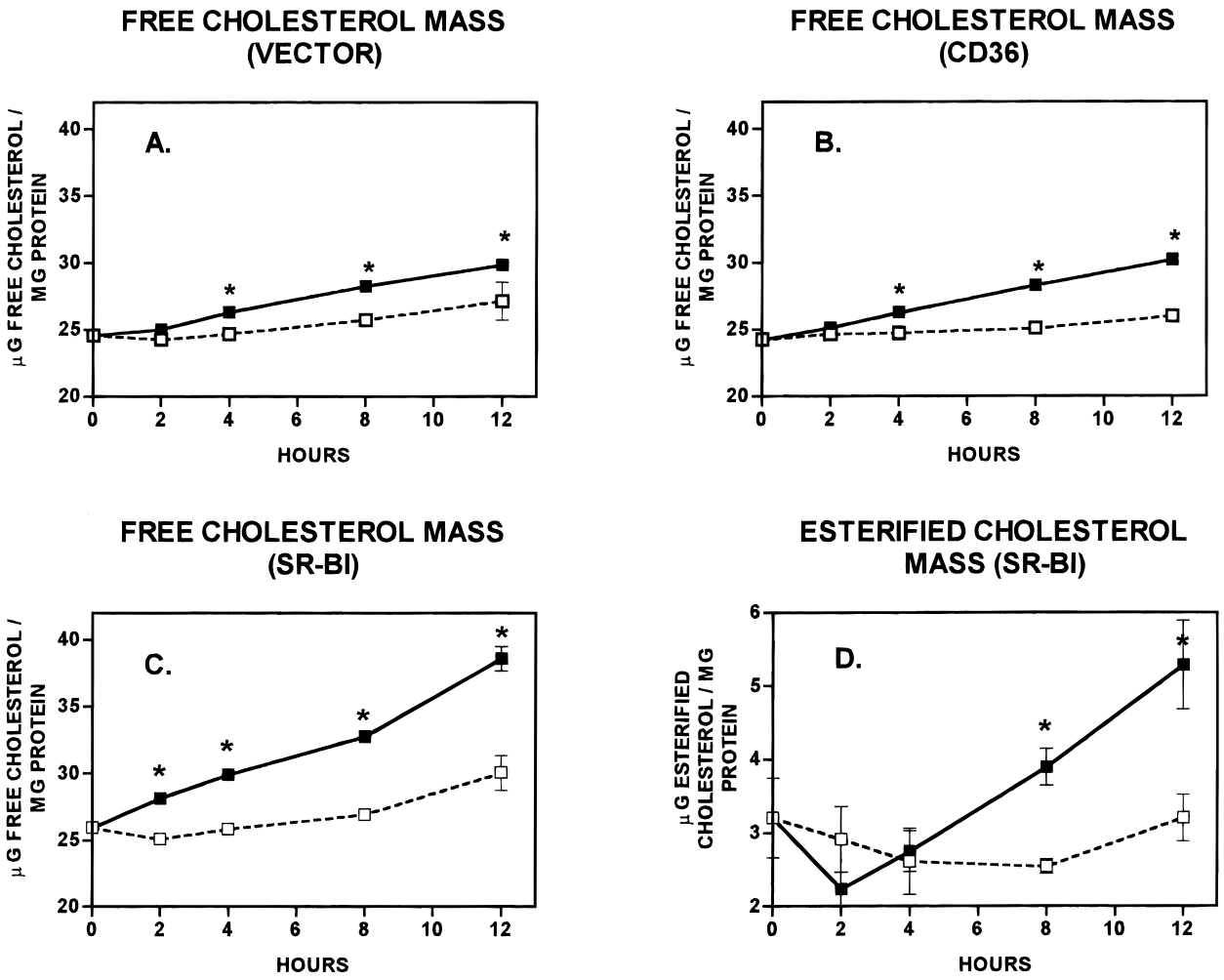


FIGURE 2: Free and esterified cholesterol mass in COS-7 cells exposed to control and FC-enriched HDL. COS-7 cells were transfected as described in Figure 1. Monolayers were then incubated with control HDL or HDL that had a 4-fold enrichment in FC. HDL preparations were added at 200 μg protein/mL. Masses were determined by GLC analysis as described in "Methods". Open symbols are data from control HDL and solid symbols are from FC-enriched HDL. Values are average ± SD, $n = 3$, $* = p < 0.05$ compared to zero time cholesterol masses.

HDL (Figure 2D). Thus, as illustrated by Figure 2, exposure of SR-BI-expressing cells to HDL results in an increased deposition of both FC and EC when compared to vector transfected cells. This excess deposition of cholesterol is relatively small if the HDL contains normal levels of free cholesterol, but is greatly accentuated if the HDL is modified to contain excess FC.

The effect of SR-BI expression to increase the fractional esterification of HDL [^{3}H]FC could effect a specific alteration in cholesterol trafficking or could simply be a result of increasing the amount of cholesterol in the cell. Previous work has suggested that cholesterol esterification is sluggish until a threshold of cellular FC is attained (*41*) an effect that can be due to the sigmoidal substrate dependence of ACAT (*42*). To distinguish between these possibilities we used two procedures to load the same amount of [^{3}H]FC into SR-BI-expressing and vector transfected cells under conditions where there was no uptake of EC. In the first, MCD plus [^{3}H]FC/PC dispersions was the FC donor; in the second, FC was provided in apo AI/phospholipid/[^{3}H]FC disks. Before the start of each experiment, transfected COS-7 cells were incubated with DMEM containing DLP for 18 h. This treatment normalized the cholesterol levels in COS-7 cells transiently expressing SR-BI (11.5 ± 0.7 μg FC/mg protein, 1.3 ± 0.5 μg EC/mg protein) or vector, 11.0 ± 0.6 μg FC/mg protein, EC not detected). The monolayers were then incubated with [^{3}H]FC/phospholipid dispersions plus cyclodextrins (0.1 mM) at 15 °C for 4 h. Under these conditions, the cyclodextrins act as a shuttle to deliver cholesterol to the plasma membrane of the cell (*26*) and internalization of the FC at the plasma membrane will not occur at 15 °C (*43*, *44*). At this concentration of MCD, the SR-BI cells incorporated slightly more [^{3}H]FC than vector transfected cells (Figure 3A). The media containing MCD was then removed and replaced with serum-free DMEM for 24 h (37 °C) to allow for internalization and esterification of the [^{3}H]FC. Figure 3B demonstrates that there was no difference in the amount of the [^{3}H]FC esterified in the presence or absence of SR-BI.

Since cyclodextrins are nonphysiological compounds and may not access the same membrane domains as lipoproteins, we designed an experiment that employed apo AI phospholipid disks containing [^{3}H]FC as cholesterol donors. DLP-treated monolayers of SR-BI-expressing or vector transfected COS-7 cells were incubated with apo-AI/phospholipid/[^{3}H]-FC disks at the concentrations indicated in Table 2 for 24 h at 37 °C. The media was removed from the cells, cellular lipids were extracted as described in "Methods" and analyzed for total FC incorporated (influx) and the amount of the FC esterified (Table 2). Similar to the cyclodextrin experiment

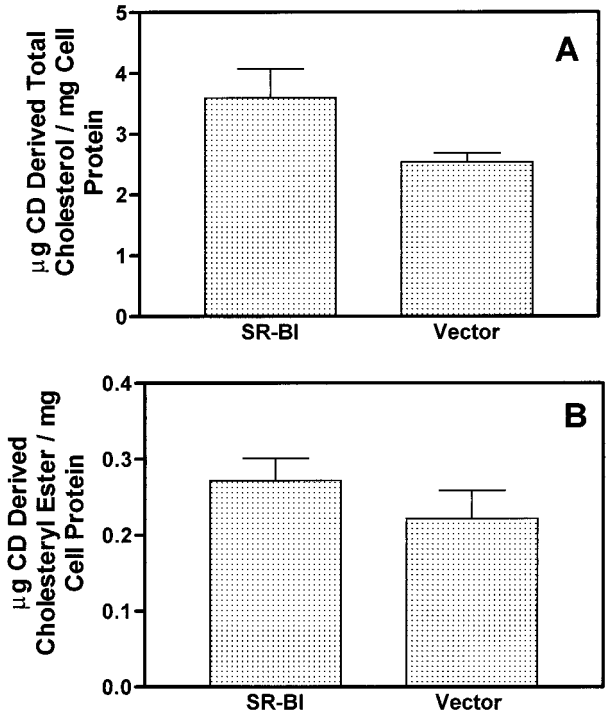


FIGURE 3: Esterification of FC delivered by methyl β-cyclodextrins (MCD). COS-7 cells were transiently transfected with SR-BI or vector as described in "Methods" and grown on DLP for 18 h to normalize the initial cell cholesterol content. Media containing [^{3}H]-FC and phospholipid dispersions (250 μg cholesterol/mL) plus MCD (0.1 mM) were added to the cells for 4 h at 15 °C. This incubation was followed by an incubation with DMEM alone at 37 °C for 24 h. Lipids were extracted from monolayers, separated by thin-layer chromatography, and quantitated by liquid scintillation counting. Total MCD-derived FC incorporated into the cells is shown in Figure 3A and the amount of this FC esterified is shown in Figure 3B. Results are the mean ± SD (n = 3).

Table 2: Influx and Esterification of FC from apo-AI/PL/FC Discs

cell[a]	apo-AI/PL/FC[b] μg prot./mL	influx ng/mg prot./24 h	esterification ng/mg prot./24 h
vector	80	3845 ± 85	111 ± 10
	250	5857 ± 101	127 ± 11
SR-BI	10	2690 ± 64	31 ± 4
	25	4334 ± 88	40 ± 5
	50	4994 ± 88	68 ± 16
	80	5851 ± 178	87 ± 9

[a] Time 0 cholesterol contents (μg/mg protein) = vector 27.0 ± 1.3 FC, 4.2 ± 1.6 EC; SR-BI 29.0 ± 0.4 FC, 6.3 ± 0.3 EC. [b] Molar composition = protein/FC/PL, 1:10.5:95.

described above, there were similar amounts of FC incorporated into the cells and there was no enhanced esterification in the cells expressing SR-BI compared to the vector transfected cells (Table 2). Therefore, we conclude that SR-BI does not selectively facilitate FC esterification.

Effect of SR-BI on the Sensitivity of Membrane Cholesterol to Cholesterol Oxidase. In previous work, we observed an SR-BI mediated re-distribution of cellular cholesterol pools as evidenced by an increase in FC susceptibility to oxidation by an exogenous cholesterol oxidase in SR-BI-expressing cells (*1*). The enzyme cholesterol oxidase, which converts cholesterol to cholestanone, has been used extensively to probe plasma membrane cholesterol content and domain distribution (*45*−*50*). A number of technical variations have been employed, and the interpretation of the data obtained with each of these techniques is sometimes controversial.

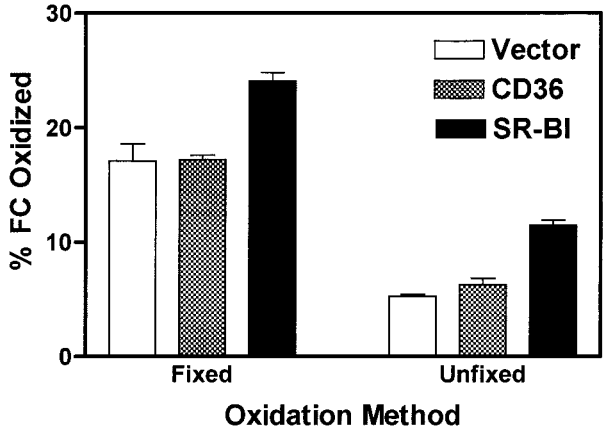
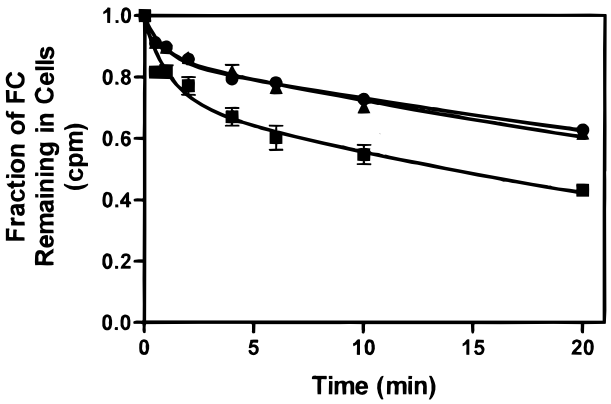


FIGURE 4: Comparison using two methods of cholesterol oxidation of SR-BI effects on the size of the oxidizable pool of cellular cholesterol. COS-7 cells were transiently transfected with SR-BI, vector, or CD36 as described in "Methods" and grown for two days in medium containing [^{3}H]cholesterol. Two cholesterol oxidase methods, using fixed and nonfixed cells, were employed. Lipids were extracted from monolayers, separated by thin-layer chromatography, and quantitated by liquid scintillation counting. Results are the mean ± SD (n = 3).

In a previous study on vector transfected and SR-BI transfected COS-7 cells, we observed that the expression of SR-BI increased the size of the pool of cell cholesterol that was converted to cholestanone upon treatment with cholesterol oxidase (*1*). In this study, the cells were fixed with glutaraldehyde before exposure to the enzyme (*1*). Recently, a cholesterol oxidase procedure has been described, which eliminates the fixation step, uses a cholesterol oxidase from a different source and modifies the incubation conditions (*32*). This protocol has been reported to primarily oxidize the plasma membrane cholesterol located in caveolae (*32*). SR-BI is, at least in part, located in caveolae (*16*), and these organelles have been linked to the flux of cholesterol between cells and lipoproteins (*51*). Therefore, we compared the cholesterol oxidase sensitivity of cholesterol in vector transfected and SR-BI transfected cells using the 2 different oxidation protocols. As can be seen from Figure 4, both protocols, with and without prior fixation, revealed that SR-BI expression increased the size of the cholesterol oxidase sensitive pool. However, the differential between vector transfected and receptor positive cells was consistently greater when the "caveolae" protocol was employed (41% increase using the "fixed" cell protocol vs 117% increase using the "unfixed" protocol, Figure 4). We have previously shown using glutaraldehyde-fixed cells that the expression of CD36 does not increase the size of the oxidase sensitive pool. In the present studies, we observed no CD36-mediated increase in the oxidase sensitive pool using either procedure (Figure 4).

Effect of SR-BI on the Kinetics of Cholesterol Efflux to 2-Hydroxypropyl-β-cyclodextrins. Our previous studies on SR-BI-mediated free cholesterol flux, and the results obtained when plasma membrane cholesterol pools were probed with cholesterol oxidase, suggest that the expression of SR-BI results in changes in the organization of the cholesterol in the plasma membrane. As an alternate way to examine membrane cholesterol domains, we quantitated the efflux of [^{3}H]cholesterol from vector-transfected, CD36- or SR-BI-expressing COS-7 cells to a high concentration of 2-hydroxypropyl-β-cyclodextrin (2OHCD). At high concentrations, cyclodextrins remove cellular cholesterol rapidly under



	SR-BI	Vector	CD36
Fast 1/2 time (min)	0.82	0.67	0.61
Slow 1/2 time (min)	25.29	44.76	38.58
Fast pool size	25.00	14.00	13.00
Slow pool size	73.00	85.00	87.00

FIGURE 5: Free cholesterol efflux from transfected COS-7 cells to 2OHCD. COS-7 cells were transiently transfected with SR-BI (■), vector (●) or CD36 (▲) and labeled with [^{3}H]cholesterol as described in "Methods". The cells were treated with trypsin and put into suspension with 50 mM 2OHCD for up to 20 min. Cholesterol efflux was determined by dividing the FC ^{3}H counts in the medium after efflux by the amount that was originally in the cells at time zero. Results are the mean ± SD ($n = 6$, pooled data from two separate experiments).

conditions where the rate-limiting step for efflux is the movement of cholesterol molecules out of the plasma membrane (*34*). The FC efflux kinetics exhibited by many cell types exposed to cyclodextrins are indicative of two kinetic pools, with half-times ($t_{1/2}$) values of approximately 30 s and 30−60 min (*34*, *52*). In addition, the sizes of these fast and slow pools differ among cell types (*34*, *52*). COS-7 cells were pre-labeled with [^{3}H]cholesterol in the presence of the ACAT inhibitor CP113818 so that all of the [^{3}H]-cholesterol in the cells at the initiation of the efflux phase of the experiment was present as FC. The inhibition of ACAT avoided the confounding possibility that the hydrolysis of cellular [^{3}H]EC would influence efflux kinetics (*53*). In addition, the 2OHCD used in these studies contained sufficient unlabeled cholesterol (2OHCD/cholesterol = 8:1) to facilitate exchange of cell cholesterol with cyclodextrin cholesterol without changing net cell cholesterol mass (*33*). This avoided the possibility that efflux kinetics reflected changes in membrane lipid distribution produced upon cholesterol depletion (*33*, *34*). As can be seen from Figure 4, we consistently observed a pattern in which the expression of SR-BI increased the size of the fast pool (approximately 2-fold) with a reciprocal reduction in the size of the slow pool. In addition, SR-BI expression increased the rate of cholesterol loss from the slow pool. The expression of CD36 had no effect on FC efflux to 2OHCD, yielding kinetics similar to those obtained with vector transfected cells (Figure 5). In a large number of our previous experiments in which SR-BI and CD36 were compared, and in which HDL-binding assays were performed, CD36 was expressed equal to or greater than SR-BI. Thus, the fact that in CD36-expressing cells we do not see a change in the kinetics of FC efflux to cyclodextrins and sensitivity to cholesterol oxidase as we do in SR-BI-expressing cells cannot be attributed lack of expression of the CD36 receptor in the transfected cells.

DISCUSSION

Much of the recent research on the function of SR-BI has been focused on its interaction with HDL and on its role in mediating the selective uptake of HDL EC, particularly in endocrine tissues and the liver (*2*, *4*, *9*, *10*, *13*, *17*). Recently, it has been demonstrated that SR-BI is playing a broader role in cell cholesterol metabolism and can influence a number of metabolic parameters including a stimulation in the bidirectional flux of FC between cells and HDL (*5*, *19*). The enhancement of FC flux is not a result of the specific binding of HDL to SR-BI as indicated by the observation that cholesterol efflux to neutral SUV that do not bind to SR-BI was stimulated by expression of the receptor, and that there was only a minor increase in cholesterol flux from COS-7 cells expressing CD36, a related receptor that binds HDL (*1*). The possibility that the increased cholesterol flux produced by SR-BI expression was linked to changes in cell plasma membrane composition or organization was supported by preliminary data indicating an increase in the size of the cell cholesterol pool that became susceptible to the action of cholesterol oxidase (*1*). In the present investigation, we have obtained further evidence that SR-BI is affecting cellular cholesterol organization and deposition.

Quantitation of the cholesterol content of transiently transfected COS-7 cells and then grown for 2 days in medium supplemented with 10% FBS revealed that vector transfected and SR-BI-expressing cells had essentially the same FC mass. However, the receptor positive cells had significantly more EC (Figure 1). Since there would be a number of different lipoprotein sources for this excess cholesterol when cells are grown in complete serum, we exposed SR-BI negative and positive cells to human HDL_3 after the monolayers had been transfected and maintained on delipidized serum protein. In addition, two different preparations of HDL were used, one containing a normal level of FC and another enriched approximately 4-fold with FC. This comparison was made because we had previously demonstrated that FC-enriched HDL increased the cholesterol content of a number of cells, and that this response appeared to be accentuated in Fu5AH hepatoma cells that have very high expression levels of SR-BI (*23*). The expression of SR-BI stimulated the influx of the HDL [^{3}H]cholesterol when compared to the vector transfected and CD36-expressing cells, and this influx was greater with FC-enriched HDL (Table 1). Interestingly, the expression of SR-BI produced a dramatic increase in the fractional esterification of HDL-derived FC (Table 1). SR-BI-linked enhanced esterification was observed when the cells were exposed to either normal or FC-enriched HDL. Thus, a greater fraction of the FC incorporated from HDL into SR-BI-expressing cells becomes available to ACAT. To address whether this increase in esterification is linked to a direct effect of SR-BI expression; for example, by increasing cholesterol transport from plasma membrane to endoplasmic reticulum, or simply a reflection of an increased pool size of cholesterol derived from both enhanced FC and EC uptake, we introduced FC directly into the plasma membrane via cyclodextrins (Figure 3) and apo-AI/phospholipid/FC disks (Table 2), and followed the subsequent esterification of the FC. In these experiments, we eliminated the complication of SR-BI-mediated EC selective uptake by using cholesterol donors that only contain FC. Our results indicate that there is not a fundamental difference in the mechanism of FC esterification in SR-BI-expressing cells. Rather this increase in cellular EC appears to be a function of selective

uptake of and increased cholesterol flux from serum and/or HDL, resulting in a larger pool of cholesterol available for esterification by ACAT. This interpretation agrees with the observation that the ACAT activity−substrate concentration curve is sigmoidal (*42*) and that a critical level of cholesterol has to be available to ACAT to stimulate esterification (*41*). In addition, our data is consistent with the recent study by Stangl et al. (*54*), who demonstrated enhanced esterification by SR-BI-expressing CHO cells incubated with high levels of microcrystalline cholesterol aggregates, but no elevated esterification if low concentrations of cholesterol were supplied to the cells.

An examination of the cholesterol contents of the cells exposed to normal HDL demonstrated that incubation of vector-transfected, CD36-expressing, and SR-BI-expressing cells with normal HDL had only a small effect on FC content, producing a minor increase after 12 h of incubation (Figure 2, A and B). In contrast, FC-enriched HDL progressively stimulated cholesterol accumulation in all cells throughout the 12 h incubation (Figure 2, A and B). The relatively minor change in FC mass in SR-BI transfected cells exposed to normal HDL (Figure 2, A and B), coupled with the increased influx of HDL FC is a reflection of a system in which the expression of SR-BI increases both the influx and efflux arms of a bidirectional flux system, consistent with our previous observations (*1*). Incubation with FC-enriched HDL shifts the cell−HDL cholesterol gradient to favor net influx, and this unbalanced bidirectional flux is accelerated by SR-BI expression, resulting in rapid net accumulation of cell FC. The combination of SR-BI expression and HDL cholesterol enrichment, which produced the enhanced esterification of HDL-derived FC (Table 1), resulted in a net accumulation of cell EC mass (Figure 2C). It should be noted that SR-BI-expressing cells consistently contained small, but significant, concentrations of EC, even after growth on DLP for 18 h. In contrast, vector transfected or CD36-expressing COS-7 cells had essentially no EC. Previous studies have demonstrated that selective uptake of EC does occur when CD36 is present on the cell surface (*17*). However, the amount of selective uptake in CD36 positive cells is relatively small compared to that which occurs when SR-BI is present.

Since we have previously obtained data indicating that the changes in cell/HDL cholesterol flux, which are linked to SR-BI expression, are not a result of HDL binding to the receptor (*1*), other SR-BI-related changes have been examined. We have used two different approaches to establish that expression of SR-BI changes the lipid organization of the plasma membrane. The first approach was to demonstrate that the pool of membrane cholesterol susceptible to oxidation by cholesterol oxidase increases upon SR-BI expression. We previously demonstrated this phenomenon by employing an oxidation protocol, with glutaraldehyde fixed cells (*1*). It is thought that this technique may make available a larger pool of membrane cholesterol than when unfixed cells are studied (*49*). As previously observed (*1*), cholesterol oxidation with fixed cells was greater when SR-BI was expressed (Figure 4). This same result was obtained when a second oxidation protocol was employed with unfixed cells, and conditions that are thought to primarily oxidize cholesterol contained in caveolae (*32*) (Figure 4). The difference in the fractional oxidation between SR-BI positive and negative cells was greater with this protocol than when using fixed cells.

The second approach to establish that SR-BI expression changed membrane lipid organization was to measure the kinetics of cholesterol efflux from SR-BI positive and negative cells exposed to a high concentration of cyclodextrin. Figure 5 illustrates that the expression of SR-BI changes the kinetics of cholesterol efflux, primarily by increasing the size of the fast pool, whereas expression of CD36 had little or no effect. At the present time, we cannot link the kinetic pools with structural elements in the plasma membrane. Thus, these two pools may represent cholesterol in the inner or outer leaflets of the membrane, or they may reflect lateral domains in the membrane. An attractive hypothesis that is consistent with the cholesterol oxidase data discussed above, and the reported localization of SR-BI in caveolae (*16*, *55*), is that the fast pool is cholesterol in caveolae or detergent resistant domains (*16*, *51*, *56*, *57*). A large fraction of the SR-BI-enhanced cholesterol flux is not a result of a binding event, but appears to relate to a change in plasma membrane lipid domain structure (*1*). Since caveolae and lipid rafts have been linked both to the flux of FC (*51*) and to the intracellular transport of cholesterol between the plasma membrane and intracellular sites (*58*), SR-BI may be mediating changes in organization of lipid in the plasma membrane or increasing the number of caveolae or lipid rafts present on the cell surface (*59*). Whether these changes in membrane organization are responsible for SR-BI-mediated selective uptake of HDL EC is unknown; however, selective uptake of HDL EC appears to be much more dependent on lipoprotein-receptor binding than is the flux of FC (*60*).

Finally, it is now apparent that SR-BI is a more ubiquitous receptor than was originally thought, and that low level expression can be observed with a number of different cell types including macrophage cell lines and primary macrophages from animals and humans (*5*, *10*, *11*, *19*, *61*). In addition, SR-BI is expressed in atherosclerotic lesions in the apo E-deficient mouse (*5*) and in humans (*61*). Thus, the ability of SR-BI to enhance FC flux may impact on cholesterol removal from lesion macrophages as well as on FC uptake into hepatocytes at the terminal step of the reverse cholesterol transport pathway.

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