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Scavenger receptor BI (SR-BI) mediates free cholesterol flux independently of HDL tethering to the cell surface

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Abstract **In addition to its effect on high density lipoprotein (HDL) cholesteryl ester (CE) uptake, scavenger receptor BI (SR-BI) was recently reported to stimulate free cholesterol (FC) flux from Chinese hamster ovary (CHO) cells stably expressing mouse SR-BI, a novel function of SR-BI that may play a role in cholesterol removal from the vessel wall where the receptor can be found. It is possible that SR-BI stimulates flux simply by tethering acceptor HDL particles in close apposition to the cell surface thereby facilitating the movement of cholesterol between the plasma membrane and HDL. To test this, we used transiently transfected cells and compared the closely related class B scavenger receptors mouse SR-BI and rat CD36 for their ability to stimulate cholesterol efflux as both receptors bind HDL with high affinity. The results showed that, although acceptor binding to SR-BI may contribute to efflux to a modest extent, the major stimulation of FC efflux occurs independently of acceptor binding to cell surface receptors. Instead our data indicate that SR-BI mediates alterations to membrane FC domains which provoke enhanced bidirectional FC flux between cells and extracellular acceptors.**—de la Llera-Moya, M., G. H. Rothblat, M. A. Connelly, G. Kellner-Weibel, S. W. Sakr, M. C. Phillips, and D. L. Williams. **Scavenger receptor BI (SR-BI) mediates free cholesterol flux independently of HDL tethering to the cell surface.** ***J. Lipid Res.*** **1999.** 40: **575–580.**

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In contrast to the well-defined receptor pathway for uptake of cholesterol from low density lipoprotein (LDL) (1), the specific interactions between HDL and cell surface receptors have not been well documented. SR-BI, a class B scavenger receptor, is the first cell surface protein that has been convincingly characterized as an HDL receptor (2). SR-BI binds HDL with high affinity and has been shown to play an important role in cholesterol metabolism as it mediates the selective uptake of cholesteryl ester (CE) from HDL in transfected cells (2). SR-BI is the receptor responsible for the uptake of HDL-CE and its delivery to the steroidogenic pathway in adrenocortical cells (3). Furthermore, inactivation of the SR-BI gene in mice alters plasma HDL metabolism and reduces adrenal gland CE accumulation, results consistent with a major role for SR-BI in cholesterol metabolism in vivo (4).

In addition to its effect on HDL-CE uptake, we recently reported that SR-BI stimulates free cholesterol (FC) flux from Chinese hamster ovary (CHO) cells stably expressing mouse SR-BI (5, 6), a novel function of SR-BI that may play an important role in cholesterol removal from the vessel wall where the receptor can be found (5). We now report experiments designed to investigate the mechanism by which SR-BI stimulates cellular FC efflux and influx. It is possible that SR-BI stimulates flux simply by tethering HDL particles in close proximity to the cell surface thereby facilitating the movement of cholesterol between the plasma membrane and HDL. To test this, we used transiently transfected cells and compared the closely related class B scavenger receptors SR-BI and CD36 for their ability to stimulate cholesterol efflux as both receptors have about 30% homology, bind HDL with high affinity (2, 7), and can be easily expressed in COS cells where they have similar K_d values (10–20 μg/ml) for HDL (8, 9). These studies demonstrate that tethering HDL particles to the cell surface is not sufficient to account for the stimulation of cholesterol efflux from cells expressing SR-BI. Instead, our data indicate that SR-BI mediates alterations to membrane cholesterol domains which provoke enhanced bi-directional cholesterol flux between cells and extracellular acceptors.

Abbreviations: HDL, high density lipoproteins; CE, cholesteryl ester; SR-BI, scavenger receptor class B type I; FC, free cholesterol; CHO, Chinese hamster ovary cells; LDL, low density lipoproteins; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PS, phosphatidylserine; PBS, phosphate-buffered saline; FBS, fetal bovine serum; BSA, bovine serum albumin; ACAT, acyl CoA-cholesterol acyl transferase; SUV, small unilamellar vesicles.

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MATERIALS AND METHODS

Materials

Human serum was obtained from healthy, normolipemic volunteers. HDL_3 ($1.125 < \rho < 1.21$ gm/ml) was isolated from human serum by sequential centrifugation (10). Human apoA-I was obtained from isolated human HDL as previously described (11). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) and phosphatidylserine (PS) were purchased from Avanti Polar Lipids Inc. and used to prepare small unilamellar vesicles (SUV) by sonication as previously described (6). Similar procedures were used to make SUV that contained both phosphatidylserine (PS) and POPC (50% mol/mol) (12). [1,2-^{3}H]cholesterol and [^{125}I]iodine were purchased from NEN Life Science Products. Phosphate-buffered saline (PBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Bio-Whittaker. Fetal bovine serum (FBS), enzymes, and antibiotics for cell culture were obtained from Sigma. Fatty acid-poor bovine serum albumin (BSA) was obtained from Intergen. Compound 58-035 was a gift from Sandoz. Tissue culture flasks were obtained from Corning Glass Works. All filtration products were obtained from Millipore. Other materials and reagents were obtained as noted.

Routine maintenance and transient transfection of COS-7 cells

COS-7 cells were maintained in DMEM supplemented with 10% FBS, 1 mm sodium pyruvate, and antibiotics in T-75 flasks and were sub-cultured 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 95% air/5% CO_2 incubator. Cells were transfected with a mixture of 10 μg of the desired plasmid diluted in serum-free DMEM, and 30 μl of Fugene-6 (Boehringer-Manheim) prepared in a sterile polystyrene tube (Falcon #2058). This mixture was added drop-wise to the plated cells. After incubation (18–24 h, 37°C), transfected cells were trypsinized, pooled, suspended in growth medium, and re-plated in multiwell plates as needed for experiments. In general, one 100-mm plate yields sufficient cells for one 12-well plate. For efflux experiments, transfected cells were re-plated in labeling medium (i.e., medium containing [^{3}H]cholesterol as described below) and incubated at least another 24 h. Experiments were typically carried out 48 h after transfection.

Plasmids were prepared as follows. The mouse SR-BI (mSR-BI) coding region was amplified from pSR-BI#77 (obtained from M. Krieger, M.I.T.) with the following primer pair: 5′-GACCGAATT CCAATTGCCGTCTCCTTCAGGTCCTGAGC-3′ and 5′-GACCG GATCCAGATCTGCGGACAGGTGTGACATCTGG-3′. The resulting PCR product was restricted with Mfe I and Bgl II and ligated into an EcoRI and Bgl II restricted pSG5 vector (Stratagene, Inc.) to produce pSG5(mSR-BI). The expression plasmid for rat (r) CD-36, pSG5(rCD36), which contains the rat CD36 coding region, was previously described (13). Plasmids were prepared using Endotoxin-free Qiagen Maxi-prep kits and sequenced throughout the SR-BI coding region to confirm the correct fragment insertion and ensure that no point mutations had been generated during the amplification process. DNA sequencing was performed by the automated sequencing facility at SUNY Stony Brook. Reactions were prepared using a dye termination cycle sequencing kit and analyzed on an Applied Biosystems Model 373 DNA Sequencer with an Excel Upgrade as recommended by the manufacturer (PE Applied Biosystems).

Assay of cellular cholesterol efflux and influx

Cell cholesterol efflux was assayed using a modification of a published method (14). Briefly, the release of radioactive cholesterol to a given acceptor was measured by scintillation counting of filtered aliquots of acceptor-containing medium and expressed as the fraction of the total isopropanol-soluble label in the cells (cell lipids) plus the label released to the medium. All efflux assays were done in triplicate. Transfected COS-7 cell monolayers were pre-labeled by incubation for 18–24 h at 37°C in a CO_2 incubator with DMEM supplemented with 5% FBS radiolabeled with [^{3}H]cholesterol and an acyl CoA:cholesterol acyltransferase (ACAT) inhibitor (58-035, 1 μg/ml) as previously described (15). In preliminary experiments it was determined that, in the presence of 58-035, over 98% of cellular cholesterol is found as free cholesterol (FC). Prior to efflux measurement, the cell monolayers were gently washed twice in DMEM supplemented with 1% BSA and once with DMEM. Acceptors were then added to the medium and efflux was monitored as described. In addition, in all experiments, fractional efflux was corrected for the small amount of radioactivity released to DMEM without an acceptor present. FC influx was assayed using replicate wells of transfected cells prepared exactly the same but exposed to "labeling medium" without any isotope. HDL was labeled with radioactive FC by exchanging [^{3}H]cholesterol (50–75 μCi) from glass fiber filters onto which the radioactive cholesterol had been dried under N_2. Influx of HDL-FC was measured by incubating unlabeled transfected cells with radiolabeled HDL and was calculated as the fractional appearance of the HDL- FC radioactivity in cell lipids.

Iodination of lipoproteins and apoproteins

Proteins were labeled using the iodine monochloride method as described by Bilheimer, Eisenberg, and Levy (16). Briefly 3 mg of protein was labeled using approximately 1 mCi carrier-free Na-[^{125}I] (DuPont NEN). The mixture was then passed through a 10-ml Sephadex G-25 column (Pharmacia PD-10) equilibrated with saline–EDTA buffer and the appropriate fractions were sampled for gamma counting and Lowry protein assay to determine specific activities.

Assay of specific binding

Transfected cells, plated in 6-well plates, were rinsed in serum-free DMEM chilled to 4°C. Ligand was diluted in chilled serum-free DMEM supplemented with 0.5% BSA and was incubated with the cells for 1.5 h at 4°C to measure total binding. Total binding was measured at ligand concentrations close to the published K_d values for SR-BI (25 μg/ml for HDL and 20 μg/ml for apoA-I). Non-specific binding was measured in duplicate incubations that contained a 20-fold excess of unlabeled ligand. Both total and non-specific binding were measured in triplicate. After incubation the cells were washed 4× with PBS supplemented with 0.1% BSA and 1× with PBS and lysed with 0.1 N NaOH at room temperature. Radioactivity in cell lysates was measured by gamma counting after which aliquots were taken for protein determination. Specific binding was calculated by subtraction of non-specific from total binding and the mass of ligand bound was obtained from the specific activity determined for each ligand.

Cholesterol oxidase treatment

Cholesterol oxidase treatment was essentially as described by Slotte and Bierman (17). Transfected cells were plated in 12-well plates and labeled with [^{3}H]cholesterol (3 μCi/well) as for efflux assays but without compound 58-035. Labeled cells were chilled on ice, washed twice with ice-cold PBS, and fixed with 1% glutaraldehyde for 10 min on ice. Cells were then washed twice with ice-cold PBS. Cholesterol oxidase (1 U/ml streptomyces enzyme, Sigma) was added and cells were incubated 30 min at 37°C. Lipids were extracted with isopropanol and radioactive cholesterol and cholestanone were separated using thin-layer chromatog-

raphy (hexane: 96 ml, methanol: 15 ml, ethyl ether: 8 ml) and quantified.

Statistical analysis

Results are presented as means and standard deviations of at least triplicate determinations. Significant differences were established by unpaired *t*-tests using Graph-Pad Prism. Rate constants were estimated using non-linear regression analysis following the model described by Johnson et al. (18).

RESULTS

SR-BI-mediated FC efflux

Expression of SR-BI in transiently transfected COS-7 cells stimulated efflux of cellular FC to human HDL_3 (**Fig. 1**) when compared to transfection with vector DNA, confirming our earlier result obtained with stably transfected CHO cells. In addition, expression of SR-BI in COS cells stimulated efflux of FC to other phospholipid-containing FC acceptors such as diluted human serum (data not shown) and POPC-SUV (Fig. 1). The stimulation of efflux to POPC-SUV suggests that receptor-dependent stimulation of efflux may not be due to direct receptor–acceptor interactions because SR-BI has been reported not to bind neutral phospholipids significantly (12, 19). We have examined the role of acceptor–receptor interaction further in two ways.

First, Fig. 1 shows that FC efflux from SR-BI-expressing COS-7 cells, both to HDL_3 (Fig. 1A) and POPC-SUV (Fig. 1B), was stimulated over a large range of acceptor concentrations. The concentration-dependence curve of efflux from SR-BI-expressing cells to HDL fits best to a biphasic function showing half-maximal efflux rates at both a low (30 ± 8 μg/ml) and a high (1131 ± 630 μg/ml) concentration of HDL protein. The half-maximal value of 30 μg/ml is similar to the reported K_d for HDL binding to SR-BI (2) suggesting that this component of FC efflux may be linked to SR-BI acceptor binding. However, this would not appear to be the case for the low efficiency component showing half-maximal efflux at 1130 μg/ml. Interestingly, the curve fitting the efflux-dependence for POPC-SUV showed only a single component with a half-maximal efflux rate occurring at a concentration of 224 ± 28 μg/ml POPC (Fig. 1B). Note that SR-BI-expressing cells showed marked FC efflux to POPC-SUV in the range of POPC-SUV concentrations (0–150 μg/ml) where binding of neutral POPC-SUV to SR-BI has not been detected (19). This result suggests that SR-BI-mediated stimulation of FC efflux to POPC-SUV does not involve binding of acceptor vesicles to SR-BI.

Second, to further examine the relationship between acceptor binding to SR-BI and FC efflux, we examined FC efflux to POPC-SUV (400 μg/ml) in comparison to POPC:PS-SUV (400 μg/ml). Published studies have demonstrated that, in contrast to neutral SUV, negatively charged vesicles bind well to SR-BI (12, 19). Therefore, if vesicle binding to SR-BI is important for the SR-BI-mediated stimulation of FC efflux, FC efflux to the negatively charged vesicles should occur much more readily than to the neutral vesicles. The results showed that SR-BI expression increased FC efflux to both types of vesicles to a similar extent (POPC-SUV, control cells = $5.0 \pm 0.1\%$/4 h, SR-BI cells = $23.5 \pm 0.5\%$; POPC:PS-SUV, control cells = $6.2 \pm 0.2\%$, SR-BI cells = $18.7 \pm 0.2\%$). These results provide strong evidence that acceptor binding to SR-BI is not necessary for SR-BI-mediated FC efflux.

As cholesterol flux has been described as a bi-directional process, we used HDL labeled with [^{3}H]cholesterol to monitor the influx of HDL-FC into control and SR-BI-expressing COS cells. **Figure 2** shows the results of this experiment, and indicates that SR-BI promotes the overall rate of both efflux (Fig. 2A) and influx (Fig. 2B), as both efflux and influx rates (Fig. 2C) were stimulated.

Relationship between acceptor binding and efflux

To better understand the mechanism by which SR-BI stimulates cholesterol efflux from COS-7 cells, we compared SR-BI to CD36, a closely related class B scavenger receptor (20) which binds HDL with a similar high affinity (8, 9). In these experiments both cholesterol efflux from

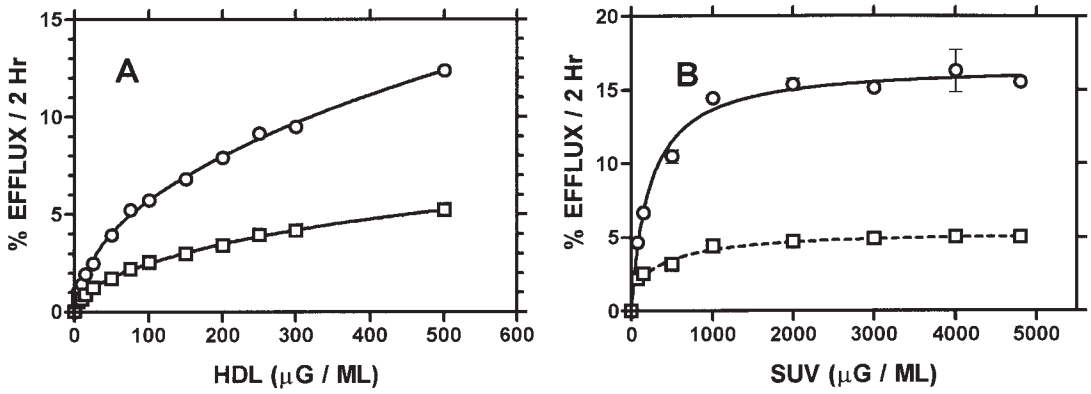

Fig. 1. Dependence of FC efflux on acceptor concentration. SR-B1 and vector-transfected COS-7 cells were prepared as described. Cells were exposed to increasing concentrations of either human HDL or POPC-SUV. After a 2-h incubation, efflux was assayed as described. The curves were obtained from non-linear regression fits of either a two-site or one-site hyperbolic function using Graph-Pad Prism Software. Open circles, SR-BI cells; open squares, control (vector-transfected) cells.

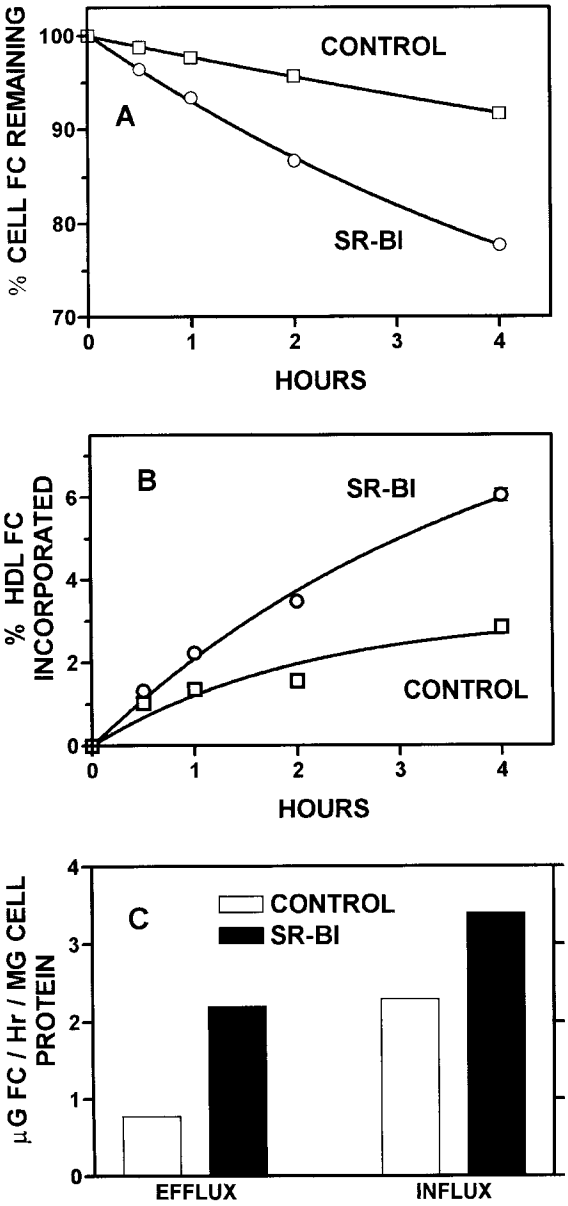

Fig. 2. Time-course and kinetics of bi-directional FC flux. SR-BI and vector-transfected COS-7 cells were prepared as described. The time course of [^{3}H]cholesterol release from cells to HDL (250 µg protein/ml) or its uptake from pre-labeled HDL (250 µg protein/ml) was followed. At each time-point individual cell monolayers were harvested and fractional efflux of cell-FC or uptake of HDL-FC was quantified as described in Methods. The curves shown in panels A and B were analyzed using non-linear regression fits to mono-exponential rate equations (18) to obtain estimates of the kinetic constants. Kinetic constants were normalized to cell protein values (panel C).

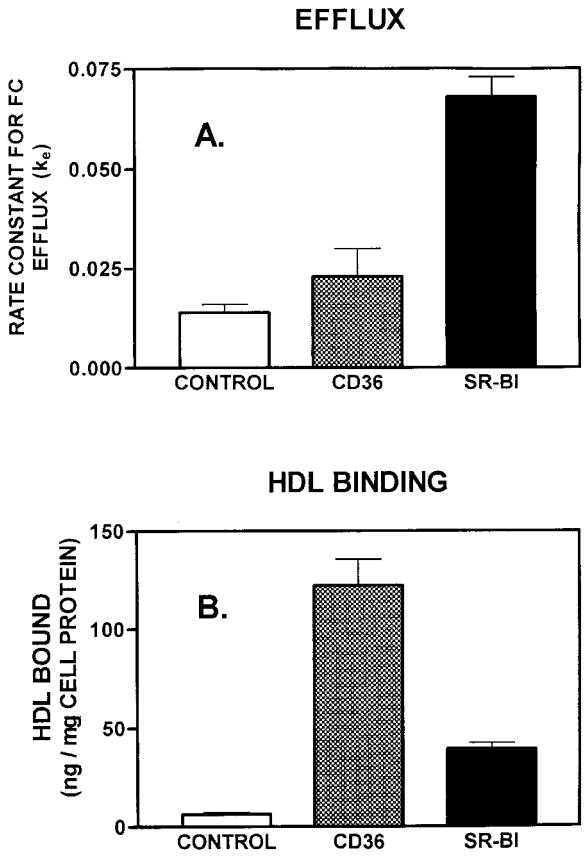

Fig. 3. Relationship between lipoprotein binding and FC efflux. Transfected COS-7 cells expressing SR-BI, CD36, or vector DNA were prepared as described. Efflux of cellular [^{3}H]cholesterol to human HDL (25 µg protein/ml) was measured as in Fig. 2. As described in Methods, total binding at 4°C was obtained at the same lipoprotein concentrations used for efflux in parallel cell cultures. Specific binding was measured as the difference between total and non-specific binding (plus 20-fold excess unlabeled ligand). The values for efflux are the average from four experiments (Fig. 3A). The values for binding (Fig. 3B) are from a single representative experiment as there was a large variation in binding between individual experiments (control, 6–15 ng HDL/mg cell protein; SR-BI, 27–88; CD36, 25–549).

COS-7 cells to HDL and specific lipoprotein binding were measured in parallel cell cultures at a lipoprotein concentration close to the published value for high-affinity binding to SR-BI and CD36 (2, 8, 9). Results for both binding and efflux assays from a representative experiment are shown in **Fig. 3**. SR-BI expression increased the first order rate constant for FC efflux approximately 500% (Fig. 3A). In contrast, CD36 expression increased the rate constant for FC by only 60% (Fig. 3A), despite the fact that, in each of these experiments, expression of CD36 increased HDL binding as much as or more than SR-BI (Fig. 3B). These results show that the efficient binding of HDL_3 to CD36 was not sufficient to produce a large stimulation of cellular FC efflux from cells expressing this receptor (compare Figs. 3A and 3B).

A number of cell types have been shown to release FC to lipid-free apolipoproteins (21–24). Therefore, to investigate the contribution of lipid-free apolipoprotein binding in the SR-BI-dependent stimulation of FC efflux, we measured both FC efflux and specific binding of lipid-free ^{125}I-labeled apoA-I (20 µg/ml) in transfected COS cells. Although apoA-I binding was increased in SR-BI transfected cells (control = 42.6 ± 11.2 ng/mg cell protein; SR-BI = 84.4 ± 17.6 ng/mg cell protein, n = 6, P = 0.001) there was no stimulation of cholesterol efflux to this apolipoprotein (control = 0.26 ± 0.13 %/2 h; SR-BI = 0.20 ±

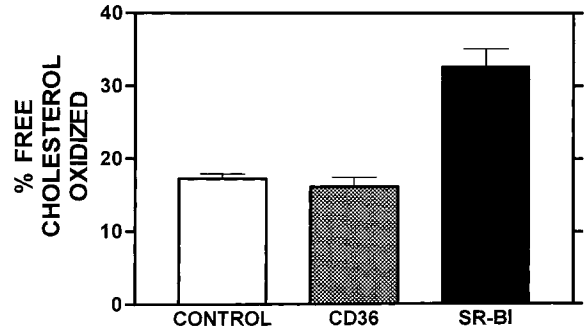

Fig. 4. SR-BI-dependent increase in susceptibility to cholesterol oxidase. Transfected cells expressing SR-BI, CD36, or vector DNA were prepared as described. Cells pre-labeled with [^{3}H]cholesterol were fixed and treated with cholesterol oxidase as described in Methods. Radioactive cholestanone and cholesterol were separated by thin-layer chromatography and each species was quantified by scintillation counting. Results are presented as mean and standard deviation with n = 12 and represent the % of the cellular radiolabeled free cholesterol converted to cholestanone.

0.12 %/2 h, n = 6). In the same experiment, SR-BI expression stimulated FC efflux to HDL as described above (data not shown).

SR-BI modified membrane cholesterol pools

As the experimental results described above clearly demonstrated that acceptor-binding to a class B scavenger receptor is not sufficient to stimulate efflux, experiments were done to investigate the possibility that SR-BI expression affected other parameters that may lead to stimulation of FC release from cells, such as the accessibility of membrane cholesterol to an external acceptor. To do this we took advantage of cholesterol oxidase treatment, which has been shown to monitor changes in the distribution of cholesterol among membrane domains (25). As shown in **Fig. 4**, expression of SR-BI significantly increased the amount of cellular cholesterol that could be oxidized, indicating that SR-BI expression increased the availability of cholesterol to the oxidase.

DISCUSSION

These experiments demonstrate that the SR-BI-dependent stimulation of cholesterol flux is not simply due to the fact that SR-BI binds to HDL and tethers it in close proximity to the cell surface, thus facilitating the exchange of lipids between HDL and the plasma membrane. One line of experimental evidence is that expression of CD36, a class B scavenger receptor homologous to SR-BI, had only a modest effect (60% increase) on the rate of cholesterol release from transiently transfected COS-7 cells. This modest effect of CD36 may represent the extent to which binding HDL to the cell surface can facilitate efflux whereas the far greater efflux promoted by SR-BI suggests that this receptor has other effects in addition to the cell surface binding of HDL. Three other findings support this conclusion. First, in the various transient transfection experiments with SR-BI, the rate of FC efflux was remarkably similar from experiment to experiment (range k_e = 0.062–0.073 fractions/h) despite the fact that the increases in HDL binding varied greatly (range 27–88 ng HDL /mg cell protein). Second, SR-BI markedly stimulated FC efflux to POPC-SUV over a wide concentration range that includes concentrations at which POPC-SUV binding to SR-BI has not been detected in other studies (13, 20). Third, we saw no significant difference in SR-BI-mediated FC efflux to neutral POPC-SUV which do not bind to SR-BI and negatively charged POPC:PS-SUV which do bind. These data indicate that, although high affinity binding of an acceptor such as HDL to SR-BI may contribute to efflux to some extent, a major stimulation of FC efflux occurs independently of direct SR-BI acceptor interactions.

Interestingly, FC efflux to lipid-free apoA-I was not stimulated by SR-BI expression despite the fact that SR-BI enhances apoA-I binding. This result has two implications. First, it indicates that the efflux of FC to lipid-poor apolipoproteins is not an SR-BI-mediated event. Second, it provides further evidence that the phospholipid content of cholesterol acceptors plays an important role in cholesterol efflux from cells expressing SR-BI (6, 26). Whether phospholipids passively provide a surface to absorb cholesterol desorbed from the plasma membrane or play a regulatory role remains to be resolved.

If tethering of HDL, or other cholesterol acceptors, in close apposition to the cell surface does not play a major role in the stimulation of cholesterol efflux caused by expression of SR-BI, then other mechanisms must be responsible for this effect. One possibility is that SR-BI creates a favorable hydrophobic environment or membrane domain that efficiently promotes the bidirectional movement of cholesterol; this effect is likely responsible for the selective uptake of CE from HDL molecules bound with high affinity to SR-BI (5, 6). As there is experimental evidence that some SR-BI localizes to caveolae (27), another possibility is that insertion of SR-BI in the plasma membrane causes a generalized redistribution of membrane cholesterol to caveolar domains. The enhanced sensitivity of plasma membrane cholesterol to cholesterol oxidase due to SR-BI expression (Fig. 4) may be a reflection of such a redistribution. It is possible that redistribution of membrane cholesterol plays an important role in the mechanism of SR-BI-stimulated cholesterol release and represents a novel mechanism for modulating membrane cholesterol movement.

In conclusion, our results demonstrate that SR-BI expression plays an important role in FC flux and can promote FC flux by a mechanism where cell surface binding of the acceptor is not the major component. Rather, our results point to a more subtle effect of SR-BI involving significant shifts in the distribution of plasma membrane FC pools. These effects of SR-BI may play important roles in both the efflux of FC from vascular cells, the first step in reverse cholesterol transport, and the influx of FC from HDL to hepatocytes, the last step of this process.

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