SR-BI Undergoes Cholesterol-stimulated Transcytosis to the Bile Canaliculus in Polarized WIF-B Cells*1

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The scavenger receptor BI (SR-BI) is highly expressed in hepatocytes, where it mediates the uptake of lipoprotein cholesterol, promotes the secretion of cholesterol into bile, and protects against atherosclerosis. Despite a strong correlation between the hepatic expression of SR-BI and biliary cholesterol secretion, little is known about SR-BI trafficking in response to changes in sterol availability. Using a well characterized polarized hepatocyte cell model, WIF-B, we determine that in cholesterol-depleted cells, SR-BI is extensively located on the basolateral surface, where it can access circulating lipoproteins. However, in response to cholesterol loading, SR-BI undergoes a slow transcytosis to the apical bile canaliculus independently of lipoprotein binding and new protein synthesis. In cholesterol-replete WIF-B cells, SR-BI that resides on the canalicular membrane is dynamically associated with defined microdomains and does not rapidly recycle to and from the subapical or basolateral regions. Taken together, these data demonstrate that hepatic SR-BI transcytosis is regulated by cholesterol and suggest that SR-BI has a stationary function on the bile canaliculus.

High density lipoproteins (HDL)2 have a functional role in the protection against cardiovascular disease. HDL mediates both cholesterol removal from lipid-laden macrophages and delivery to the liver for subsequent biliary secretion in a process termed “macrophage reverse cholesterol transport.” The cholesterol removal is mediated, in part, by the well established HDL receptor, scavenger receptor class BI (SR-BI) (1) (reviewed in Ref. 2). SR-BI is a two-transmembrane domain cell surface glycoprotein with short intracellular N- and C-terminal domains (3). The receptor is ubiquitously expressed at low levels and is highly expressed in steroidogenic, intestinal, and hepatic tissues (1). SR-BI binds a large array of ligands, including HDL and native or modified low density lipoproteins (LDL).

Hepatic SR-BI protects against atherosclerosis by promoting the final stages of macrophage reverse cholesterol transport (5). In contrast to the holo-particle uptake of the LDL receptor pathway, SR-BI mediates cholesterol, cholesteryl ester, and phospholipid uptake via a selective pathway whereby lipids are transferred down their concentration gradient through a hydrophobic channel into the membrane (6). Importantly, if the concentration gradient is reversed, SR-BI can also perform cholesterol efflux (7).

Although lipid delivery to and from lipoprotein particles occurs mainly on the plasma membrane, both SR-BI and HDL have been shown to internalize into and recycle from endocytic compartments (8–10). SR-BI also localizes on the apical domain in gall bladder epithelial cells (11), testicular Sertoli cells (12), isolated primary mouse hepatocytes (13), primary mouse hepatocyte couplets (8), and hepatic tissues sections (14) and has been shown to undergo regulated transcytotic movement in polarized Madin-Darby canine kidney cells (15).

Our laboratory has recently demonstrated that in primary mouse hepatocytes, SR-BI achieves efficient selective uptake of cholesteryl esters in the absence of endocytosis (16). These studies, confirmed by others (17–19), indicate that endocytosis and recycling are not required for selective uptake by SR-BI. Thus, the functional implications of the hepatic trafficking of SR-BI remain unclear. Given its polarized distribution, there has been speculation that SR-BI may mediate selective sorting and secretion of HDL-derived cholesterol into the bile. This is an attractive hypothesis, given that both gene deletion (20) and overexpression (14) of SR-BI reciprocally affect in vivo biliary cholesterol secretion. However, there is currently no link between the basolateral selective uptake function of SR-BI in polarized hepatocytes and the in vivo data demonstrating that hepatocyte SR-BI expression correlates with biliary cholesterol secretion. Instead, the ATP binding cassette (ABC) half-transporters ABCG5 and ABCG8, which localize exclusively to the bile canalculus, have been shown to be rate-limiting for cholesterol secretion into bile (21). However, ABCG5/G8 expression does not always correlate with biliary cholesterol secretion, indicating that other cellular processes may be involved (22). The above data suggest that SR-BI might participate with ABCG5/G8 at the bile canalculus in the regulation of cholesterol secretion.

Therefore, to gain further insight into the hepatic function and trafficking of SR-BI, we studied the movement of SR-BI-YFP in a well characterized polarized hepatocyte cell model.
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(WIF-B) (23). WIF-B cells grow in monolayers, mimic functional hepatic cells, and form bile canalicularkike spaces (BC) between adjacent cells (23). Each BC is spatially restricted by tight junctions, rendering it inaccessible to surrounding media (24). With this system, we demonstrate that SR-BI undergoes transcytosis to the BC in response to cellular cholesterol availability, thereby providing new insights into the atheroprotective function of this lipoprotein receptor.

EXPERIMENTAL PROCEDURES

Cells and Reagents—WIF-B cells were a kind gift from Dr. Ann Hubbard (The Johns Hopkins University, Baltimore, MD). Cytochrome, 2-deoxyglucose, NaN₃, filipin, and holotransferrin were from Sigma. MβCD was a gift from Cerestar Inc. Antibodies used were anti-YFP (JL-8) (BD Biosciences), anti-MDR1 (NB 400-104 and NB400-134; Novus Biologicals), monoclonal anti-MDR1 (C219; Signet Laboratories).

Cloning—Human SR-BI (Cla-1 (CD-36 LIMPII-analogous-1)) was cloned and found to be consistent with published GenBank™ sequence gi:33620766. For SR-BI-YFP adenovirus (Ad), SR-BI-YFP was subcloned into pShuttle-CMV and recombined with the pAd-Easy1. The Ad was then generated, scaled up, and purified (Q-biogene). SR-BI-YFP adenovirus (6.7 × 10¹⁰ plaque-forming units/ml) was added at a multiplicity of infection of 50 (~9.7 × 10⁶ plaque-forming units/well) and incubated for 16–20 h.

Lipoprotein Analysis—FBS serum components and human lipoproteins (from a single normolipemic donor) were isolated by sequential density ultracentrifugation and labeled for biochemical analysis as previously reported (16).

Fluorescence Microscopy—For analysis of the subcellular distribution of SR-BI-YFP, WIF-B cells were seeded on 35-mm dishes with attached glass coverslips (Fisher) and infected with SR-BI-YFP. The coverslips were then mounted in a temperature-controlled chamber (37 °C) in regular growth media supplemented with 20 mM HEPES (pH 7.4). The cells were visualized with an Olympus ×100 oil immersion objective, numerical aperture 1.4, at 1 Airy unit on an Olympus IX80 laser-scanning confocal microscope operated by FV1000 software, version 1.4a. The YFP was excited with the 488- or 514-nm line of a multiple-line argon laser, the rhodamine red-X and Cy3 transferrin were excited with the 543-nm line of a helium/neon green laser, the Alexa 647 was excited with the 633-nm line of helium/neon red laser, and the filipin was excited with a 405-nm laser. For experiments with filipin, cells were washed, fixed, and quenched as described above. Fixed cells were then incubated with 5 mg/ml filipin in PBS for 1.5 h in the dark, rinsed, and mounted with Mowial. All images shown demonstrate cells that are representative of moderate infection efficiencies and that have been obtained from at least two independent experiments.

Immunofluorescence—Differentiated WIF-B cells were washed twice quickly with PBS and incubated with 200 µl of 3.3% paraformaldehyde for 10 min. The paraformaldehyde was removed, and the cells were rinsed once with phosphate-buffered saline (PBS) and then quenched for 30 min with 50 mM NH₄Cl. The NH₄Cl was removed, and the cells were permeabilized with 1 ml of ice-cold acetone or methanol for 10 s. The cells were rinsed and blocked for 1 h with 1% BSA, PBS. The primary antibody (1:200 for SR-BI, 1:50 for MDR1) in 1% BSA, 1% horse serum, PBS was incubated for 1 h. The cells were washed three times for 15 min each with 1% BSA, 0.1% Tween 20, PBS. The secondary antibodies (rhodamine red-X goat anti-mouse (MDR1; 1:200), Alexa 647 goat anti-mouse (MDR1; 1:100), Alexa 647 goat anti-rabbit (SR-BI; 1:200), and Alexa 488 goat anti-rabbit (SR-BI; 1:200) were incubated in 1% BSA, 1% horse serum, PBS for 1 h. The cells were washed three times for 15 min each with 1% BSA, 0.2% Tween 20, PBS, rinsed with distilled water, and mounted with Mowial.

Transcytosis Experiments—The BC localization of SR-BI-YFP was determined in 20–60 BC in each of three coverslips for each condition using confocal laser-scanning microscopy. The canalicular localization of SR-BI-YFP was determined by estimating the percentage of the BC surface area where SR-BI-YFP and MDR1 colocalized. This was achieved by individually analyzing each BC through multiple z planes. The observations were made using a laser power between 0.1 and 3% without saturating cellular fluorescence. Each BC was scored as having <20% colocalization, 20–80% colocalization, or >80% colocalization with MDR1. The ranges were chosen to minimize subjectivity and emphasize the shifts in localization observed. Although the counting method does not consider the quantity of SR-BI-YFP undergoing translocation, the amounts of SR-BI-YFP fluorescence on the BC paralleled the percentage of SR-BI/MDR1 colocalization.

MβCD Cholesterol Loading Transcytosis Experiment—After the percentage of BC with >80% SR-BI/MDR1 colocalization was determined for each time point as described above, the data were fitted to a Boltzmann sigmoidal curve. For determining the rate of transcytosis, the 60 min time point was considered to be the start of transcytosis, and the data were fitted to a hyperbolic equation.

Antibody Transcytosis Experiment—We followed a previously documented antibody transcytosis experiment protocol (25) using a 1:40 dilution of anti-SR-BI (NB 400-134 blocking antibody).

Fluorescence Recovery after Photobleaching (FRAP) and Selective Photobleaching—We used standard equations for normalizing FRAP and selective photobleaching (26). The specified areas were bleached with the 514-nm line of the multiple argon laser at 30–50% laser power in no more than 10 s. Fluorescence intensities from the areas of interest (background, bleached area, and total cellular fluorescence) were calculated by the FV1000 software and were exported to Microsoft Excel to calculate normalized fluorescence. Images were graphed in Graphpad Prism 4.0 software.

Cy3-Transferrin Labeling for FRAP—Iron-loaded holotransferrin was first purified by high pressure liquid chromatography on a Sephacryl S-200 column (BD Biosciences) and labeled by monoreactive Cy3 dye as described by Harder et al. (16). For FRAP studies, cells were preincubated with 20 µg/ml Cy3-transferrin for at least 30 min prior to photobleaching to ensure the endocytic recycling compartment was saturated. Cells were then photobleached and imaged for fluorescence recovery to that region with 20 µg/ml fresh Cy3-transferrin in the media.
Energy Depletion Experiments—Cells were preincubated with energy depletion media (50 mM 2-deoxyglucose and 5 mM NaN$_3$) at 37 °C for 30 min to ensure complete inhibition of endocytosis as described by Harder et al. (16). Cells were then incubated with fresh energy depletion media and analyzed for FRAP for up to 2 h.

Immunoelectron Microscopy (EM)—Immunogold labeling of SR-BI-YFP was performed on WIF-B cells infected overnight with the SR-BI-YFP adenovirus. 2 × 10 cm plates of confluent cells were washed with PBS and carefully scraped into 10 ml of PBS. The cells were then removed with a large disposable plastic transfer pipette (to avoid shearing the sheets of cells) and centrifuged at 300 G for 3 min to pellet the cells. The supernatant was removed, and ~5 ml of a 0.5% glutaraldehyde, 4% paraformaldehyde (pH 7.2–7.4) mix was added to the 500-µl fluffy white pellet. The solution was mixed and left for 2 h at
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**FIGURE 2.** 15% FBS treatment enhances the localization of SR-BI to the BC independently of changes in protein expression. A and B, WIF-B cells expressing SR-BI-YFP were incubated with either 0% FBS or 15% FBS overnight. BC are denoted by white asterisks. Bars, 20 μm. C, lysates from cells incubated with 0, 5, or 15% FBS were separated by 8% SDS-PAGE and analyzed by Western blot analysis with antibodies against endogenous SR-BI and SR-BI-YFP that do not cross-react. D, WIF-B cells infected with SR-BI-YFP Ad were serum-starved for 20 h, preincubated with 300 μM cycloheximide for 1 h, and incubated with either 0% FBS media or 15% FBS media with cycloheximide for 4 h. Cells were then immunostained with anti-MDR1/rhodamine red-X, and the BC colocalization with SR-BI was determined. n = 3 (50 BC each), S.E. *, p = 0.001.

WIF-B cells showed characteristic basolateral (BL) distribution indicating proper cell surface expression (Fig. 1C, white arrows). Cells mock-infected with green fluorescent protein adenovirus further confirm the specificity of the SR-BI-YFP distribution (supplemental Fig. 1, A–C). In ~20% of cells, we also noted colocalization of SR-BI-YFP with the BC marker multidrug-resistant protein 1 (MDR1 or P-glycoprotein) (27) (Fig. 1D). In undifferentiated cells, MDR1 localized exclusively to the BL surface. However, when cells were differentiated, MDR1 was restricted to the BC (supplemental Fig. 1, D and E). In these studies, the exclusion of MDR1 from the BL surface was used as a marker to confirm that WIF-B cells were fully differentiated. SR-BI-YFP also localized on the microvilli of the BL membrane and on the bile canaliculus by EM (Fig. 1, F and H, respectively). By EM, the BC contained numerous microvilli and were identifiable by flanking electron dense tight junctions (supplemental Fig. 1F, black arrows).

Both the quantity of SR-BI-YFP on the BC and the number of BC displaying SR-BI-YFP and MDR1 colocalization varied greatly among individual cells (e.g. see Fig. 1, C versus D). Our preliminary studies indicated that the extent to which SR-BI-YFP localized to the BC was related to the amount of FBS available to the cells. To investigate this further, we incubated cells with 0 or 15% FBS for 20 h. Whereas SR-BI-YFP did not localize to the BC in the absence of FBS (Fig. 2A, white asterisks), SR-BI-YFP displayed extensive localization to the BC in the 15% FBS-treated cells (Fig. 2B, white asterisks). To determine whether the observed SR-BI translocation required new protein synthesis, we examined the protein levels of SR-BI (Fig. 2C) and performed a cycloheximide chase experiment (Fig. 2D). With cycloheximide, 58.2 ± 8% of observed BC in the 15% FBS-treated cells displayed greater than 80% colocalization of SR-BI-YFP and MDR1 compared with 7.1 ± 2.6% colocalization in the 0% FBS-treated cells (Fig. 2D). No changes were observed in the protein expression of either endogenous or tagged SR-BI, indicating that translocation of SR-BI to the BC does not require new protein synthesis.

We next confirmed that translocation of endogenous SR-BI also occurs in FBS-loaded cells (Fig. 3). Similar to SR-BI-YFP, we see extensive basolateral labeling of endogenous SR-BI in WIF-B cells that were serum-starved for 20 h (Fig. 3A), whereas...
after a 5-h 15% FBS incubation, there was extensive colocalization of SR-BI and MDR1 on the BC (Fig. 3B). For these experiments, the secondary antibody alone indicated that the antibodies were specific and that there was little background fluorescence (Fig. 3C).

Having determined that the addition of FBS promotes the translocation of SR-BI to the BC, we sought to identify which components of FBS were responsible for this effect. We found very little translocation of SR-BI-YFP with bovine lipoprotein-deficient serum or with the amount of LDL normally found in 15% FBS, whereas there was significant translocation with bovine HDL and human HDL (supplemental Fig. 2).

Since the small amounts of LDL in FBS (28) may not have been sufficient to stimulate transcytosis, we sought to determine whether LDL at higher concentrations could stimulate SR-BI translocation. We incubated WIF-B cells with either serum-free media or 15% FBS for 5 h (B). Cells were then fixed and immunostained with anti-SR-BI/Alexa 488 and anti-MDR1/Alexa 647. C, cells were also probed with both secondary antibodies alone. Endogenous SR-BI, similar to SR-BI-YFP, also undergoes translocation in response to serum loading in WIF-B cells. Bars, 10 μm.

Although our data suggested that it was the cholesterol status of the cell that caused translocation, we had not ruled out the possibility that lipoprotein binding was required for SR-BI translocation. Therefore, we determined whether we could also stimulate SR-BI-YFP translocation by MβCD cholesterol loading (29). We demonstrated that a 3-min incubation of 1 mM MβCD (1:8 cholesterol/MβCD) loaded sufficient amounts of cholesterol to cause SR-BI translocation to the same extent as that achieved with LDL loading at 5 h (Table 1). In addition, because the lipoprotein method of cholesterol loading occurs gradually over time, it was difficult to assess when sufficient cholesterol had accumulated to cause SR-BI translocation by stimulating translocation with MβCD cholesterol and by counting the percentage of BC with greater than 80% colocalization (SR-BI/MDR1), we were able to determine that SR-BI undergoes a delayed transcytosis in response to cholesterol loading (Fig. 5A). To determine the rate of transcytosis, the 60 min time point was considered to be the start of transcytosis, and the data were fitted to a single exponential function (30) (Fig. 5B). The rate constant for the translocation of SR-BI to the BC was 0.0202 ± 0.0068 min⁻¹, which corresponded to a halftime of 34.4 min.

To confirm that SR-BI was indeed moving from the basolateral to the apical membranes, we used an antibody against the extracellular domain of SR-BI to surface-label the basolateral extracellular domain of the receptor. Following a 30-min 4 °C incubation of intact differentiated WIF-B cells with the antibody, the medium was washed, and the cells were warmed to 37 °C for 1.25-, 2.5-, and 4-h chases with 165 μg/ml LDL loading. Cells were then fixed, permeabilized, blocked, and labeled with an Alexa 647 secondary antibody. After the 4 °C labeling, the antibody remained exclusively on the cell surface (Fig. 6A).
and did not have access to the BC membrane (Fig. 6B, white asterisks). At 1.25 h, the antibody signal had decreased substantially, and there were more intracellular puncta, but there was no localization of the antibody on the BC (data not shown).

Only after 2.5 h did we observe localization of both SR-BI-YFP and the antibody on some BC (Fig. 6C, i). This delayed translocation with LDL cholesterol loading paralleled the time course for translocation with MβCD cholesterol loading. The antibody (to mouse SR-BI) did not recognize the human SR-BI-YFP but recognized the endogenous SR-BI. Interestingly, both tags localized to the BC but remained partially segregated within the membrane. This observation is probably due to species-specific differences in SR-BI protein structure.

To gain further insight into how the gradient of cholesterol between the basolateral and apical membrane in cholesterol-

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**TABLE 1**

| Loading method | Free cholesterol content |
|----------------|--------------------------|
| 20 h 0% FBS    | 100 ± 8                  |
| 20 h 0% FBS, 4 h 165 µg/ml LDL | 125 ± 11              |
| 20 h 0% FBS, 1 mM MβCD for 3 min | 161 ± 7               |

* Cells were healthy and remained polarized.
loaded cells may modulate the transcytosis of SR-BI, we incubated both differentiated and undifferentiated WIF-B cells with or without 15% FBS and monitored cholesterol distribution with filipin (Fig. 7). We found that in undifferentiated cells treated with 15% FBS, there was bright filipin staining on the plasma membrane and bright staining on densely packed subapical vesicles (Fig. 7A). On the other hand, in similar cells treated with 0% FBS media, we observed filipin staining present in dispersed puncta throughout the cytoplasm and not brightly detected on the BL membrane (Fig. 7B). When specifically examining cholesterol loading in polarized cells, 15% FBS-treated cells had bright filipin staining on the BC, whereas the 0% FBS-treated cells did not (Fig. 7C versus D). To determine if SR-BI expression is responsible for this movement, we examined the filipin staining in cells with and without overexpression of SR-BI-YFP (Fig. 7E). On the basis of filipin staining, the overexpression of SR-BI does not appear to change the cholesterol distribution on the BC, suggesting that SR-BI transcytosis to the BC parallels or follows that of cholesterol.

Having demonstrated that SR-BI undergoes cholesterol-dependent stimulated transcytosis to the BC in polarized WIF-B cells, we attempted to gain insight into the function of SR-BI transcytosis by examining the kinetics of its transportation.

Since the receptor resides both within intracellular vesicle compartments and on the BC, we tested whether the intracellular pool is actively recycling (31) or remains stable (13) by performing selective photobleaching studies on the intracellular subapical pool or on the BC-localized SR-BI-YFP. Following the photobleaching of the complete subapical region of the cell, we tracked the fluorescence recovery of SR-BI-YFP over time (Fig. 8, A and C, green lines). Relative to the known recycling of the transferrin receptor that recovered to nearly 80% of prebleach fluorescence (Fig. 8, B and C, red lines), there was very little or no recovery of SR-BI-YFP from the BL membrane pool to the intracellular vesicles and BC membrane within the first 8 min of imaging.

To determine if SR-BI-YFP on the canalicular membrane rapidly equilibrates with subapical SR-BI-YFP, we specifically photobleached the BC membrane and examined the fluorescence recovery (Fig. 9, A and C, green lines). To distinguish between lateral diffusion on the BC and vesicular recovery of SR-BI, we repeated the experiment using energy depletion (28)
to inhibit vesicular trafficking (Fig. 9, B and C, red lines). Both control and energy depletion experiments displayed similar fluorescence recovery, indicating that there was very little BC-associated SR-BI-YFP turnover in the time course examined. These experiments indicate that after SR-BI-YFP is transcytosed to the BC in a cholesterol-loaded cell, it does not rapidly recover to either subapical vesicles or to the BC.

Finally, SR-BI-YFP fluorescence within the BC membrane appeared to form rather punctate lateral enrichments, within a single confocal plane. These “microdomains,” also seen colocalizing with MDR-1 in supplemental Fig. 2A, often appear to be the only sites of SR-BI localization on the BC. To further examine the nature of the canalicular SR-BI-YFP microdomains, we performed FRAP studies on individual microdomain segments of the canalicular membrane (Fig. 10, A and C, green lines). Following photobleaching, SR-BI-YFP rapidly recovered to the same microdomain (full recovery in ~3 min). This recovery was comparable with the recovery rate of SR-BI-YFP on the BL membrane (Fig. 10, B and C, red lines). Taken together, our results indicate that these enriched microdomains are stable canalicular structures with which SR-BI-YFP dynamically associates.

**DISCUSSION**

The present study provides new insight into the regulation of hepatic SR-BI trafficking. Using both fluorescence and electron microscopy, we demonstrate that SR-BI localizes to the bile canaliculus and describe the canalicular microenvironment in which SR-BI resides. Most importantly, we establish a link between cellular sterol availability and hepatic SR-BI trafficking. We find that cholesterol loading promotes SR-BI transcytosis from the basolateral membrane to the bile canaliculus. This transcytosis occurs with both MβCD and lipoprotein cholesterol loading, suggesting that the direct plasma membrane binding of HDL to SR-BI and SR-BI-mediated signaling (32) are not required for SR-BI transcytosis.

These findings are in contrast to the only other reported study examining the regulation of SR-BI transcytosis (15). Experiments performed by Burgos et al. (15) in top/bottom polarized Madin-Darby canine kidney cells revealed basolateral
to apical transcytosis of SR-BI that was enhanced rather than decreased by basolateral plasma membrane cholesterol depletion with MβCD. The reverse effect of cholesterol availability in WIF-B and Madin-Darby canine kidney cells suggests that polarized cells have unique regulatory mechanisms for receptor transcytosis.

Our data suggest that SR-BI transcytosis is tightly tied to plasma membrane free cholesterol levels and possibly to SR-BI raft cholesterol levels. This idea is supported by the increased transcytosis observed following high concentration LDL incubations (Fig. 4A). It is also supported by our observation that HDL and LDL pools with similar levels of FC stimulate similar amounts of transcytosis (Fig. 4B) and that SR-BI transcytosis parallels the concentration of FC on the BC (Fig. 7). The slow time course for translocation is very similar to that observed for another transcytotic raft protein, 5'-nucleotidase. The 5'-nucleotidase is a glycosylphosphatidylinositol-anchored protein that associates with and uses membrane rafts for its slow transcytotic itinerary (36). It takes over 3.5 h to achieve steady state apical distribution (33). The clathrin-independent trafficking of 5'-nucleotidase provides a precedent for raft-mediated SR-BI transcytosis (33). This is consistent with the clathrin-independent endocytosis observed for SR-BI (34). However, it is also possible that SR-BI transcytosis is not dependent on plasma membrane cholesterol per se. As such, future studies will address the contribution of cellular signaling events leading to SR-BI transcytosis and further characterize its intracellular itinerary.

In addition to determining that SR-BI transcytosis is uniquely regulated in hepatocytes, our studies provide valuable new insight into the role of SR-BI in reverse cholesterol transport. Currently, three different, but not mutually exclusive, mechanisms attempt to explain the correlation between SR-BI expression and biliary cholesterol secretion. The first intuitive hypothesis is that the basolateral expression of SR-BI alters biliary cholesterol secretion by modulating the pool of cholesterol available for secretion or the signals that stimulate secretion without the need for SR-BI on the BC (35). Second, Silver et al. (8) and Wustner et al. (31) have proposed that selective depletion of cholesterol destined for transcytotic delivery occurs as HDL undergoes recycling in hepatocytes. Third, Sehayek et al. (13) suggested that BC-localized SR-BI mediates cholesterol efflux to phospholipid micelles.

The first mechanism is supported by data demonstrating only partial decrease in biliary cholesterol secretion in SR-BI (−/−) mice (36) and by data demonstrating that ABCG5/G8 expression is down-regulated in SR-BI (−/−) mice (37). In addition, earlier experiments failed to confirm localization of SR-BI on the bile canaliculus (38, 39). However, our studies using sufficient molecular resolution clearly demonstrate the presence of SR-BI on the canicular membrane (Fig. 1). We find that cholesterol loading, cholesterol depletion, and the levels of SR-BI protein all affect the amount of SR-BI localized on the BC. Furthermore, we show that upon cholesterol depletion, SR-BI rapidly disappears from the BC (Fig. 4C). Therefore, the inconsistent BC localization of SR-BI in other studies may be due to cellular cholesterol depletion, sample manipulations, or the levels of SR-BI expressed. Importantly, our findings suggest that the role of SR-BI in facilitating biliary cholesterol secretion is not limited to its function in promoting hepatocyte cholesterol uptake.

The second mechanism proposed to explain the correlation between SR-BI expression and biliary cholesterol secretion is supported by studies demonstrating selective depletion of lipoprotein cholesterol during recycling (8, 10, 31, 40, 41). This process requires active transport of cholesterol in lipoproteins or cholesterol-enriched domains. In our studies, we observe a delayed and slowed transcytosis of SR-BI in contrast to the rapid transcytosis seen by other recycling cargo like transferrin (Fig. 5B). Furthermore, SR-BI transcytosis is independent of lipoprotein binding (Figs. 5 and 6), has a lag phase of about 60 min (Fig. 5A), and is not specific to HDL (Fig. 4, A and B). Additionally, FRAP experiments suggest that, once localized to the BC, SR-BI does not rapidly recycle to or from the basolateral surface (Figs. 8 and 9). Although it is possible that small amounts of cholesterol are actively transcytosed in SR-BI rafts, such transportation is unlikely to account for the net transcytosis of cholesterol. SR-BI has also been shown to exist in detergent-resistant microdomains or lipid rafts (10, 15, 42, 43).
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Given that rafts have long been studied as transcytotic vehicles (44), it is possible that small amounts of cholesterol are actively transported across the cell in rafts. However, based on the slow rate of SR-BI transcytosis (Fig. 5) and the slow rate of SR-BI recovery to the BC in the lipid-loaded state (Figs. 8 and 9), it is unlikely that this trafficking accounts for significant amounts of selective cholesterol transcytosis. Notably, Wustner et al. (31) also report a rapid nonvesicular translocation of a cholesterol analogue (dehydroergosterol-DHE) to the BC that does not involve active transport of cholesterol, suggesting that active transcytosis is not required. There is also currently no evidence to suggest that intracellular depletion of lipoproteins directs the intracellularly acquired cholesterol to a different compartment than cell surface-acquired cholesterol. Furthermore, we have recently shown that endocytosis and recycling of HDL are not required for efficient selective uptake (16, 17). Overall, our recent studies do not support the role of rapid transcytotic HDL cholesterol delivery as the mechanism leading to the secretion of significant amounts of biliary cholesterol.

The third mechanism suggesting that canalicular SR-BI may affect cholesterol into the bile (13) is consistent with a known function of SR-BI as a bidirectional channel (6). Our data indicate that SR-BI has a resident function in the bile canaliculus and demonstrates the cholesterol-dependent regulation of SR-BI with stable canalicular microdomains. However, there is currently no specific evidence indicating that SR-BI has a direct role in promoting cholesterol secretion at the bile canaliculus.

Alternatively, other cellular functions of SR-BI may explain the in vivo correlation between biliary cholesterol secretion and SR-BI expression. SR-BI has been shown to localize in phosphatidylcholine-enriched membranes (45). It is possible that SR-BI creates a unique lipid environment conducive to cholesterol and/or phospholipid efflux. This is in line with our data indicating that canalicular SR-BI dynamically associates with microdomains on the BC. In addition, given that SR-BI has been shown to bind cholesterol (32), its BC localization may increase the concentration of free cholesterol available for secretion into bile. SR-BI may also promote the formation of canalicular microvilli as seen in other cell types (4), resulting in an increase in the curvature of the canalicular membrane such that cholesterol is better able to desorb into the bile.

In summary, our selective photobleaching results combined with data highlighting the regulation of SR-BI transcytosis by free cholesterol strongly suggest that SR-BI has an important resident function on the bile canaliculus that is independent of its role in basolateral cholesterol selective uptake. We demonstrate that in the cholesterol-loaded state, SR-BI alters its subcellular localization toward the BC, where its function as a bidirectional lipid channel may facilitate cholesterol secretion into bile. Therefore, these studies provide a molecular explanation for the observed correlation between SR-BI expression and biliary cholesterol secretion.

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REFERENCES

1. Acton, S., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H., and Krieger, M. (1996) Science 271, 518–520
2. Lewis, G. F., and Rader, D. J. (2005) Circ. Res. 96, 1221–1232
3. Babitt, J., Trigatti, B., Rigotti, A., Smart, E. J., Anderson, R. G., Xu, S., and Krieger, M. (1997) J. Biol. Chem. 272, 13242–13249
4. Williams, D. L., Wong, J. S., and Hamilton, R. L. (2002) J. Lipid Res. 43, 544–549
5. Zhang, Y., Da Silva, J. R., Reilly, M., Billheimer, J. T., Rothblat, G. H., and Rader, D. J. (2005) J. Clin. Invest. 115, 2870–2874
6. Rodrigues, W. V., Thuhnahin, S. T., Temel, R. E., Lund-Katz, S., Phillips, M. C., and Williams, D. L. (1999) J. Biol. Chem. 274, 20344–20350
7. Yi, J., Jian, B., Wang, N., Sun, Y., Moya, M. L., Phillips, M. C., Rothblat, G. H., Swaney, J. B., and Tall, A. R. (1997) J. Biol. Chem. 272, 20982–20985
8. Silver, D. L., Wang, N., Xiao, X., and Tall, A. R. (2001) J. Biol. Chem. 276, 25287–25293
9. Wustner, D. (2005) J. Biol. Chem. 280, 6766–6779
10. Rahind, D., Bourgeois, P., Baurret, G., Huard, K., Falstrau, L., and Brissette, L. (2004) J. Cell Sci. 117, 3095–3105
11. Miquel, J. F., Moreno, M., Amigo, L., Molina, H., Mardones, P., Wistuba, I. I., and Rigotti, A. (2003) Gut 52, 1017–1024
12. Nakagawa, A., Nagaosa, K., Hirose, T., Tsuda, K., Hasegawa, K., Shiratsuchi, A., and Nakinishi, Y. (2004) Dev. Growth Differ. 46, 283–298
13. Sehayek, E., Wang, R., No, J. G., Zinchuk, V. S., Duncan, E. M., Shefer, S., Vance, D. E., Ananthnarayanan, M., Chait, B. T., and Breslow, J. I. (2003) J. Lipid Res. 44, 1605–1613
14. Kozarsky, K. F., Donahee, M. H., Rigotti, A., Iqbal, S. N., Edelman, E. R., and Krieger, M. (1997) Nature 387, 414–417
15. Burgos, P. V., Klattenhoff, C., de la Fuente, E., Rigotti, A., and Gonzalez, A. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 3845–3850
16. Harder, C. J., Vassiliou, G., McBride, H. M., and McPherson, R. (2006) J. Lipid Res. 47, 492–503
17. Nieland, T. J., Ehrlich, M., Krieger, M., and Kirchhausen, T. (2005) Biochim. Biophys. Acta. 1734, 44–51
18. Pittman, R. C., Knecht, T. P., Rosenbaum, M. S., and Taylor, C. A., Jr. (1987) J. Biol. Chem. 262, 2443–2450
19. Eckhardt, E. R., Cai, L., Sun, B., Webb, N. R., and van der Westhuyzen, D. R. (2004) J. Biol. Chem. 279, 14372–14381
20. Mardones, P., Quinones, V., Amigo, L., Moreno, M., Miquel, J. F., Schwarz, M., Miettinen, H. E., Trigatti, B., Krieger, M., VanPatten, S., Cohen, D. E., and Rigotti, A. (2001) J. Lipid Res. 42, 170–180
21. Yu, L., Li-Hawkins, J., Hammer, R. E., Berge, K. E., Horton, J. D., Cohen, J. C., and Hobbs, H. H. (2002) J. Clin. Invest. 110, 671–680
22. Geuken, E., Visser, D. S., Leuvenhink, H. G., de Jong, K. P., Peeters, P. M., Slooff, M. J., Kuipers, F., and Porte, R. J. (2005) Hepatology 42, 1166–1174
23. Ihrke, G., Neufeld, E. B., Meets, T., Shanks, M. R., Cassio, D., Laurent, M., Schoer, T. A., Pagano, R. E., and Hubbard, A. L. (1993) J. Cell Biol. 123, 1761–1775
24. Shanks, M. R., Cassio, D., Lecoq, O., and Hubbard, A. L. (1994) J. Cell Sci. 107, 813–825
25. Ihrke, G., Martin, G. V., Shanks, M. R., Schrader, M., Schoer, T. A., and Hubbard, A. L. (1998) J. Cell Biol. 141, 115–133
26. Goodwin, J. S., and Kenworthy, A. K. (2005) Methods 37, 154–164
27. Sai, Y., Nies, A. T., and Arias, I. M. (1999) J. Cell Sci. 112, 4353–4354
28. Bauchart, D., Durand, D., Laplaud, P. M., Forgez, P., Goulinet, S., and Chapman, M. J. (1989) J. Biol. Chem. 264, 1499–1514
29. Racchi, M., Baetta, R., Salvietti, N., Iannetta, P., Franceschini, G., Paoletti, R., Fumagalli, R., Govoni, S., Trabucchi, M., and Soma, M. (1997) Biochem. J. 322, 893–898
30. Ghosh, R. N., Gelman, D. L., and Maxfield, F. R. (1994) J. Cell Sci. 107, 2177–2189
31. Wustner, D., Mondal, M., Huang, A., and Maxfield, F. R. (2004) J. Lipid Res. 45, 427–437
32. Assanasen, C., Mineo, C., Seetharam, D., Yuhanna, I. S., Marcel, Y. L., Connelly, M. A., Williams, D. L., Llera-Moya, M., Shaul, P. W., and Silver, D. L. (2005) J. Clin. Invest. 115, 969–977
33. Schell, M. J., Maurice, M., Stieger, B., and Hubbard, A. L. (1992) J. Cell Biol. 119, 1173–1182
34. Eckhardt, E. R., Cai, L., Shetty, S., Zhao, Z., Szanto, A., Webb, N. R., and van der Westhuyzen, D. R. (2006) J. Biol. Chem. 281, 4348–4353
35. Schwartz, C. C., Halloran, L. G., Vlahcevic, Z. R., Gregory, D. H., and Swell, L. (1978) Science 200, 62–64
36. Trigatti, B., Rayburn, H., Vinals, M., Braun, A., Miettinen, H., Penman, M., Hertz, M., Schrenzel, M., Amigo, L., Rigotti, A., and Krieger, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9322–9327
37. Van, E. M., Twisk, J., Hoekstra, M., Van Rij, B. T., Van der Lans, C. A., Bos, I. S., Kruijt, J. K., Kuipers, F., and van Berkel, T. J. (2003) J. Biol. Chem. 278, 23699–23705
38. Mardones, P., Pilon, A., Bouly, M., Duran, D., Nishimoto, T., Arai, H., Kozarsky, K. F., Altayo, M., Miquel, J. F., Luc, G., Clavey, V., Staels, B., and Rigotti, A. (2003) J. Biol. Chem. 278, 7884–7890
39. Stangl, H., Graf, G. A., Yu, L., Cao, G., and Wyne, K. (2002) J. Endocrinol. 175, 663–672
40. Delamatre, J. G., Sarphie, T. G., Archibold, R. C., and Hornick, C. A. (1990) J. Lipid Res. 31, 191–202
41. Kambouris, A. M., Roach, P. D., Calvert, G. D., and Nestel, P. J. (1990) Arteriosclerosis 10, 582–590
42. Camarota, L. M., Chapman, J. M., Hui, D. Y., and Howles, P. N. (2004) J. Biol. Chem. 279, 27599–27606
43. Peng, Y., Akmentin, W., Connelly, M. A., Lund-Katz, S., Phillips, M. C., and Williams, D. L. (2004) Mol. Biol. Cell 15, 384–396
44. Nyasae, L. K., Hubbard, A. L., and Tuma, P. L. (2003) Mol. Biol. Cell 14, 2689–2705
45. Parathath, S., Connelly, M. A., Rieger, R. A., Klein, S. M., Abumrad, N. A., Llera-Moya, M., Iden, C. R., Rothblat, G. H., and Williams, D. L. (2004) J. Biol. Chem. 279, 41310–41318