Scavenger receptor class B type I reduces cholesterol absorption in cultured enterocyte CaCo-2 cells[1]

Lei Cai,[2,*] Erik R. M. Eckhardt,[2,*] Wei Shi,[*] Zhenze Zhao,[*] Munira Nasser,[†] Willem J. S. de Villiers,[†] and Deneys R. van der Westhuyzen[3,*]

Department of Internal Medicine,[*] Divisions of Endocrinology and Molecular Medicine, and Gastroenterology,[†] University of Kentucky Medical Center, Lexington, KY 40536; and Department of Veterans Affairs Medical Center, Lexington, KY 40511

Abstract **Scavenger receptor class B type I (SR-BI) mediates selective uptake of cholesteryl esters from HDL as well as efflux of cellular free cholesterol to HDL. It is unclear whether the receptor is involved in intestinal cholesterol absorption. We addressed this issue by studying [^{3}H]cholesterol flux in differentiated CaCo-2 cells incubated at their apical side with mixed taurocholate/phosphatidylcholine/cholesterol micelles. Biotinylation and HDL binding experiments showed predominant apical expression of endogenous and overexpressed SR-BI. Mixed micellar cholesterol saturation affected the magnitude and direction of cholesterol flux with significant net uptake only from supersaturated micelles and net efflux from unsaturated micelles. Incubation with micelles that depleted cellular cholesterol resulted in a decrease of SR-BI protein, whereas incubation with cholesterol-loading micelles resulted in a significant increase of SR-BI protein. Apical cholesterol uptake by CaCo-2 cells was increased in the presence of a SR-BI-blocking antibody and by partial inhibition of SR-BI expression with small inhibitory RNA. Adenovirus-mediated overexpression of apical SR-BI did not affect cholesterol uptake but stimulated apical cholesterol efflux, even to supersaturated mixed micelles. Partial inhibition of SR-BI with small inhibitory RNA reduced apical cholesterol efflux. Our data argue against a direct role for SR-BI in micellar cholesterol uptake. However, SR-BI might be involved in cholesterol absorption by facilitating cholesterol efflux to micelles.**—Cai, L., E. R. M. Eckhardt, Z. Zhao, M. Nasser, W. J. S. de Villiers, and D. R. van der Westhuyzen. **Scavenger receptor class B type I reduces cholesterol absorption in cultured enterocyte CaCo-2 cells.** ***J. Lipid Res.*** **2004.** 45: **253–262.**

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The intestine is of major importance in whole body cholesterol homeostasis because it synthesizes, secretes, absorbs, and excretes significant amounts of the sterol. Each day, the luminal side of the organ is exposed to ~1 g of cholesterol, which is mainly derived from bile and, to a lesser extent, from the diet and sloughed intestinal cells (1, 2). Approximately 50% of this cholesterol pool is absorbed by the enterocyte (1, 2). The physiological importance of intestinal cholesterol absorption is illustrated by the fact that its inhibition dramatically reduces serum LDL levels in humans (3) and decreases the incidence and severity of atherosclerosis in animal models (4).

Intestinal cholesterol absorption requires solubilization of the sterol in micelles by the detergent action of bile salts and phospholipids. Such mixed micelles serve as the principal reservoir from which cholesterol monomers then diffuse across the unstirred water layer that covers the microvilli (5, 6). It is not known at present how cholesterol monomers are subsequently transported across the apical membrane and whether transport proteins facilitate this movement. One protein that has been suggested to act as an intestinal cholesterol receptor is scavenger receptor class B type I (SR-BI) (7), a protein originally described as an HDL receptor mediating the selective uptake of cholesteryl ester from HDL particles (8–10). SR-BI is indeed expressed on the apical surface of enterocytes (7, 11–13), although exposure to HDL at this membrane would be minimal. Hauser and colleagues (7) reported that uptake of micellar cholesterol by rabbit brush border membrane vesicles (BBMVs) could be competitively inhibited by apolipoprotein A-I (apoA-I), a ligand for SR-BI, and by a blocking antibody against SR-BI. Furthermore, influx of micellar cholesterol into CaCo-2 cells, a widely used model for enterocytes, could be inhibited by HDL

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Abbreviations: apoA-I, apolipoprotein A-I; eypc, egg yolk phosphatidylcholine; MBSTOG, MES-buffered saline with 1% Triton X-100 and 1.75% *n*-octyl-β-D-glucopyranoside; SR-BI, scavenger receptor class B type I; tc, taurocholate.

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[2] L. Cai and E. R. M. Eckhardt contributed equally to this work.

[3] To whom correspondence should be addressed.
e-mail: dvwest1@uky.edu

(7, 14). Recent studies of mice lacking functional SR-BI, however, showed either a slight increase in intestinal cholesterol absorption (15) or no change at all (16), leading to the conclusion that SR-BI was not involved in intestinal cholesterol absorption.

Recent developments suggest that cholesterol absorption rates may not be controlled at the level of uptake but rather by active apical efflux of cholesterol by proteins belonging to the important group of ATP binding cassette (ABC) transporters, namely ABCA1 [the *Tangier* gene product (17)] and heterodimers of the half-transporters ABCG5/ABCG8 [the *sitosterolemia* gene products (18–21)]. Interestingly, SR-BI is known to stimulate bidirectional cholesterol flux (22). Although it can stimulate uptake of free cholesterol from various donor particles (23), it also significantly enhances the efflux of cellular cholesterol to various acceptors, including HDL (24–26), phospholipid vesicles (24), or cyclodextrins (27). It is not known if mixed micelles can act as cholesterol acceptors for SR-BI-mediated efflux.

We sought to study whether SR-BI affects cholesterol uptake from or efflux to mixed micelles in CaCo-2 cells. Because the degree of micellar cholesterol saturation strongly affects the propensity of the micelle to "donate" cholesterol monomers (28), unsaturated as well as supersaturated micellar solutions were used. Furthermore, we studied how alterations in SR-BI expression and function affect cholesterol flux across the apical membrane. Our results strongly suggest that apically expressed SR-BI does not facilitate cholesterol uptake from mixed micelles but rather enhances efflux, even to supersaturated micelles.

MATERIALS AND METHODS

Cell culture

CaCo-2 cells (American Type Culture Collection, Manassas, VA) were grown in 250 ml tissue culture flasks with HEPES/carbonate-buffered DMEM supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10% FBS, 50 U/ml penicillin G, and 50 μg/ml streptomycin (Gibco, Grand Island, NY). Cells were subcultured once per week using a 1:5 split ratio. As indicated, cells were seeded on either 12-well cell culture plates (1×10^5 cells/cm^2; Corning Corp., Corning, NY) and grown until 2–3 days after confluence or on permeable (Transwell®) membranes (growth area, 4.7 cm^2; 0.4 μm pores; Costar, Cambridge, MA) until the transepithelial resistance had reached 250 Ω. Overexpression of SR-BI was achieved by incubating cells for 24 h with 1,000 particles/cell of AdSRBI, an adenoviral vector containing the coding sequences for murine SR-BI (29). AdNull (provided by Dr. D. J. Rader, University of Pennsylvania) is a recombinant adenovirus containing no transgene.

Preparation of mixed micelles

Stock solutions of taurocholate (tc; Sigma, St. Louis, MO; more than 99% pure as assessed by thin layer chromatography) were prepared in methanol; stock solutions of egg yolk phosphatidylcholine (eypc; Avanti Polar Lipids, Alabaster, AL) and cholesterol (Sigma) were prepared in chloroform. Required amounts of each stock solution were transferred to a sealable glass tube and mixed in a vortex. Solvents were evaporated under a mild stream of N_2 before 4–16 h of lyophilization. The lipid film was stored under N_2 at −20°C. Approximately 1 h before the start of the experiment, the lipid film was hydrated in serum-free DMEM containing 0.5% fatty acid-free BSA (Sigma) and incubated at 37°C in a rotating incubator. Solutions were filtered through a 0.45 μm cellulose acetate filter (Nalgene, Rochester, NY) before adding them to the cells. For uptake experiments, for each μmol of cholesterol, ~1 μCi of [1α,2α(n)-^{3}H]cholesterol (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added to the organic lipid solution before solvent evaporation.

RNA inhibition

Double-stranded small inhibitory RNA (siRNA) specifically targeted against the sequence 5′-AAGCAGCAGGTCCTTAAGAAC of human SR-BI was synthesized using Ambion's Silencer RNA construction kit (Ambion, Austin, TX). The sequence of the sense RNA strand was 5′-GCAGCAGGUCCUUAAGAACUU, and that of the antisense strand was 5′-GUUCUUAAGGACCUGCUGCUU. Specificity for SR-BI was confirmed by a Basic Local Alignment Search Tool search. As soon as transmembrane resistance had reached 250 Ω, siRNA (1.5 ng/mm^2) was transfected into CaCo-2 cells using Ambion's SiPort Lipid reagent. Cells were used 24 h after transfection.

Cholesterol uptake/efflux measurement

For experiments in which mass transfer of cholesterol was measured upon incubation with mixed micelles, cells were first washed twice with serum-free DMEM and incubated at 37°C with 1 ml of mixed micellar solution in serum-free DMEM containing 0.5% BSA for 24 h. After the incubation, cells were placed on ice and washed twice with 1 ml of ice-cold PBS (pH 7.4) containing 1 mg BSA/ml and twice with ice-cold PBS (pH 7.4). Cellular cholesterol was extracted by incubating cells with 1 ml of hexane-isopropanol (3:2) for 30 min. The residue was dissolved in 1 ml of 0.1 N NaOH for estimation of protein content. The hexane-isopropanol extract was dried under N_2, and the lipid film was redissolved in $CHCl_3$ with 0.5% Triton X-100. After evaporation of $CHCl_3$, pellets were redissolved in water and total cholesterol was determined enzymatically with commercially available kits (Wako Chemicals, Osaka, Japan) using similarly treated standards. Cellular cholesterol and cholesteryl ester content was measured by thin-layer chromatography (28). Cholesterol and cholesteryl ester spots were scraped from the plates, solubilized in hexane-isopropanol, dried under N_2, resuspended in isopropanol, and quantified with the CII cholesterol kit from Wako. For efflux experiments, cells were incubated with DMEM containing 10% FBS and 0.2 μCi/ml [^{3}H]cholesterol (Amersham Pharmacia Biotech) during 24 h, treated with adenovirus or siRNA in the presence of [^{3}H]cholesterol for an additional 24 h, washed three times with PBS containing 1 mg/ml BSA, and then equilibrated in serum-free medium containing 0.5% BSA for 16 h. Thereafter, cells were incubated with unlabeled micellar solutions for 2 h at 37°C. Radioactivity in the supernatant was compared with the sum of radioactivity in cell extracts and the supernatant. Monolayer integrity was verified during the assay period by monitoring transepithelial resistance. For experiments in which the rate of uptake rather than the mass transfer of cholesterol was measured, cells were incubated for 2 h with DMEM containing 0.5% BSA and [^{3}H]cholesterol-labeled mixed micelles. Cells were lysed in 0.1 N NaOH, and part was dissolved in scintillation fluid (Packard Biosciences, Meriden, CT) and counted with a β-counter after neutralization with HCl. Cholesterol uptake is expressed as nanomoles per milligram of protein per hour.

Where applicable, blocking antibody (prepared by injecting a rabbit with recombinant adenoviral particles expressing mouse

SR-BI) was added to the assay medium at a concentration of 300 μg/ml.

Biotinylation

Biotinylation was carried out as described elsewhere (30), with slight modifications. CaCo-2 cells were grown to confluence on permeable membranes until the transepithelial resistance had reached 250 Ω. Culture dishes were placed on ice and washed five times, from both apical and basolateral sides, with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-CM). EZ-Link™ Sulfo-NHS-LC Biotin (Pierce, Rockford, IL) was added to either the apical or basolateral side or to both sides (0.5 mg/ml in PBS-CM). Cells were incubated for 20 min on ice, and the biotin solution was then replenished, followed by another 20 min incubation. Cells were washed three times for 2 min each with Tris-buffered saline to quench free biotin (once) and PBS-CM (twice). Membranes were excised and transferred to individual dishes, and cells were solubilized with cold MES-buffered saline with 1% Triton X-100 and 1.75% *n*-octyl-β-D-glucopyranoside (MBSTOG) supplemented with Complete-Mini® protease inhibitors (Roche Diagnostics, Mannheim, Germany). Protein concentrations of cell lysates were estimated with a bicinchoninic acid assay (Pierce), and 10 μg aliquots of cell lysate were then incubated with an equal volume of immobilized streptavidin (Oncogene Research Products, Cambridge, MA) for 16 h at 4°C. The supernatant, free of biotinylated surface proteins (as revealed by repeated precipitation with streptavidin agarose; data not shown), was analyzed by Western blotting.

Immunoblotting

Cultured cells were cooled on ice and lysed in cold MBSTOG. Cell lysates were centrifuged at 13,000 *g* for 10 min and protein content was measured. After reducing SDS-PAGE, samples were transferred by electroblotting onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked overnight at 4°C in PBS containing 5% nonfat dry milk and 0.5% Tween® 20 (Sigma) followed by incubation with anti-human SR-BI antibody directed against the cytoplasmic C terminus of human SR-BI (29). SR-BI was quantified by enhanced chemiluminescence detection (Amersham Pharmacia Biotech) and autoradiography. Autoradiograms were analyzed with a densitometer and ImageQuant software, version 1.2 (Molecular Dynamics, Sunnyvale, CA).

Selective cholesteryl ester uptake measurements

Human HDL (d = 1.063–1.21 g/ml) fractions were isolated from fresh human plasma by density gradient ultracentrifugation as previously described (31). Human HDL_3 (d = 1.13–1.18 g/ml) was obtained from total HDL by density gradient fractionation. All isolated fractions were dialyzed against 150 mM NaCl/0.01% EDTA, sterile filtered, and stored under nitrogen at 4°C. HDL was iodinated by the iodine monochloride method (32) to a specific activity of 100–200 cpm/ng protein. ^{125}I-labeled HDL was further labeled with nonhydrolyzable [1α,2α(n)-^{3}H]cholesteryl oleoyl ether ([^{3}H]CEt) using partially purified cholesteryl ester transfer protein (CETP) as described (33) with the following modifications. [^{3}H]CEt dissolved in dichloromethanol was dried in a 12 × 75 cm glass borosilicate tube (20 μCi/mg HDL protein), after which HDL and CETP were added. After 16 h of incubation at 37°C, HDL was reisolated by ultracentrifugation at a density of 1.21 g/ml. The specific activity of the [^{3}H]HDL ranged from 10 to 30 dpm/mg protein. Cell association assays, as previously described (29), were performed in medium with 0.5% BSA instead of serum and radiolabeled lipoprotein. CaCo-2 cells grown to confluence on permeable membranes were incubated at 37°C for 2 h with 10 μg/ml double-labeled HDL_3 added to either the apical or the basolateral side. Medium was removed, and cells were washed four times with cold 50 mM Tris-HCl/150 mM NaCl (pH 7.4) containing 0.2% BSA followed by two washes in the same buffer without BSA. Cells were then solubilized with 0.1 N NaOH for 60 min at room temperature, and proteins and radioactivity were measured in the lysate. ^{125}I represented HDL protein association according to the protein tracer, and ^{3}H represented HDL protein association according to the cholesteryl ether tracer. The noniodide, trichloroacetic acid-soluble degraded material in cell media was assayed as described (34). Values are expressed as apparent HDL protein uptake assuming the uptake of intact holoparticles. Selective uptake is defined as ^{3}H − (^{125}I cell associated + ^{125}I degraded) and represents the uptake of cholesteryl ester that cannot be accounted for by the internalization of intact particles. SR-BI-specific HDL association studies were performed using CHO cells (CHO ldlA7 cells) stably transfected with human SR-BI (CHO-SRBI cells) and nontransfected CHO cells as previously described (29).

Statistics

Results are expressed as means ± SD. Differences between groups were determined by means of ANOVA and were calculated using Prism software, version 3.0, from Graphpad (San Diego, CA).

RESULTS

SR-BI is mainly expressed at the apical membrane of differentiated CaCo-2 cells

SR-BI expression markedly differed between undifferentiated, preconfluent cells and differentiated, postconfluent CaCo-2 cells (data not shown), in agreement with published data (11). SR-BI expression in preconfluent cells was relatively low, although detectable by immunoblotting**.** In postconfluent cells grown on either standard dishes or permeable membranes, expression levels were approximately 10-fold higher (data not shown). The cellular distribution of SR-BI in differentiated CaCo-2 cells was studied by biotinylation. In this approach, polarized cells grown on permeable membranes were biotinylated on ice at either the apical or the basolateral side. The amount of total cell SR-BI was then compared, by immunoblotting, with the amount of SR-BI that remained in the cell extract after precipitation of all biotinylated proteins (**Fig. 1A**). The results revealed that the large majority of SR-BI was expressed on the surface of the cell with predominant apical expression, in line with SR-BI distribution in intestinal cells (7, 12, 13) but in contrast to the even distribution across apical and basolateral membranes in CaCo-2 cells as reported by Cai et al. (11). Figure 1B shows that the association and selective uptake of HDL was markedly higher at the apical surface than at the basolateral surface. The cell association of HDL cholesteryl ester delivered at the apical surface greatly exceeded the amount that could be accounted for by the binding or uptake of whole HDL particles, indicating a substantial amount of selective cholesteryl ester uptake. Little HDL protein degradation was observed (∼10% of the amount of cell-associated HDL), indicating relatively low rates of endocytic uptake. From the basolateral surface, however, no selec-

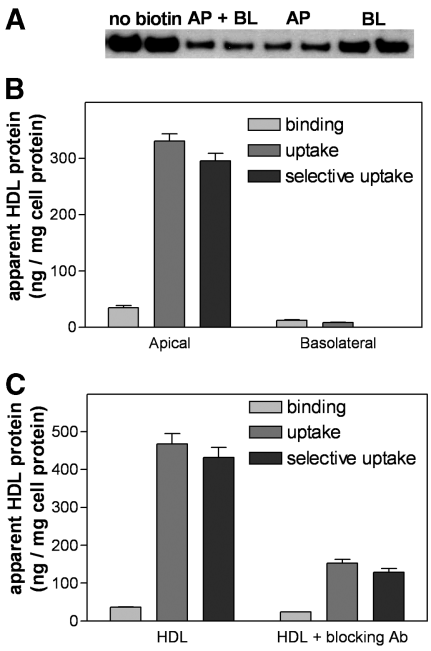

Fig. 1. Expression of scavenger receptor class B type I (SR-BI) protein in CaCo-2 cells. A: Biotinylation at 4°C of basolateral (BL) and/or apical (AP) surfaces of differentiated CaCo-2 cells grown on membranes, followed by precipitation of biotinylated proteins from 5 μg of cell extract with streptavidin-agarose and immunoblotting of remaining proteins. B: HDL association and cholesteryl ester uptake from apical or basolateral surfaces of differentiated CaCo-2 cells grown on permeable membranes. Cells were incubated with double-labeled human HDL_3 (10 μg/ml) for 2 h at 37°C. Cell-associated ^{125}I-HDL, [^{3}H]cholesteryl ether uptake, and calculated cholesteryl ester selective uptake were determined. Values are expressed as nanograms of HDL protein per milligram of cell protein assuming the association/uptake of intact particles. C: SR-BI dependence of selective cholesteryl ester uptake from HDL particles. CaCo-2 cells grown on standard dishes until 3 days after confluence were incubated with human HDL_3 alone (10 μg/ml) or with HDL_3 together with the SR-BI-blocking antibody (Ab; 300 μg/ml). Values in B and C represent means ± SD of three data points from a representative experiment. Differences between the apical and basolateral sides, as well as with and without blocking antibody, reached statistical significance for all three parameters ($P < 0.01$). The biotinylation experiment was repeated twice with similar results.

tive uptake of cholesteryl ester was observed, despite the presence of detectable amounts of SR-BI at this surface. The explanation for this phenomenon is not known. SR-BI accounted for the large majority of the selective uptake from the apical side, as demonstrated by the significant inhibition of selective lipid uptake by the SR-BI-blocking antibody (Fig. 1C). In addition to the blocking capacity clearly illustrated in Fig. 1C, the antibody effectively inhibits SR-BI-mediated association of HDL in CHO cells (see below). These results, as well as confocal laser scanning microscopy (data not shown), indicate a predominantly apical SR-BI expression in CaCo-2 cells.

Micellar cholesterol saturation strongly affects the direction and rate of transmembrane cholesterol flux

Cells were incubated for 24 h with micellar solutions composed of 9.7 mM of the bile salt tc, 6.5 mM of the phospholipid eypc, and various concentrations of free cholesterol. CaCo-2 cells exposed to these micellar solutions survived 24 h incubations, as indicated by microscopic observation, the lack of detachment of cells from the support, and the similar cellular protein content of cell lysates of the various groups (data not shown).

Figure 2 shows the total cholesterol content in CaCo-2 cells incubated for 24 h with micelles with 0, 0.5, or 1.5 mM (0, 3, or 8.6 mol%) cholesterol. Only solutions with 8.6 mol% cholesterol were supersaturated with cholesterol, as indicated by the presence of small unilamellar vesicles after filtration. After the incubation, cells exposed to the unsaturated micellar solutions became cholesterol depleted, whereas cells exposed to the supersaturated micellar solutions showed a marked (~2-fold) increase in total cellular cholesterol levels. The variations in total cellular cholesterol content were largely the result of changes in free cholesterol. Cholesteryl ester constituted 6.1 ± 3.8%, 22.1 ± 9.1%, 8.5 ± 3.0%, and 6.6 ± 2.3% of total cholesterol in cells treated with no micelles, 0% cholesterol, 3% cholesterol, and 8.6% cholesterol micelles, respectively (n = 3 experiments). Experiments carried out with cells on permeable membranes, with or without basolateral cholesterol acceptor (human HDL_3), and with cells grown on standard dishes until 3 days after confluence yielded similar results (data not shown). These results show that the degree of micellar cholesterol saturation strongly affects the net directional movement of cholesterol across the apical membrane of the enterocyte.

SR-BI overexpression has no effect on cholesterol uptake from micelles

SR-BI has been proposed to facilitate intestinal cholesterol absorption (7). Therefore, we hypothesized that SR-BI overexpression should enhance apical cholesterol uptake in CaCo-2 cells. To test this, we overexpressed SR-BI

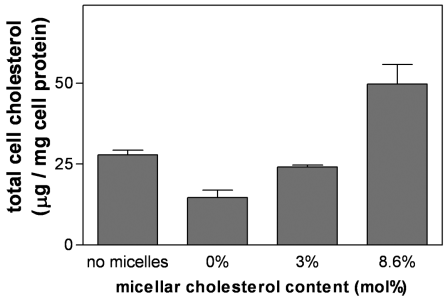

Fig. 2. Effect of micellar cholesterol content on cellular cholesterol levels. CaCo-2 cells were grown on standard dishes until 3 days after confluence and were incubated with mixed micellar solutions [9.7 mM taurocholate (tc), 6.5 mM egg yolk phosphatidylcholine (eypc), and 0, 0.5, or 1.5 mM (0, 3, or 8.6 mol%) cholesterol] for 24 h. Cell lipids and proteins were extracted and quantified as described in Materials and Methods. Values represent means ± SD of nine dishes from three independent experiments. All differences, except for the difference between 3% and no micelles, were significantly different, $P < 0.05$ (ANOVA with Bonferroni's multiple comparison test).

in confluent CaCo-2 cells by adenovirus-mediated gene transfer using AdSRBI. Twenty-four hours after AdSRBI treatment, apical SR-BI expression was significantly increased above background (2.6-fold), as shown by the increase in SR-BI immunoreactivity in streptavidin-precipitable material after biotinylation of the apical side (**Fig. 3A**). Cells were then incubated for 2 h with micelles containing [^{3}H]cholesterol at their apical side and with human HDL_3 (40 μg/ml final concentration) as a cholesterol acceptor at the basolateral side. Figure 3B shows that overexpression of SR-BI had no effect on the rate of uptake of cholesterol from micelles containing either 3 or 8.6 mol% cholesterol.

SR-BI inhibition increases cholesterol uptake from micelles

Because CaCo-2 cells already express significant amounts of endogenous SR-BI, we tested whether inhibition of SR-BI would affect apical uptake of micellar cholesterol. For this purpose, confluent CaCo-2 cells, cultured on standard dishes, were incubated with a blocking antibody directed against SR-BI together with mixed micelles that contained [^{3}H]cholesterol. This antibody effectively inhibited SR-BI-specific association in transfected CHO cells expressing human SR-BI (**Fig. 4B**). As shown above, the same blocking antibody also markedly inhibited selective uptake of cholesteryl ester from HDL particles in CaCo-2 cells (Fig. 1C). Figure 4A shows that incubation of CaCo-2 cells with the blocking antibody significantly increased cholesterol influx from micelles compared with cells incubated with preimmune serum, suggesting that the presence of SR-BI negatively affects cholesterol uptake.

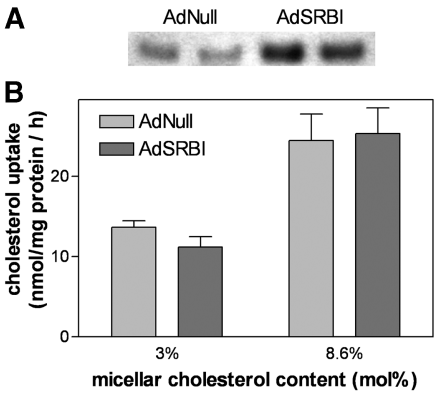

Fig. 3. Effect of overexpression of SR-BI on cholesterol uptake. Expression of SR-BI in differentiated CaCo-2 cells grown on permeable membranes was increased by treating cells with AdSRBI as described in Materials and Methods; control cells were treated with AdNull. A: Streptavidin-precipitable SR-BI immunoreactivity in 10 μg of MES-buffered saline with 1% Triton X-100 and 1.75% *n*-octyl-β-D-glucopyranoside extract of cells biotinylated at their apical membrane, showing a significant increase in apical expression of SR-BI. B: Cells were incubated for 2 h at their apical side with DMEM containing 0.5% BSA and [^{3}H]cholesterol-labeled mixed micellar solutions [9.7 mM tc, 6.5 mM eypc, and 0.5 or 1.5 mM (3 or 8.6 mol%) cholesterol]. The basolateral compartment contained human HDL_3 (40 μg/ml). Lipids were extracted from cells and cellular ^{3}H was measured. Each value represents the mean ± SD of three dishes. The experiment was performed twice with similar results.

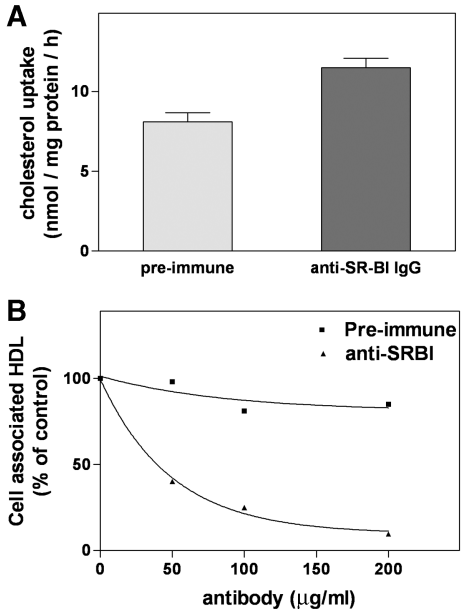

Fig. 4. Effect of SR-BI blocking on micellar cholesterol uptake. A: CaCo-2 cells were incubated with [^{3}H]cholesterol-labeled mixed micellar solutions [9.7 mM tc, 6.5 mM eypc, and 0.5 mM (3 mol%) cholesterol] for 2 h with or without 300 μg/ml SR-BI-blocking antibody or preimmune IgG. Cellular cholesterol uptake was measured as described in Materials and Methods. Each value represents the mean ± SD of three dishes. Addition of anti-SR-BI led to a statistically significant increase in uptake ($P < 0.05$, *t*-test). B: Inhibition of SR-BI-mediated HDL association in CHO-SRBI cells. Cells were incubated with ^{125}I-labeled HDL (10 μg/ml) at 37°C for 2 h in the presence of increasing concentrations of anti-SRBI antibody or preimmune IgG. SR-BI-specific cell association was measured as the difference between the values obtained for CHO and CHO-SRBI cells. Each point represents the mean of duplicate dishes.

Besides blocking SR-BI, we impaired SR-BI function by decreasing expression by means of RNA interference. Transfection with SR-BI-specific siRNA resulted in a decrease of SR-BI expression in CaCo-2 cells by an average of 36 ± 16% (n = 3), as indicated by immunoblotting (**Fig. 5**). When those cells were exposed to micellar solutions at their apical side (3 or 8.6 mol% cholesterol, 9.7 mM tc, 6.5 mM eypc), cholesterol uptake was increased compared with that in cells transfected with randomly generated RNA interference (Fig. 5).

SR-BI overexpression enhances apical cholesterol efflux to mixed micelles

The results described above indicate that SR-BI does not play a direct role in the uptake of micellar cholesterol. Nevertheless, SR-BI might influence cholesterol uptake by affecting efflux of cholesterol toward mixed micelles, analogous to the proposed mode of action of ABCG5/ABCG8 (18–20). To test this hypothesis, we studied the effect of SR-BI overexpression on efflux of cellular cholesterol across the apical membrane to mixed micelles. Differentiated CaCo-2 cells, grown on permeable membranes, were

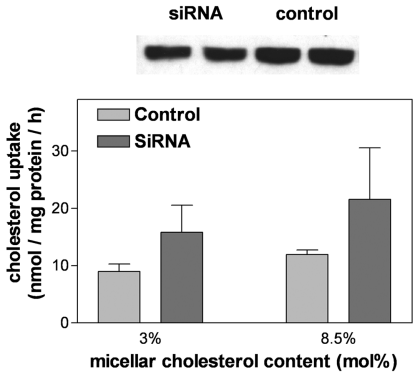

Fig. 5. Effect of inhibition of SR-BI expression on micellar cholesterol uptake. CaCo-2 cells grown on permeable membranes were transfected with either randomly generated ("scrambled") small inhibitory RNA (siRNA) or with siRNA targeted against human SR-BI for 24 h. Cells were then incubated with [^{3}H]cholesterol-labeled mixed micellar solutions [9.7 mM tc, 6.5 mM eypc, and 0.5 mM (3 mol%) cholesterol] for 2 h, and cellular cholesterol uptake was measured as described in Materials and Methods. Values represent means ± SD of three dishes. Similar results were obtained in three separate experiments. Differences between control and siRNA reached statistical significance for both types of micelles ($P < 0.05$, *t*-test). The upper panel shows the immunoblot analysis of SR-BI expression in similarly treated dishes (10 μg protein/well). Expression was consistently decreased by siRNA treatment in three independent experiments, each carried out in duplicate, by an average of 36 ± 16%.

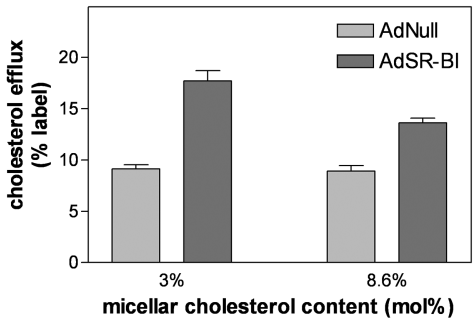

Fig. 6. Effect of overexpression of SR-BI on cellular cholesterol efflux in CaCo-2 cells. Differentiated CaCo-2 cells on permeable membranes were incubated for 24 h with [^{3}H]cholesterol in DMEM containing 10% BSA. AdNull (control) or AdSRBI (to overexpress SR-BI) then was added, and cells were incubated for an additional 24 h. Thereafter, cells were equilibrated in DMEM containing 0.5% BSA for 16 h and subsequently incubated at their apical side with mixed micelles [9.7 mM tc, 6.5 mM eypc, and 0.5 or 1.5 mM (3 or 8.6 mol%) cholesterol] and at the basolateral side with human HDL_3 (40 μg/ml) for 2 h. The radiolabel in the medium was expressed as the percentage of initial label associated with the cells. Each value represents the mean ± SD of three wells; differences between control and siRNA reached statistical significance for both types of micelles ($P < 0.05$, *t*-test). The experiment was repeated three times with similar results.

incubated with AdSRBI or AdNull for 24 h in the presence of [^{3}H]cholesterol. Apical SR-BI expression increased markedly, similar to levels shown in Fig. 3A (data not shown). Thereafter, cells were incubated at the apical compartment with mixed micelles and at the basolateral compartment with human HDL_3 (final concentration 40 μg/ml). **Figure 6** shows that overexpression of SR-BI significantly increased the efflux of [^{3}H]cholesterol to both unsaturated (3 mol% cholesterol) and, interestingly, supersaturated (8.6 mol%) micelles. A similar enhancement of cellular cholesterol efflux was observed in CHO cells expressing SR-BI (CHO-SRBI cells) compared with CHO cells lacking SR-BI (data not shown). These results show that SR-BI facilitates cholesterol efflux from CaCo-2 cells and may therefore influence cholesterol absorption. The apparent inconsistency in response to SR-BI overexpression between the observed increase in cholesterol efflux and the lack of effect on cholesterol uptake (Fig. 3) is likely attributable to the fact that the cholesterol uptake assay measures the rate of cholesterol influx rather than the net rate of cellular cholesterol accumulation.

SR-BI inhibition decreases apical cholesterol efflux to mixed micelles

Because overexpression of SR-BI facilitated the efflux of cellular cholesterol toward mixed micelles, we tested whether partial inhibition of SR-BI would decrease efflux. Differentiated CaCo-2 cells, grown on permeable membranes, were transfected with siRNA specific against SR-BI or with a randomly generated siRNA preparation (control). Transfection with SR-BI-specific siRNA resulted in a small yet significant decrease of SR-BI expression (57 ± 9.4% inhibition, n = 3) compared with control values. Cells were labeled with [^{3}H]cholesterol and then incubated with mixed micelles at their apical side and with human HDL_3 (40 μg/ml) on their basolateral side. As shown in **Fig. 7**, inhibition of SR-BI expression significantly decreased apical cholesterol efflux.

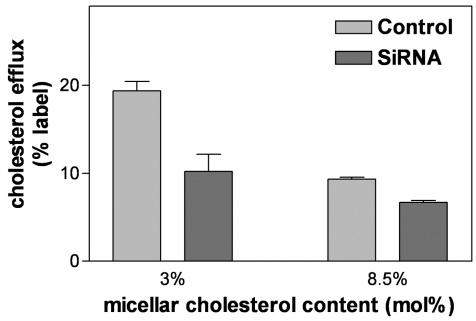

Fig. 7. Effect of inhibition of SR-BI expression by means of RNA interference on cholesterol efflux capacity. Differentiated CaCo-2 cells grown on permeable membranes were labeled with [^{3}H]cholesterol for 48 h. Cells were then transfected with SR-BI-specific siRNA or scrambled, randomly generated siRNA using Ambion's Si-Port Lipid reagent in DMEM containing 0.5% BSA for 24 h. Cells were incubated for 2 h at the apical side with mixed micelles [9.7 mM tc, 6.5 mM eypc, and 0.5 or 1.5 mM (3 or 8.6 mol%) cholesterol] and on their basolateral side with 40 μg/ml human HDL_3. Values are means ± SD of three wells. Differences between control and siRNA reached statistical significance for both types of micelles ($P < 0.05$, *t*-test). The experiment was performed twice with similar results.

SR-BI protein levels correlate with cellular cholesterol levels

Because SR-BI overexpression did not stimulate cholesterol uptake but significantly stimulated efflux, and because SR-BI inhibition reduced efflux capacity, we hypothesized that the function of apically expressed SR-BI might be to prevent cholesterol uptake and/or to enhance the apical efflux of acquired cholesterol. To test the hypothesis that SR-BI expression might itself be regulated by the level of cellular cholesterol, we measured SR-BI protein levels in CaCo-2 cells treated with different micellar solutions known to modulate cell cholesterol levels. Results showed that SR-BI expression in CaCo2 cells was regulated under the same conditions in which cellular cholesterol content was altered by treatment with micelles. **Figure 8A** shows a representative immunoblot of cells treated with identical micellar solutions as in Fig. 2. The level of expression of SR-BI protein followed the same pattern as total cellular cholesterol, with decreased expression in cells that were depleted of cholesterol and increased expression in cells that were loaded with cholesterol (Fig. 8B).

DISCUSSION

SR-BI is a HDL receptor (8–10) and is highly expressed in organs that display the greatest HDL binding and cholesteryl ester uptake, such as the liver and steroidogenic tissues. Recently, it was shown that SR-BI is also expressed in significant amounts in the small intestine (7, 8, 11–13, 17), with the highest levels in duodenum and jejunum (11, 13, 17), where cholesterol absorption is most efficient (1, 2). Furthermore, SR-BI is mainly expressed on the apical side of the enterocyte (7, 11–13). This expression pattern of SR-BI and its ability to also facilitate the influx of free cholesterol (23) make it an attractive candidate receptor for the absorption of free dietary and biliary cholesterol. Hauser et al. (7) reported that protein-mediated uptake of cholesterol from micelles by rabbit BBMVs decreased when a SR-BI-blocking antibody or free apoA-I was present. The same group recently estimated the contribution of SR-BI to the absorption process at ~25% (14). On the other hand, mice that lack functional SR-BI showed either unaltered (16) or slightly increased intestinal absorption (15), and it was concluded that SR-BI did not play a role in intestinal cholesterol absorption. To shed more light on these apparent inconsistencies, we studied cholesterol uptake from micelles using CaCo-2 cells, a generally accepted model for intestinal epithelium (35).

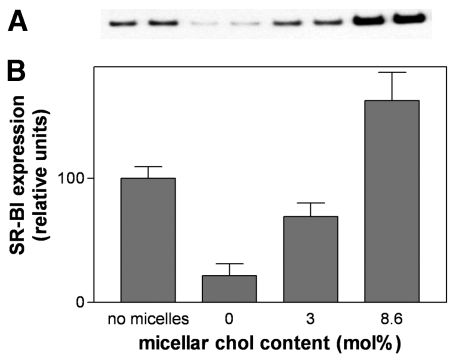

Fig. 8. Effect of micellar cholesterol (chol) loading on SR-BI expression. CaCo-2 cells were grown on standard dishes until 3 days after confluence and were incubated with mixed micellar solutions [9.7 mM tc, 6.5 mM eypc, and 0, 0.5, or 1.5 mM (0, 3, or 8.6 mol%) cholesterol] for 24 h. Cellular cholesterol levels from the same experiment are shown in Fig. 2. A: The upper immunoblot (10 μg protein/well) depicts SR-BI levels from one experiment, and the bar graph (B) shows mean relative SR-BI expression levels from three independent experiments, each carried out in triplicate dishes. Differences between all groups reached statistical significance [n = 3, one-way ANOVA with Bonferroni's multiple comparison, $P < 0.05$ or $P < 0.01$ (0% versus 8.6%)].

We first assessed the expression of SR-BI in CaCo-2 cells. Whereas subconfluent CaCo-2 cells did not express significant amounts of SR-BI, postconfluent cells did so abundantly. Using a biotinylation approach, we found that SR-BI was localized predominantly at the surface of differentiated CaCo-2 cells and that most surface SR-BI was confined to the apical compartment. This was confirmed by confocal microscopy (data not shown) and the fact that SR-BI-specific binding of HDL occurred predominantly at the apical surface. These findings are in contrast to the even distribution of SR-BI among both membranes in CaCo-2 cells recently reported by Cai et al. (11). Differences might be attributable to the different methods used or to cell clone differences. We also showed that SR-BI in CaCo-2 cells is functionally active, because HDL-derived cholesteryl esters were selectively absorbed. Despite significant, although lower, binding of HDL to the basolateral surface, SR-BI-mediated selective uptake occurred almost exclusively at the apical surface. The reason for the almost complete absence of selective uptake by SR-BI at the basolateral surface is not clear.

Cholesterol uptake was studied by incubating CaCo-2 cells with mixed micellar solutions. Bile salt concentrations in the human small intestine average 14.5 mM (36). Therefore, we used concentrations that were significantly higher than those described in the literature, typically 5 mM (14, 37, 38), which does not exceed the critical micellar concentration of 4–6 mM (39) for this bile salt. The high bile salt concentration appeared not to affect cell viability, even after 24 h of incubation. The degree of cholesterol saturation of the mixed micelles was varied by altering the amounts of cholesterol. Our findings show that CaCo-2 cells that were incubated with cholesterol-unsaturated micelles became cholesterol depleted; only incubation with cholesterol-supersaturated solutions, in which part of the cholesterol and phospholipids were present in vesicles, resulted in net cellular accumulation of cholesterol. This is not unexpected, because solutions that contain only micelles at equilibrium are by definition unsaturated (40) and can solubilize additional cholesterol.

We then studied the role of SR-BI in cholesterol uptake from mixed micelles. Surprisingly, we did not observe a decrease in cholesterol uptake, even from supersaturated micelles, when a SR-BI-blocking antibody was added. This

is in contrast to earlier reports showing inhibition of uptake of free and esterified cholesterol by rabbit BBMVs in the presence of either anti-SR-BI IgG (14) or the SR-BI ligand apoA-I (7). In fact, our SR-BI-blocking antibody actually slightly increased cholesterol uptake. This discrepancy may be explained by certain differences in the experimental approaches. We were unable to block SR-BI-mediated HDL binding and selective uptake in CHO cells when using the same anti-SR-BI antibody (directed against the extracellular domain of SR-BI) that was previously used to block cholesterol uptake in BBMVs (14). In contrast, our SR-BI-blocking antibody was highly effective in blocking receptor activity in CHO and CaCo-2 cells. Furthermore, Hauser et al. (7) used relatively low cholesterol concentrations in their micellar solutions (4 μM, as opposed to up to 1.5 mM in present study), which could perhaps result in a reequilibration of labeled cholesterol between micelles and HDL (which was added as an inhibitor of SR-BI function), thus leading to an apparent decrease in [^{3}H]cholesterol uptake. In support of our observations, Jourdheuil-Rahmani et al. (41) showed that uptake of cholesterol from micellar solutions by BBMVs was enhanced in the presence of the SR-BI ligand biliary anionic peptide factor (a protein with strong resemblance to apoA-I) or in the presence of purified apoA-I. It should be noted, however, that bile salt concentrations in that study were rather low (5 mM), so it cannot be excluded that part of the lipids and apoproteins were forming HDL-like particles from which cholesterol could be absorbed by SR-BI-mediated selective uptake. In agreement with that possibility was the observation that an antibody directed against the extracellular loop of SR-BI abolished cholesterol uptake (41).

Additional findings presented in our study further indicate that SR-BI does not directly contribute to micellar cholesterol uptake in CaCo-2 cells. Adenovirus-mediated overexpression of SR-BI in CaCo-2 cells did not stimulate the influx of cholesterol from mixed micelles. Partial suppression of SR-BI protein by siRNA also did not decrease the uptake of micellar cholesterol. In fact, as was the case for antibody-mediated inhibition of SR-BI, uptake was somewhat increased. Thus, our observations argue against a direct role for SR-BI in the uptake of micellar cholesterol.

Besides mediating the selective uptake of cholesteryl esters from HDL, SR-BI can also facilitate the efflux of free cholesterol toward HDL (24–26, 42) and other acceptors, such as cyclodextrins (27) or phospholipid vesicles (24, 42). We observed that SR-BI overexpression strongly enhanced apical cholesterol efflux to micelles, even to supersaturated micelles. Partial suppression of SR-BI expression with siRNA decreased effluxing capability. To our knowledge, this is the first time that bile salt/phospholipid micelles have been identified as potential acceptors for SR-BI-mediated cholesterol efflux. In facilitating cholesterol efflux, SR-BI could act analogous to or in cooperation with ABC transporters, such as ABCG5/ABCG8 heterodimers, which decrease intestinal cholesterol absorption (20). Although ABCA1 also has been implicated in intestinal cholesterol efflux (17), recent studies show that ABCA1 is expressed predominantly on the basolateral surface of CaCo-2 cells (43, 44) and that it functions to mediate cellular cholesterol efflux from the basolateral surface to HDL or apoA-I (44), analogous to cultured gallbladder epithelial cells (45) or chicken intestinal cells (46). Therefore, SR-BI and ABCA1 are localized on different sides of the polarized CaCo-2 cell and differ in the direction to which they efflux cellular cholesterol. In the case of ABCA1, efflux basolaterally would not necessarily affect absorption from the apical side (44). On the other hand, SR-BI-mediated efflux from the apical surface would decrease net absorption. Intestinal SR-BI may also be involved in apical secretion of cholesterol from basolaterally acquired HDL cholesterol, similar to the model for biliary cholesterol secretion proposed by Silver et al. (47).

Our finding that SR-BI expression in CaCo-2 cells correlates with cellular cholesterol levels further suggests that SR-BI may play a regulatory role in cholesterol absorption. We observed that cholesterol loading of CaCo-2 cells markedly induces expression of SR-BI, whereas cholesterol depletion leads to a significant decrease. This is in contrast to the situation in adrenal cells, in which SR-BI appeared to be upregulated when cells were depleted of cholesterol (48), and might reflect cell-specific functions. Data on the regulation of intestinal SR-BI expression are scarce. Mice and rats with defects in biliary lipid secretion, either as the result of targeted gene disruption or by bile diversion, were shown to have decreased intestinal SR-BI expression (13), whereas hamsters on a high-sucrose diet with strongly increased biliary cholesterol secretion showed a marked increase of SR-BI expression in the small intestine (49). Recently, it was shown that SR-BI expression in hepatoma cells is stimulated by liver X receptor α (LXRα) (50), a nuclear receptor that is also expressed in the intestines. Cholesterol-derived oxysterols could theoretically activate LXRα in CaCo-2 cells and thus account for increased SR-BI expression. ABCA1, which is also under the control of LXR, is indeed upregulated in CaCo-2 cells by oxysterols or LXR-specific ligands (44). Likewise, LXR activation leads to upregulation of ABCG5/ABCG8 in mouse intestine (51). We speculate that SR-BI is also upregulated in the intestine during exposure to high amounts of cholesterol, which then would allow the enterocyte to efflux excess cholesterol back to the lumen.

Taken together, our data indicate that SR-BI does not act as a receptor for micellar cholesterol; rather, they support a model in which intestinally expressed SR-BI may modulate cholesterol absorption by facilitating cholesterol excretion toward mixed micelles.

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