Class B Scavenger Receptor-Mediated Intestinal Absorption of Dietary β-Carotene and Cholesterol†

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ABSTRACT: There is now a general consensus that the intestinal absorption of water-insoluble, dietary lipids is protein-mediated, but the assignment of protein(s) to this function is still a matter of debate. To address this issue, we measured β-carotene and cholesterol absorption in wild-type and SR-BI knockout mice and the uptake of these lipids in vitro using brush border membrane (BBM) vesicles. From the comparison of the in vivo and in vitro results we conclude that both BBM-resident class B scavenger receptors, SR-BI and CD36, can facilitate the absorption of β-carotene and cholesterol. SR-BI is essential for β-carotene absorption, at least in mice on a high fat diet. This is due to the fact that the absorption of β-carotene is restricted to the duodenum and SR-BI is the predominant receptor in the mouse duodenum. In contrast, SR-BI may be involved but is not essential for cholesterol absorption in the small intestine. The question of whether SR-BI contributes to cholesterol absorption in vivo is still unresolved. Transfection of COS-7 cells with SR-BI or CD36 confers on these cells lipid uptake properties closely resembling those of enterocytes and BBM vesicles. Both scavenger receptors facilitate the uptake of dietary lipids such as β-carotene, free and esterified cholesterol, phospholipids, and fatty acids into COS-7 cells. This lipid uptake is effected from three different lipid donor particles: mixed bile salt micelles, phospholipid small unilamellar vesicles, and trioleoylglycerol emulsions which are all likely to be present in the small intestine. Ezetimibe, a representative of a new class of drugs that inhibit intestinal cholesterol absorption, blocks SR-BI- and CD36-facilitated uptake of cholesterol into COS-7 cells.

Extensive studies of cholesterol metabolism have revealed many of the mechanisms involved in the regulation of whole body cholesterol homeostasis. However, a major lacuna is the definition of the pathway involved in the uptake of cholesterol, and other hydrophobic molecules such as nonpolar vitamins (e.g., β-carotene), from the lumen of the small intestine into epithelial cells (enterocytes). Intestinal cholesterol and β-carotene absorption is important in both human health and disease. There is a clear correlation between the efficiency of cholesterol absorption and the plasma low-density lipoprotein (LDL) cholesterol level (*1*) and between elevated plasma LDL−cholesterol and the probability of developing atherosclerosis (*2*). In the case of β-carotene, consumption of this nutrient is related to a reduced risk of heart disease and cancer (*3*, *4*).

The classical view is that intestinal absorption of cholesterol (*5*, *6*) and other dietary lipids including fat-soluble, nonpolar vitamins (*3*) is a multistep process involving the solubilization of dietary lipids and their hydrolytic products in mixed bile salt micelles, diffusion of these micelles through the aqueous phase of the intestinal lumen to the brush border membrane (BBM),[1] and finally passive diffusion down a concentration gradient of individual lipid molecules across the BBM into the cytoplasm of enterocytes. In contrast to this simple diffusion model, kinetic evidence obtained with model systems such as BBM vesicles (BBMV) and Caco-2 cells showed that the uptake into the BBM of cholesterol and other hydrophobic, dietary lipids is protein-mediated

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[1] Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; apoA-I, apolipoprotein A-I; BBM, brush border membrane; BBMV, brush border membrane vesicles; CD36, cluster determinant 36; CE, cholesteryl ester; POPC, 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine; NPC1L1, Niemann−Pick C1-like 1; SR-BI, scavenger receptor class B, type I; SUV, small unilamellar vesicle.

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(*7*, *8*). Such a mechanism is supported by the finding that both macromolecules such as proteins, α- and β-peptides (*9*−*11*), and small molecules such as ezetimibe (*12*, *13*) can inhibit intestinal cholesterol uptake/absorption. A protein-facilitated lipid uptake mechanism is also consistent with the observation of a pronounced interindividual variability in cholesterol absorption efficiency in both animals and humans that has been attributed to genetic factors at the enterocyte level (*1*, *14*). It is noteworthy that there is also interindividual variability in the response to dietary β-carotene (*15*), and the absorption is enhanced if the diet contains more fat (*3*). In addition, the fact that β-carotene uptake into CaCo-2 cells exhibits isomer specificity suggests that carotenoid uptake by intestinal cells is a facilitated process (*16*).

At present, the identity of the BBM transport protein(s) mediating the uptake of dietary lipids, though of immediate interest, is still a matter of debate. On one hand, we showed in model systems such as BBMV that both scavenger receptor class B, type I (SR-BI) (*17*), and CD36 (*18*) facilitate the uptake of free and esterified cholesterol and other dietary lipids. Consistent with this, both SR-BI and CD36 are expressed in the small intestines of animals (*17*, *19*−*23*) and humans (*18*, *24*). Several publications have appeared recently providing further evidence for SR-BI being involved in intestinal cholesterol absorption (*12*, *25*−*28*). On the other hand, the involvement of SR-BI in cholesterol uptake has been questioned because intestinal cholesterol absorption was shown to be unaffected by deletion of the SR-BI gene in mice (*12*, *29*). There is also evidence that SR-BI is important for the transport of carotenoids; thus, a gene encoding a protein that is homologous to SR-BI is essential for the cellular uptake of carotenoids in *Drosophila* (*30*).

Here, we address this issue of the identity of BBM lipid transport proteins by combining in vivo feeding studies with in vitro experiments. We show that SR-BI facilitates β-carotene absorption in the mouse small intestine.

EXPERIMENTAL PROCEDURES

Materials

C57BL/6 mice (aged 8−12 weeks) were obtained from Jackson Laboratories (Bar Harbor, ME). SR-BI knockout (SR-BI$^{-/-}$) mice on a mixed C57BL/6 × 129/SV background, originally described by Rigotti et al. (*31*), were generated by crossing either male and female SR-BI $^{+/-}$ mice or male SR-BI$^{-/-}$ and female SR-BI $^{+/-}$ mice. SR-BI $^{+/+}$ mice obtained in this fashion were used as control animals. Genotypes were determined by polymerase chain reaction as described (*31*). All animal studies were approved by the Institutional Animal Care and Use Committee of the Children's Hospital of Philadelphia.

Radioactively labeled lipids were purchased as follows: [4-^{14}C]cholesterol (51 mCi/mmol), [1,2-^{3}H]cholesterol (45 Ci/mmol), and choline methyl[^{14}C]sphingomyelin (52 mCi/mmol) from Perkin-Elmer Life Sciences (Boston, MA); [1α,2α(N)-^{3}H]cholesteryl oleyl ether (CE) (49 Ci/mmol) from Amersham Pharmacia Biotech (Piscataway, NJ); [5,6-^{3}H]-αβ-sitostanol (50 Ci/mmol) from American Radiolabeled Chemicals Inc. (St. Louis, MO). [6,7,6′,7′-^{14}C]-β-carotene (185 μCi/mg) was a gift of Dr. W. Cohn, Roche Vitamins (Basel), and its purity was better than 95% as determined by analytical HPLC. 1-Palmitoyl-2-oleoyl-*sn*-phosphatidylcholine (POPC) and egg phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Alabaster, AL) and Lipid Products (Nutfield, Surrey, U.K.), respectively. Trioleoylglycerol, cholesterol, cholesteryl oleate, and sodium taurocholate were purchased from Sigma. Apolipoprotein A-I was isolated from human HDL as described (*32*). Ezetimibe, 1-(4-fluorophenyl)-(3*R*)-[3-(4-fluorophenyl)-(3*S*)-hydroxypropyl]-(4*S*)-(4-hydroxyphenyl)-2-azetidinone, was synthesized as described (*33*). However, the yields obtained for most of the reaction steps were significantly lower than those reported previously. Consequently, several reactions were optimized in order to obtain a reasonable overall yield. The final product was purified by preparative HPLC on Nucleosil 100-5, VP 250/21. The purity of ezetimibe used in this study was >98% as determined by analytical HPLC on Nucleosil 100-5 as well as 1D and 2D 500 MHz NMR. Polyclonal rabbit anti-human CD36 IgG (pAb300) raised against amino acids 1−300 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal anti-human CD36 IgM raised against the entire CD36 was from Sigma. Three polyclonal rabbit anti-SR-BI antibodies were used, two anti-mouse SR-BI (IgG) antibodies with the epitope corresponding to amino acids 230−380 (pAb150) and another one to the C-terminal 15 amino acids (pAbI15) obtained from Novus Biologicals (AbCam, Cambridge, U.K.) and an anti-human SR-BI antibody raised against a C-terminal peptide (pAb589) from Genosys (Cambridge, U.K.) (*10*). A polyclonal rabbit anti-actin antibody raised against a C-terminal fragment was obtained from Sigma. The monoclonal anti-human sucrase isomaltase antibody (IgG) was a gift from Prof. Hans-Peter Hauri of the Biocenter of the University of Basel.

Methods

Intestinal Lipid Absorption in Mice. Mice were fed either a basal diet (Teklad LM 485; North Penn Feeds, Inc., Hatfield, PA) containing 5 wt % fat and no cholesterol or a high-fat, high-cholesterol diet (Purina Mouse Chow 5015 supplemented with 7.5 wt % cocoa butter and 1.25 wt % cholesterol obtained from the same manufacturer) for a period of 3 weeks. Mice were housed in a temperature- and humidity-controlled animal facility with 12 h light−dark cycles. At the end of the feeding period, animals were transferred to metabolic cages in the afternoon before the experiment. On the following morning, mice were fasted for 6 h, and 2 h before the start of the dark cycle the animals were given 0.1 mL of corn oil containing 1 μCi of either [^{14}C]-β-carotene or [^{14}C]cholesterol and 0.4 μCi (8 pmol) of [^{3}H]-αβ-sitostanol by stomach gavage. To prepare the gavage, aliquots of [^{14}C]cholesterol or [^{14}C]-β-carotene (handled in dim light under nitrogen) and [^{3}H]-αβ-sitostanol in organic solvent were mixed with an appropriate volume of corn oil, and the organic solvent was removed by slowly bubbling N_2 gas through the mixture for 3−4 h. [^{3}H]-αβ-Sitostanol was included in the corn oil as a nonabsorbable sterol in order to correct for the recovery of nonabsorbed fecal cholesterol or β-carotene. The mice were returned to their metabolic cages where they had free access to their diet and water. Feces were collected for 24 h. Total feces were weighed and homogenized in water (10% w/v). An aliquot of an appropriate dilution of this homogenate was

extracted with an equal volume of chloroform/methanol (2:1 v/v) to determine the amount of radioactive sterols or β-carotene excreted in the feces by liquid scintillation counting. Cholesterol and β-carotene absorption efficiencies were calculated using the fecal dual-isotope ratio method (*34*). When ezetimibe was used as an inhibitor, the appropriate amount (10 mg/kg body weight) was dissolved in 0.15 mL of corn oil and administered by gavage to the mice 30 min before the gavage of corn oil containing the radioactive lipids. After 24 h, the animals were anesthetized by intraperitoneal injection of 150 mg of ketamine and 10 mg of xylazine per kilogram of body weight (Research Biomedical Instruments, Natick, MA) and sacrificed. The small intestines were removed within 5 min of death and divided into four sections: part A (duodenum) = proximal 5 cm, part B (proximal jejunum) = subsequent 5 cm, part C (distal jejunum) = subsequent 10 cm, and part D (ileum) = subsequent 10 cm. Each part was cut longitudinally, rinsed with ice-cold buffer A [0.01 M Tris, pH 7.3, 0.3 M D-mannitol, 5 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1 mM phenylmethanesulfonyl fluoride, 1 μg/mL antipain, and 0.02% sodium azide] and immediately frozen in liquid N_2. Blood samples were collected via the vena cava, and serum cholesterol levels were measured enzymatically using Sigma Diagnostic Commercial Kit No. 352.

Brush Border Membrane Vesicles. BBMV were prepared from samples of frozen mouse intestine by a scaled-down version of the Mg^{2+} precipitation method (*35*). Prior to use BBMV were subjected to a routine quality control (*18*). The uptake of β-carotene, cholesterol, and CE was determined at room temperature over a period of 20 min unless otherwise stated, and the kinetics of lipid uptake into the BBM of BBMV from different donor particles [mixed sodium taurocholate micelles, phospholipid small unilamellar vesicles (SUV), and POPC-stabilized trioleoylglycerol emulsions] were monitored using established procedures (*7*, *18*, *36*). Digestion of BBMV with proteinase K was carried out as described previously (*7*). The levels of SR-BI and CD36 protein present in BBMV were determined by fractionating a total of 50 μg of BBMV protein on 7.5% SDS−PAGE and immunoblotting with anti-mouse SR-BI (pAbI15) and anti-human CD36 antibodies (pAb300), respectively (*10*, *17*, *18*). Both SR-BI and CD36 bands were standardized to the actin band of BBMV subjected to the same immunoblot procedure using an anti-actin antibody. The same results were obtained when SR-BI and CD36 were standardized to sucrase isomaltase.

Cell Culture. Kidney COS-7 were maintained and transiently transfected with either SR-BI or CD36 cDNA or the vector DNA alone, as described before (*37*). Uptake of radioactive lipid from POPC SUV, trioleoylglycerol/POPC emulsions, or mixed bile salt micelles was measured using established procedures (*38*). The micelles were prepared by dissolving sodium taurocholate, cholesterol, and POPC at a molar ratio of 10/0.1/1 in minimal essential medium. POPC/apoA-I discoidal complexes were prepared as described before (*38*). To study the effects of ezetimibe on lipid uptake, the COS-7 cells were pretreated for 1 h with ezetimibe dissolved in dimethyl sulfoxide (DMSO) and then placed in fresh medium containing the lipid donor particles. The final volume of DMSO in the extracellular medium was 0.5% v/v, the resulting DMSO concentration having no effect on lipid uptake.

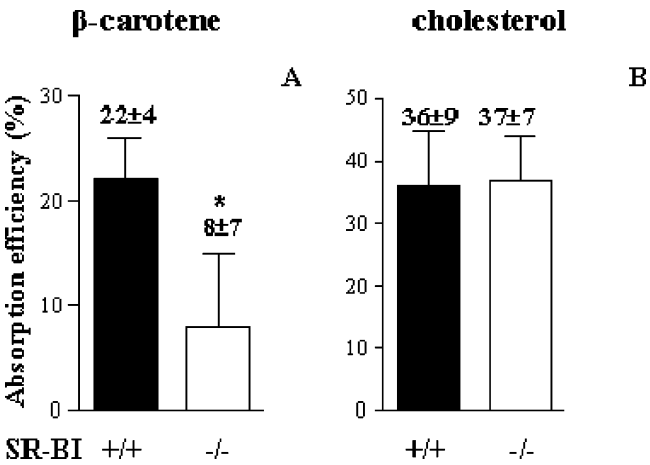

FIGURE 1: Effects of SR-BI gene deletion on β-carotene (panel A) and cholesterol intestinal absorption (panel B) in mouse (in vivo) experiments. (A) The efficiency of β-carotene absorption was measured in age- and sex-matched wild-type (SR-BI$^{+/+}$, solid bar) and SR-BI knockout (SR-BI$^{-/-}$, open bar) mice (four males and four females, 9−12 months of age, in each group) fed a high-fat, high-cholesterol diet. The absorption efficiencies are reported as the mean ± SD (n = 8), and the asterisk indicates a significant difference between the two groups (p < 0.001, Anova followed by the Mann−Whitney test). (B) SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (four males and four females, 3−6 months of age, in each group) were used to measure cholesterol absorption efficiencies expressed as the mean ± SD (n = 8).

Statistical Analysis. Results are expressed as the mean ± one standard deviation (SD). Statistical evaluation was performed using Excel and SPSS version 11.5. ANOVA, Student's t test, and Mann−Whitney test were used as appropriate.

RESULTS AND DISCUSSION

In Vivo and in Vitro Comparisons of Intestinal Uptake of β-Carotene and Cholesterol. Lipid uptake is defined here as the transfer of dietary lipids from a lipid donor particle (mixed bile salt micelle, SUV, or lipid emulsion) to the BBM and is regarded as the first step of the multistep lipid absorption process. The absorption of both β-carotene and cholesterol in mouse feeding (in vivo) experiments using wild-type and SR-BI knockout mice (Figure 1) was compared to in vitro uptake of these two lipids using BBMV (Figure 2).

Figure 1A shows that the absorption of β-carotene is significantly reduced in SR-BI$^{-/-}$ mice (8 ± 7%) compared to SR-BI$^{+/+}$ mice (22 ± 4%, p < 0.001) when fed a high-fat, high-cholesterol diet. The β-carotene absorption efficiency observed with SR-BI$^{-/-}$ mice under these conditions was comparable to that measured with SR-BI$^{+/+}$ mice fed a basal diet. Thus, for SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (n = 8 per group) fed a basal diet the β-carotene absorption efficiencies were 9 ± 5% and 5 ± 3%, respectively, and these values were not significantly different from one another. These findings are interpreted to indicate that (I) SR-BI is essential for β-carotene absorption in the small intestine of mice fed a high-fat, high-cholesterol diet and (II) the effective uptake of β-carotene requires fat in the diet otherwise very low levels of absorption result (*3*). In contrast to β-carotene

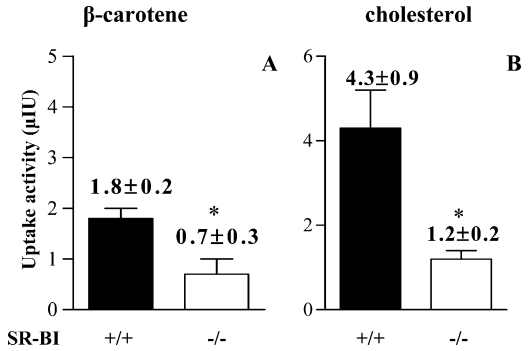

FIGURE 2: β-Carotene and cholesterol uptake in mouse BBMV. (A) β-Carotene uptake was measured after 20 min of incubation at room temperature of egg PC SUV as the donor (2 μg of total lipid/mL) containing 1 mol % [^{14}C]-β-carotene with duodenal BBMV (0.15 mg of protein/mL) as the acceptor. Duodenal BBMV were prepared from SR-BI$^{+/+}$ (solid bars) and SR-BI$^{-/-}$ mice (open bars) treated as described in Figure 1. The uptake activities are presented as the mean $\pm$ SD ($n = 3$), and the asterisk indicates a significant difference between the two groups ($p = 0.002$, two-tailed, unpaired Student's t test). (B) Uptake of [1α,2α(N)-^{3}H]cholesterol was measured using egg PC SUV as the donor and duodenal BBMV prepared from SR-BI$^{+/+}$ (solid bars) and SR-BI$^{-/-}$ mice (open bars) as the acceptor. Experimental conditions are described in panel A. The uptake activities are presented as the mean $\pm$ SD ($n = 4$) and are significantly different ($p = 0.0007$; cf. panel A).

absorption (Figure 1A), the absorption of cholesterol (Figure 1B) was identical within the error of the measurement in SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice, confirming literature reports (*12*, *29*). This result was interpreted in the literature in two ways: (I) as evidence that SR-BI is not involved in intestinal cholesterol absorption (*12*, *39*) and (II) that SR-BI may be involved but is not essential for cholesterol absorption (*29*). The data presented here are consistent with the second interpretation (see below).

By comparing the in vivo experiments (Figure 1) with in vitro experiments (Figure 2), important insights are gained into the mechanism of intestinal lipid uptake. Figure 2 shows that the uptake activities of both β-carotene (0.7 $\pm$ 0.3 versus 1.8 $\pm$ 0.2 μIU, $p = 0.002$) and cholesterol (1.2 $\pm$ 0.2 versus 4.3 $\pm$ 0.9 μIU, $p < 0.001$) were significantly lower in SR-BI$^{-/-}$ mice compared to wild-type mice. This reduction is apparently due to the lack of SR-BI in the duodenum of SR-BI$^{-/-}$ mice, indicating that SR-BI facilitates the uptake into the BBM of hydrophobic molecules such as β-carotene and cholesterol.

Since our previous in vitro studies of lipid uptake were carried out almost exclusively with BBMV derived from total rabbit small intestine, we wished to compare the β-carotene uptake in mouse and rabbit BBMV. The results of β-carotene uptake measurements using rabbit BBMV as the acceptor and three different kinds of donor particles are compiled in Table 1. Facilitated β-carotene uptake was observed with all three kinds of donor particles. Comparison of β-carotene uptake in intact and proteinase K-treated BBMV (Table 1) indicates that the uptake of the fat-soluble vitamin is receptor-mediated. From an inspection of this table, it is obvious that β-carotene uptake was most efficient from mixed taurocholate micelles. The pseudo-first-order rate constant derived from the kinetics of β-carotene uptake from mixed bile salt micelles was about 3 orders of magnitude larger than the corresponding rate constants obtained with SUV of egg phosphatidylcholine and trioleoylglycerol emulsions. This finding is consistent with published data showing that the kinetics of cholesterol uptake into rabbit BBMV was 3−4 orders of magnitude faster when mixed bile salt micelles rather than egg lysophosphatidylcholine micelles were used as the donor (*40*). ApoA-I has been shown to inhibit sterol uptake into rabbit BBMV (*9*, *10*). In the presence of apoA-I (0.15 mg/mL), or the anti-SR-BI antibody pAb150 at 0.1 mg/mL, β-carotene uptake was significantly inhibited (Table 1), consistent with the data in Figures 1 and 2.

Figures 1 and 2 imply the involvement of SR-BI in both β-carotene and cholesterol absorption/uptake. If SR-BI is indeed involved in intestinal cholesterol absorption/uptake, the data in Figure 1B warrant closer scrutiny. A possible explanation of the result of this animal experiment is that the lack of SR-BI in SR-BI$^{-/-}$ mice is compensated for by alternate mechanisms such as the activity of other transport proteins. The second scavenger receptor, CD36 (*18*), present in the BBM, Niemann−Pick C1-like 1 (NPC1L1) protein (*39*), and other yet unidentified proteins are possible candidates. These alternate transport proteins may even be upregulated in SR-BI$^{-/-}$ mice. Another possibility which might add to the difficulty in elucidating the cholesterol absorption mechanism is that cholesterol uptake into the BBM may not be the rate-limiting step in the overall cholesterol absorption process.

Distribution of Class B Scavenger Receptors, SR-BI and CD36, and Cholesterol Uptake Activity Measured in the Four Sections of the Mouse Small Intestine. The distribution of the two class B scavenger receptors, SR-BI and CD36, and the uptake of free cholesterol were measured along the gastrocolic axis. For this measurement, BBMV were prepared from the four consecutive sections of the small intestine, and the expression of SR-BI and CD36 was determined by immunoblot analysis of the BBMV (Figure 3), and the uptake of free cholesterol into the BBM of these vesicles was determined as described in Table 2. While SR-BI was reported to be most abundant in the proximal small intestine of the mouse (*21*−*23*), the distribution of CD36 in the mouse intestine has been unknown. We confirmed that SR-BI is concentrated in the duodenum showing a steep gradation of expression along the gastrocolic axis (Figure 3) (*21*−*23*). In contrast, CD36 was present in all four sections of the mouse small intestine with a low level of expression in the duodenum and a maximum in the jejunum (Figure 3). No SR-BI was detected in BBMV made from the four sections of the small intestine of SR-BI$^{-/-}$ mice.

The significant reduction of β-carotene absorption in SR-BI$^{-/-}$ mice (Figure 1A) can be rationalized by the fact that, in mouse, SR-BI is concentrated in the duodenum and CD36 in the jejunum and ileum and by postulating that the proximal small intestine is the principal region of β-carotene absorption. This is reasonable considering that in this region triacylglycerol emulsion particles still exist and that these particles are the preferred solvent of very hydrophobic lipid molecules such as β-carotene (*3*).

The uptake of free cholesterol into BBMV prepared from the four sections of the small intestine was determined as described in Table 2. Comparing BBMV from SR-BI$^{+/+}$ mice with those of SR-BI$^{-/-}$ mice, a significant reduction in cholesterol uptake was only observed in the duodenum while all other sections showed similar behavior (Table 2). This result is consistent with SR-BI being concentrated in the

Table 1: β-Carotene Uptake Determined in Rabbit BBMV

donor	rabbit BBMV	β-carotene uptake (%)[a]	inhibition (%)	time of β-carotene uptake
egg phosphatidylcholine SUV	intact	15 ± 3		120
	proteinase K treated	0−1.5	90−100	
	apoA-I treated	3	80	
	anti-SR-BI treated	4	75	
trioleoylglycerol/POPC emulsion	intact	27 ± 4		120
	proteinase K treated	0−2	90−100	
	apoA-I treated	4	85	
mixed taurocholate micelles	intact	13 ± 2		10
	proteinase K treated	2	85	
	apoA-I treated	1.5	90	

[a] The kinetics of β-carotene uptake was measured using three different kinds of donor particles and rabbit BBMV as the acceptor. Mixed bile salt micelles, SUV of egg phosphatidylcholine, and POPC-stabilized trioleoylglycerol emulsions were used as lipid donor particles, each one containing 1 mol % radioactive β-carotene. β-Carotene uptake from mixed sodium taurocholate micelles ([sodium taurocholate] = 5 mM, [POPC] = 0.5 mM, [cholesterol] = 0.05 mM), egg phosphatidylcholine SUV (0.1 mg of total lipid/mL), and trioleoylglycerol/POPC emulsion (0.1 mg of total lipid/mL) into the BBM of rabbit BBMV ([BBMV] = 2 mg of protein/mL, 5 mg of protein/mL and 5 mg of protein/mL, respectively) was measured at room temperature for the indicated times. The apoA-I concentration was 0.15 mg/mL, and that of the anti-SR−BI antibody, pAb150, was 0.1 mg/mL.

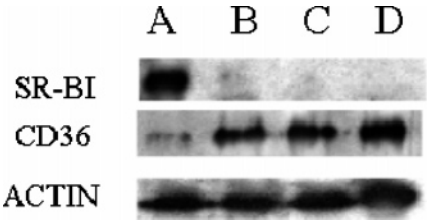

FIGURE 3: Distribution of SR-BI and CD36 along the gastrocolic axis of the mouse small intestines. Different sections of the small intestine (A = duodenum, B = proximal jejunum, C = distal jejunum, D = ileum) were collected from a pool of four mice fed a high-fat, high-cholesterol diet, and BBMV were prepared from the four sections A−D. Immunoblots were obtained as described in Methods. The apparent molecular masses of SR-BI, CD36, and actin were approximately 80, 75, and 45 kDa, respectively.

mouse duodenum (cf. Figure 3). It is in qualitative agreement with the results in Figure 2B; the quantitative difference in the cholesterol uptake values between Table 2 and Figure 2B is due to different experimental conditions.

The following series of experiments show that the cholesterol uptake measured in all four sections of the mouse small intestine is protein-mediated. Proteinase K treatment of all four types of BBMV made from either SR-BI$^{+/+}$ or SR-BI$^{-/-}$ mice (cf. Table 2) reduced the cholesterol uptake activity to 1−1.5 μIU. This value compares well to the activity of 1 μIU calculated from the first-order rate constant $k_1 = 0.08 \pm 0.02$ h^{-1} for passive diffusion of cholesterol in an aqueous medium, e.g., between two populations of SUV (*7*). The cholesterol uptake measured with BBMV prepared from the jejunum and ileum where practically no or very little SR-BI was present exceeded passive diffusion by a factor of about 10 (Table 2). This observation indicates that proteins other than SR-BI are responsible for cholesterol uptake. A possible candidate is CD36, the second scavenger receptor evidently present in the BBM (*18*, *19*, *23*, *24*). Interestingly, the distribution of CD36 along the mouse small intestine is complementary to that of SR-BI with little CD36 in the duodenum and the major part of the protein being present in the jejunum and ileum (Figure 3). The following experiments using anti-CD36 antibodies suggest that CD36 has the ability to facilitate intestinal cholesterol uptake, as proposed in ref *18*. Cholesterol uptake in the presence of the anti-CD36 antibody pAb300 was significantly reduced

Table 2: Uptake Activity of Free Cholesterol Determined in BBMV Prepared from Four Sections of the Small Intestine of C57 BL/6 Wild-Type (SR-BI$^{+/+}$) and Knockout (SR-BI$^{-/-}$) Mice Fed a High-Fat, High-Cholesterol Diet[a]

mouse genotype	intestinal section	cholesterol uptake activity (μIU) (−) anti-CD36 antibody	cholesterol uptake activity (μIU) (+) anti-CD36 antibody	% inhibition
SR-BI$^{+/+}$	duodenum	11 ± 3	8	20
	proximal jejunum	9 ± 1	3.1	74
	distal jejunum	11.5 ± 2	4.5	67
	ileum	10 ± 0.4	4.1	66
SR-BI$^{-/-}$	duodenum	5.5 ± 0.3	nd	
	proximal jejunum	8 ± 1	3.6	65
	distal jejunum	11 ± 3	3.9	71
	ileum	13 ± 4	4.7	69

[a] The small intestine of four SR-BI$^{+/+}$ and four SR-BI$^{-/-}$ mice was divided into duodenum, proximal jejunum, distal jejunum, and ileum as described in Methods, the corresponding sections of four mice were combined, and BBMV were prepared. The cholesterol uptake activity was measured with these BBMV (0.9 mg of protein/mL) and with egg PC SUV containing 1 mol % [^{14}C]cholesterol (0.05 mg of total lipid/mL) as the donor. Cholesterol uptake was measured in the absence (−) and presence (+) of the polyclonal anti-human CD36 antibody pAb300 (0.1 mg of protein/mL). The values for the cholesterol uptake activity (expressed as μIU = pmol of cholesterol transferred per minute and mg of BBMV protein) represent the mean ± SD of triplicate measurements in the absence of pAb300 and duplicate measurements in the presence of pAb300; nd, not determined. The inhibition (in %) was calculated from the cholesterol uptake activities corrected for passive diffusion.

with BBMV made from both SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice (Table 2). As a control, cholesterol uptake was measured in the presence of an antibody raised against sucrase isomaltase, the most abundant cell surface protein of the BBM. In contrast to pAb300, concentrations up to 0.1 mg of IgG/mL of this antibody had no effect on cholesterol uptake, indicating that the interaction of pAb300 with CD36 is specific and not an in vitro artifact. The observation that the anti-CD36 antibody has a limited effect and does not eliminate the residual cholesterol uptake activity in BBMV from SR-BI$^{-/-}$ mice can be interpreted in two ways: either the antibody only partially eliminated the CD36 activity or yet another, still unknown, protein is implicated in facilitated cholesterol uptake.

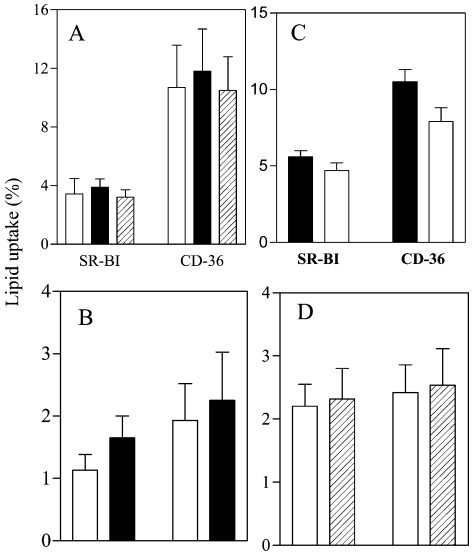

FIGURE 4: Protein-mediated lipid uptake from different donor particles into COS-7 cells transiently transfected with either SR-BI or CD36. COS-7 cells expressing either SR-BI or CD36 and control cells mock-transfected with the vector alone were incubated at 37 °C for 1 h with different donor particles, and lipid uptake was measured and expressed as the percentage of radioactivity transferred from lipid donor particles to COS-7 cells. The receptor contribution to lipid uptake was obtained by subtracting the value for control cells. (A) The lipid donor particles were SUV of 1-palmitoyl-2-oleoyl-*sn*-phosphatidylcholine (POPC) (100 μg of lipid/mL) plus 1 mol % each of cholesterol and cholesteryl oleate, dual-labeled with trace amounts of ^{14}C brain sphingomyelin (SM, open bars) and [^{3}H]cholesteryl oleyl ether (CE, solid bars) or ^{14}C-SM and [^{3}H]cholesterol (crosshatched bars). (B) The donor particles were mixed bile salt micelles consisting of sodium taurocholate (5 mM), cholesterol (50 μM), and POPC (0.5 mM) dual-labeled with trace amounts of ^{14}C-SM (open bars) and ^{3}H-CE (solid bars). (C) The donor particles were SUV of POPC (100 μg of lipid/mL) plus 1 mol % each of cholesterol and cholesteryl oleate, dual-labeled with trace amounts of ^{3}H-CE (solid bars) and [^{14}C]-β-carotene (open bars). (D) The lipid donor particles were POPC-stabilized trioleoylglycerol emulsions containing trace amounts of ^{14}C-SM (open bars) and [^{3}H]cholesterol (crosshatched bars). Values are presented as the mean ± SD of triplicate measurements from three independent experiments ($n = 9$) in panels A−C and five independent experiments ($n = 15$) in panel D. The values were compared using Anova in panel A and a two-tailed, unpaired Student's *t*-test in panels B−D; in all cases there was no significant difference ($p > 0.05$) in uptake of different lipids from a given donor particle into either SR-BI- or CD36-transfected cells.

The data in Figures 1 and 2 show that SR-BI in the duodenum is essential for β-carotene but not cholesterol absorption, consistent with the distributions of SR-BI and CD36 in the mouse small intestine (Figure 3). It should be noted that the distribution of SR-BI and CD36 in the human small intestine is significantly different from that in the mouse. SR-BI was reported to be present all along the human small intestine while CD36 was found mainly in the duodenum and jejunum (*24*).

Both Scavenger Receptors, SR-BI and CD36, Facilitate Lipid Uptake. We used COS-7 cells transiently transfected with either SR-BI or CD36 to examine lipid uptake by each receptor separately. Both SR-BI and CD36 facilitated the uptake of different lipids such as free and esterified cholesterol (Figure 4A), phospholipids including sphingomyelin (Figure 4A,B) and phosphatidylcholine (data not shown), β-carotene (Figure 4C), and long-chain fatty acids (Table 3). Transfection of COS-7 cells with either receptor typically increased lipid uptake from SUV 5−8-fold and with the other lipid donor particles 2−3-fold relative to mock-transfected cells. As shown in Figure 4, lipid uptake was facilitated from all three kinds of lipid donor particles likely to be present in the gastrointestinal tract: mixed bile salt micelles (Figure 4B), phospholipid small unilamellar vesicles (SUV) (Figure 4A,C), and particles of a trioleoylglycerol emulsion (Figure 4D). It is important to note that, in the case of the bile salt micelle, there was no facilitated uptake of taurocholate. Thus, in a 1 h incubation at 37 °C there was no difference in taurocholate uptake between control and SR-BI transfected cells, and the uptake of bile salt was <10% that of sphingomyelin. This result is in good agreement with the in vitro uptake of cholesterol into rabbit BBMV from mixed sodium taurocholate micelles as the donor. In a double-labeling experiment using taurocholate micelles labeled with [^{14}C]cholate and [^{3}H]cholesterol the uptake of cholate was negligible compared to that of cholesterol (*40*). The data in Figure 4 show that both SR-BI and CD36 can mediate the uptake of membrane-soluble lipids from all kinds of lipid donor particles. The two scavenger receptors SR-BI and CD36 behaved similarly in terms of lipid uptake (cf. Figure 4), with similar functions reflecting closely related three-dimensional protein structures. The lipid uptake properties of COS-7 cells transfected with either SR-BI or CD36 closely resembled those measured with intestinal BBMV (*7*, *17*). The transfection of COS-7 cells with either SR-BI or CD36 apparently confers on these cells lipid uptake properties closely resembling those of BBMV (*7*, *17*). Recently, NPC1L1 protein was proposed to be the intestinal BBM protein that catalyzes cholesterol uptake from the intestinal lumen to the BBM (*39*). However, in contrast to the lipid uptake properties observed with COS-7 cells transfected with a scavenger receptor as described above, overexpression of NPC1L1 protein in nonenterocyte cells failed to confer cholesterol uptake on these cells (*39*).

Table 3: Effects of Transfection of COS Cells with either SR-BI or CD36 on Fatty Acid Uptake

	fatty acid uptake (% label/well)[a]		
fatty acid	control	SR-BI	CD36
oleic acid	10.2 ± 0.6	10.2 ± 0.6	8.6 ± 3.3
lignoceric acid	0.5 ± 0.1	1.5 ± 0.2	3.5 ± 0.4

[a] Fatty acid uptake was determined by incubating COS cells, transiently transfected with either empty vector (control), SR-BI, or CD36 (see Methods), with mixed bile salt micelles containing the indicated radiolabeled fatty acid for 5 min at 37 °C. The values are the mean ± SD ($n = 3$). Lignoceric acid uptake into SR-BI- and CD36-transfected cells was significantly different from uptake into control cells ($p < 0.01$, ANOVA followed by the Dunnett test).

The SR-BI- and CD36-mediated uptake of lipids from the three kinds of donor particles into COS-7 cells was equiproportional, i.e., in the same proportion as the different membrane-soluble lipids were present initially in the donor particle (Figure 4). A possible mechanism underlying the equiproportional lipid uptake is endocytosis of the intact donor particle. However, previous studies using SR-BI-transfected COS cells rule out this possibility (*38*). Lipid uptake measurements using BBMV and different donor particles have also led to the conclusion that endocytosis is not a major mechanism (*7*, *17*). The true mechanism is very likely collision-induced lipid transfer from the donor particle

Table 4: Effects of Ezetimibe on Cholesterol Absorption in SR-BI$^{+/+}$ and SR-BI$^{-/-}$ Mice

mouse genotype	cholesterol absorption efficiency (%)[a]	
	control	(+) ezetimibe
SR-BI$^{+/+}$	65 ± 7	45 ± 10 ($p = 0.0007$)
SR-BI$^{-/-}$	68 ± 7	39 ± 5 ($p = 0.009$)

[a] Cholesterol absorption was measured by treating female mice fed a basal diet with either a corn oil gavage (control) or a corn oil plus ezetimibe gavage (see Methods). The values of absorption efficiency are the mean ± SD ($n = 4$), and the values for ezetimibe-treated animals are significantly reduced compared to the control animals.

to the BBM as previously deduced from a study of the reaction order (*7*). The extracellular domains of SR-BI and CD36 may contain a fusogenic motif consistent with the observation that fusogenic proteins are released from the BBM by proteolysis (*41*). We propose that such a motif is responsible for the binding of the donor particle to the scavenger receptor followed by nonselective transfer of the lipids of the donor particles into the cell plasma membrane. Elucidation of the detailed molecular mechanism of intestinal lipid uptake must await the determination of the three-dimensional structures of the extracellular domains of CD36 and SR-BI.

Since CD36 was suggested to be involved in the transport of long-chain fatty acids (*42*), we examined the uptake of long-chain fatty acids into COS cells transfected with either SR-BI or CD36. Uptake of oleic acid from mixed bile salt micelles was so rapid that protein-mediated uptake was undetectable in both SR-BI- and CD36-transfected COS cells (Table 3). However, as shown in this table, the uptake of the 24 carbon chain, lignoceric acid molecule from mixed bile salt micelles was facilitated by both receptors. In our model system (Figure 4), oleic acid is apparently taken up by simple passive diffusion. This still leaves open the possibility of protein-mediated uptake of oleic acid in vivo: in the lumen of the small intestine the actual concentration of free fatty acid might be much lower than in our simplified model. Due to strong binding of fatty acids to other molecules present in the lumen of the small intestine, the free fatty acid concentration might be so low that passive diffusion becomes inefficient. Under these conditions, the protein-mediated mechanism of fatty acid uptake would come into play.

Inhibitors of Intestinal Cholesterol Absorption. Further evidence for transporter activity in the small intestinal BBM comes from the existence of specific inhibitors of lipid absorption, e.g., amphipathic, helical α-peptides (*9*, *10*) and β-peptides (*11*) and small molecules such as ezetimibe (*12*). Ezetimibe was shown to effectively inhibit intestinal cholesterol absorption in various animals including mice and also in humans (*12*, *13*, *43*) and to lower LDL−cholesterol levels in serum. The data in Table 4 confirm a report in the literature (*12*) showing that ezetimibe reduces the cholesterol absorption efficiency in both C57BL/6 SR-BI$^{+/+}$ and SR-BI$^{-/-}$ mice. The positive inhibition in wild-type mice was attributed, in part, to interaction of ezetimibe with SR-BI (*12*), but the inhibition in SR-BI$^{-/-}$ mice was not explained. To account for the effect of ezetimibe in SR-BI$^{-/-}$ mice, we postulated that ezetimibe also interacts with CD36. We tested this possibility by using COS-7 cells transfected with CD36. The data presented in Figure 5A show that (I) CE uptake is

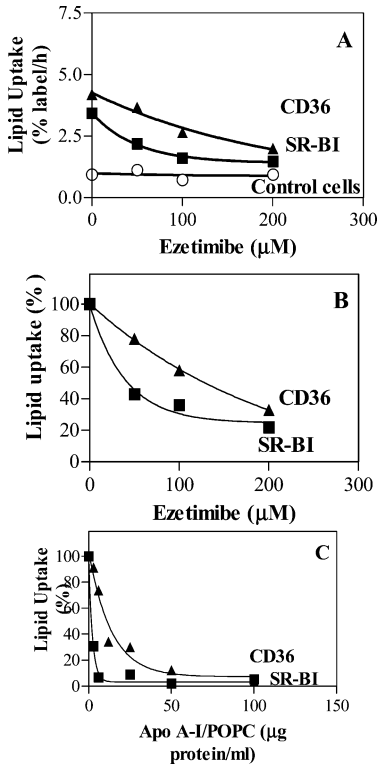

FIGURE 5: Inhibition of lipid uptake into COS-7 cells transiently transfected with either SR-BI or CD36. The effects of two inhibitors, ezetimibe and apolipoprotein (apo) A-I, on the uptake of [^{3}H]-cholesteryl oleyl ether (^{3}H-CE) from egg PC SUV (100 μg of PC/mL) into transfected COS-7 cells were measured at 37 °C as described in Figure 4. Values are the mean of duplicate measurements. (A) Effect on ^{3}H-CE uptake of preincubating the cells at 37 °C for 1 h with the indicated concentration of ezetimibe dissolved in DMSO. Key: (○) control cells; (■) SR-BI-expressing cells; (▲) CD36-expressing cells. (B) Relative effect of ezetimibe on SR-BI- and CD36-mediated ^{3}H-CE uptake. Receptor-mediated uptake was calculated by subtracting the lipid uptake values obtained with control cells from the corresponding values obtained with receptor-expressing cells shown in panel A. Lipid uptake measured in the absence of ezetimibe was set to 100% for both SR-BI- and CD36-expressing cells, and the effects of increasing concentrations of ezetimibe on lipid uptake relative to this value are shown. Key: (■) SR-BI; (▲) CD36. (C) Inhibition of ^{3}H-CE uptake in the presence of apoA-I/POPC (1/98 mol/mol) discoidal complexes. The effects on ^{3}H-CE uptake of co-incubating the donor SUV with increasing concentrations of the apoA-I/POPC complex were measured. As in panel B, the lipid uptake values were corrected for uptake into control cells and are expressed relative to the values in the absence of inhibitor. Key: (■) SR-BI; (▲) CD36.

increased by a factor of 3−4 in SR-BI- and CD36-transfected COS-7 cells relative to mock-transfected cells and (II) ezetimibe does indeed interact with both receptors, the interaction with SR-BI being stronger by a factor of 3−4 compared to that of CD36 (Figure 5B). Increasing ezetimibe concentrations progressively inhibited both the SR-BI- and the CD36-mediated lipid uptake but had no effect on the lipid uptake into mock-transfected cells (Figure 5A,B). The results are consistent with the concept that ezetimibe binds to both class B scavenger receptors and that the binding of ezetimibe to CD36 underlies the inhibition by ezetimibe in SR-BI$^{-/-}$ mice (see below).

Both SR-BI and CD36 are known to be HDL receptors (*44*−*46*), and as such, HDL is expected to compete with lipid donor particles such as bile salt micelles and SUV for binding sites of the scavenger receptors. This expectation is

borne out by experiment (Figure 5C). At apoA-I concentrations greater than 50 μg/mL (1.7 μM), the CE uptake into both SR-BI- and CD36-transfected COS-7 cells was completely inhibited. The functional motif in apoA-I responsible for the interaction with class B scavenger receptors was shown to be the amphipathic α-helix (*9*, *10*, *47*).

The Role of Scavenger Receptors in Intestinal Lipid Uptake in Mice. Both in vivo (mouse feeding) and in vitro (BBMV) experiments (Figures 1 and 2) demonstrate that the duodenal BBM-resident scavenger receptor, SR-BI, is responsible for the facilitated uptake of β-carotene. This demonstration of a functional role for SR-BI is consistent with the finding that an SR-BI homologue is essential for cellular uptake of carotenoids in *Drosophila* (*30*). Regarding cholesterol uptake, the in vitro experiments show that both SR-BI and CD36 have the ability to facilitate cholesterol uptake into the BBM whereas the in vivo experiments are ambiguous. The contribution of any such facilitation by these two class B scavenger receptors to cholesterol absorption in vivo remains to be established, e.g., by feeding experiments using SR-BI/CD36 double-knockout mice. SR-BI- and CD36-mediated lipid uptake is relatively unspecific with respect to both the lipid donor particles and the nature of the transported lipid (Figure 4); mixed bile salt micelles, SUV, and triacylglycerol emulsions, which are all likely to be present in the intestinal lumen, can serve as lipid donor particles. The different distributions of the two receptors along the small intestine suggest that their functions in vivo may differ. Assuming that, under the experimental conditions used (cf. Table 2), the contribution of passive diffusion to cholesterol uptake is about 1 μIU, it follows that SR-BI contributes largely to facilitated lipid uptake in the duodenum. According to the distribution of CD36 along the mouse small intestine (Figure 3), CD36 is expected to account for lipid uptake in the jejunum and ileum; free fatty acids and monoacylglycerols generated by lipolysis of triacylglycerols are absorbed mainly in this region (*42*). Considering that SR-BI activity peaks sharply in the duodenum and declines steeply in the jejunum, we infer that the contribution of SR-BI to total lipid uptake is relatively small in the mouse. CD36 and perhaps (an)-other BBM-resident protein(s) are likely to be the major proteins facilitating the uptake of dietary lipids in the jejunum and ileum. The functional assignment of NPC1L1 protein (*39*) has to await its unequivocal identification as an apical surface receptor of small intestinal epithelial cells. This protein might still be in contact with the BBM, particularly with the cytoplasmic surface of the membrane; however, like Niemann−Pick C1 protein, NPC1L1 may be involved in endosomal transport of cholesterol from the BBM to the endoplasmic reticulum.

The action of the newly developed inhibitor of intestinal cholesterol absorption, ezetimibe (*12*, *13*, *43*), may be explained, at least in part, by the fact that it targets SR-BI and CD36. Since ezetimibe can inhibit both receptors (Figure 5), it is likely that each of these homologous class B scavenger receptors possesses an ezetimibe binding site(s). It is important to note that ezetimibe can bind to several BBM proteins (*48*). More work is required for a full description of the mechanism of inhibition.

Both in vivo and in vitro experiments indicate that SR-BI is important for β-carotene absorption in the small intestine, at least in mice on a high-fat, high-cholesterol diet. While in vitro experiments using BBMV show that SR-BI mediates cholesterol uptake in this model, it is still unclear if and to what extent SR-BI contributes to cholesterol absorption in vivo. The in vivo proof that scavenger receptors are indeed involved in cholesterol absorption has to await the availability of the SR-BI/CD36 double-knockout mouse.

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