

# Influence of class B scavenger receptors on cholesterol flux across the brush border membrane and intestinal absorption

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**Abstract** To learn more about how the step of cholesterol uptake into the brush border membrane (BBM) of enterocytes influences overall cholesterol absorption, we measured cholesterol absorption 4 and 24 h after administration of an intragastric bolus of radioactive cholesterol in mice with scavenger receptor class B, type 1 (SR-BI) and/or cluster determinant 36 (CD36) deleted. The cholesterol absorption efficiency is unaltered by deletion of either one or both of the receptors. *In vitro* determinations of the cholesterol uptake specific activity of the BBM from the mice reveal that the scavenger receptors facilitate cholesterol uptake into the proximal BBM. It follows that cholesterol uptake into the BBM is not normally rate-limiting for the cholesterol absorption process *in vivo*; a subsequent step, such as NPC1L1-mediated transfer from the BBM into the interior of the enterocyte, is rate-limiting. The absorption of dietary cholesterol after 4 h in mice lacking SR-BI and/or CD36 and fed a high-fat/high-cholesterol diet is delayed to more distal regions of the small intestine. This effect probably arises because ATP binding cassette half transporters G5 and G8-mediated back flux of cholesterol from the BBM to the lumen of the small intestine limits absorption and causes the local cholesterol uptake facilitated by SR-BI and CD36 to become rate-limiting under this dietary condition.—Nguyen, D. V., V. A. Drover, M. Knopfel, P. Dhanasekaran, H. Hauser, and M. C. Phillips. **Influence of class B scavenger receptors on cholesterol flux across the brush border membrane and intestinal absorption.** *J. Lipid Res.* 2009. 50: 2235–2244.

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The efficiency of absorption of dietary cholesterol in the small intestine affects plasma LDL cholesterol levels and thereby cardiovascular disease risk (1, 2). Consequently, there is great interest in understanding the mechanism of intestinal cholesterol absorption and inhibiting the process to reduce plasma cholesterol levels. Ezetimibe is such an inhibitor whose target is the protein Niemann-Pick C1-like 1 (NPC1L1) (3, 4); the importance of this protein in cholesterol absorption is reflected by the fact that the absorption efficiency is greatly reduced in mice lacking the gene encoding NPC1L1 (5, 6). Experiments with knockout and transgenic mice have variously demonstrated that acylCoA:cholesterol-acyltransferase-2 (ACAT-2) (7–9) and ATP binding cassette half transporters G5 and G8 (ABCG5/G8) (10–13) also play critical roles in the cholesterol absorption pathway. The above proteins are all involved in enterocyte cholesterol homeostasis (14, 15), but factors such as lipase activity and bile salt availability in the earlier digestive phase of the pathway are also critical (for recent reviews, see Refs. 14 and 16). Current understanding of the proteins and mechanisms controlling the uptake of cholesterol from the lumen of the small intestine into and across the brush border membrane (BBM) is relatively limited. Here, we distinguish between cholesterol uptake and cholesterol absorption, defining the former as the process of cholesterol transfer from the donor particle in the lumen of the small intestine to the BBM and the latter as the cascade of transport steps

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Abbreviations: ABCG5/G8, ATP binding cassette half transporters G5 and G8; ACAT-2, acylCoA:cholesterol-acyltransferase-2; BBM, brush border membrane; BBMV, brush border membrane vesicle; BSM, mixed bile salt micelle; CD36, cluster determinant 36; HFHC, high-fat/high-cholesterol diet; NPC1L1, Niemann Pick C1-Like 1; SR-BI, scavenger receptor class B, type 1; SUV, small unilamellar vesicle; WT, wild type.

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whereby cholesterol moves from the lumen across the enterocyte to the lymph and circulatory system. Cholesterol uptake is measured *in vitro*, whereas cholesterol absorption is measured *in vivo*.

Historically, it has been realized that simple diffusion of cholesterol molecules from mixed bile salt micelles (BSM) in the lumen of the small intestine promotes cholesterol uptake into the BBM (17). Protein-facilitated uptake of cholesterol into the BBM has also been demonstrated (18). As a consequence of studies in many laboratories aimed at identifying the responsible cholesterol transporter(s), several candidate proteins have been suggested (for recent reviews, see Refs. 19 and 20). NPC1L1 has been a favored candidate (3), but recent evidence suggests that it is not essential because cholesterol uptake into BBM vesicles prepared from the small intestines of wild-type (WT) and NPC1L1<sup>-/-</sup> mice is the same (21, 22). It is now known that NPC1L1 traffics between the cell surface and intracellular compartments (15, 23–26) and plays a critical role in the internalization of BBM cholesterol through clathrin-mediated endocytosis (27, 28). The class B scavenger receptors cluster determinant 36 (CD36) (29) and scavenger receptor class B, type 1 (SR-BI) (30), which have established lipid transport capabilities, have been known for a decade or more to be located in the BBM and can potentially mediate cholesterol uptake into the BBM. Indeed, experiments with mice in which these genes are deleted have demonstrated that SR-BI is important for the absorption of  $\beta$ -carotene (31) and vitamin E (32), and CD36 is important for the absorption of very long chain fatty acids (33). However, there is no effect on cholesterol absorption of deleting either SR-BI (31, 34, 35) or CD36 (36), although both receptors have been shown to facilitate the transport of cholesterol into cells (31, 37, 38). The reasons for this paradox are not clear, but possible explanations include 1) compensation between the two receptor pathways occurs and 2) movement of cholesterol from BSM into the BBM is not rate-limiting for the cholesterol absorption process. Here we use double-knockout mice in which both SR-BI and CD36 are deleted to address these issues. The results provide insight into the steps controlling cholesterol transfer across the BBM under basal and high-fat/high-cholesterol (HFHC) diet conditions.

## EXPERIMENTAL PROCEDURES

### Materials

C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). SR-BI<sup>-/-</sup> mice on the same background, originally described by Rigotti et al. (39), were generated as described before (31). CD36<sup>-/-</sup> and CD36<sup>-/-</sup>/SR-BI<sup>-/-</sup> double-knockout mice on a C57BL/6 background were produced and characterized as described earlier (33). The Institutional Animal Care and Use Committee of The Children's Hospital of Philadelphia approved all the animal studies. A basal mouse diet containing 5 wt% fat and no cholesterol (Teklad LM-485) and an HFHC diet (Purina mouse chow 5015 supplemented with 7.5 wt% cocoa butter and 1.25 wt% cholesterol) were obtained from Animal Specialties and Provisions (Quakertown, PA). [<sup>14</sup>C] Cholesterol

(53 mCi/mmol) and [5,6-<sup>3</sup>H] $\alpha$  $\beta$ -sitostanol (50 Ci/mmol) were obtained from Perkin-Elmer Life Sciences (Boston, MA) and American Radiolabeled Chemicals (St. Louis, MO), respectively. The sources of the other lipids have been reported before (31).

### Methods

*Intestinal cholesterol absorption in mice.* Mice were housed in a temperature- and humidity-controlled animal facility with 12 h light-dark cycles and fed either a basal or HFHC diet for at least 3 weeks. The efficiency of dietary cholesterol absorption over a 24 h period was then determined as described before (31, 40) using the fecal dual isotope ratio method (41). Briefly, fasted mice were given an intragastric gavage of 0.1 ml of corn oil containing 1  $\mu$ Ci [<sup>14</sup>C] cholesterol and 0.4  $\mu$ Ci [<sup>3</sup>H] sitostanol. The animals were returned to metabolic cages where they had free access to their diet and water. Feces were collected for 24 h, and after extraction the amounts of radioactive sterols in them were determined by liquid scintillation counting. After 24 h, the mice were euthanized and samples of small intestine harvested (31). To measure the acute intestinal absorption of dietary cholesterol, the mice were maintained in metabolic cages overnight with access to only water before receipt of the intragastric gavage. The 0.1 ml bolus of corn oil contained 1  $\mu$ Ci [<sup>14</sup>C] cholesterol and 0.4  $\mu$ Ci [<sup>3</sup>H] sitostanol and 0.1 mg unlabeled cholesterol. Exactly 4 h after the gavage, the animals were euthanized and tissues (blood, liver, and small intestine) were harvested. The small intestines were divided into four sections (part A = proximal 10 cm, B = medial 10 cm, C = distal 10 cm, and D = remainder of small intestine), cut longitudinally and washed thoroughly with ice-cold buffer to remove the luminal contents. The weighed intestinal samples were dissolved in Solvable (Perkin-Elmer), and the fractions of the administered doses of [<sup>14</sup>C] cholesterol and [<sup>3</sup>H] sitostanol present were determined by liquid scintillation counting. A weighed sample of liver was analyzed in a similar fashion. Plasma samples were prepared from the blood and analyzed for radioactive sterols by liquid scintillation counting and for cholesterol levels by an enzymatic assay (33).

*Cholesterol uptake into BBM vesicles.* BBM vesicles (BBMVs) were prepared from sections of frozen mouse small intestine using procedures described before (22). Prior to use, the BBMVs were subjected to a routine quality control (42). Digestion of BBMVs with proteinase K was carried out as described previously (18). The cholesterol uptake activities at room temperature of BBMVs prepared from different sections of mouse small intestine were compared using 0.9 mg BBMVs protein/ml mixed with egg phosphatidylcholine small unilamellar vesicle (SUV) containing 1 mol% [<sup>14</sup>C] cholesterol (0.05 mg total lipid/ml) as the donor (22, 31). The time courses describing the equilibration of radiolabel between the SUV and BBMVs were analyzed as described previously (43); the kinetic equations account for the bidirectional flux of cholesterol between the donor and acceptor particles.

### Real-time PCR

The relative expression of SR-BI, CD36, NPC1L1, and ABCG5 in mouse intestinal segments was examined by the two-step real-time PCR method (Applied Biosystems, Foster, CA). Tissues from three mice per group were pooled, and the RNA was extracted using a mirVana miRNA isolation kit (Ambion, Austin, TX) and converted to cDNA in a reverse transcription reaction carried out with the High Capacity cDNA reverse transcription kit (Applied Biosystems). The subsequent real-time PCR reactions were performed in an Applied Biosystems 7500 real-time PCR system using 12.5 ng aliquots of the cDNA preparations as templates. The PCR reactions were run in triplicate using Inventoried TaqMan

Gene Expression Assays with FAM-labeled primer and probe mixes for SR-BI, CD36, NPC1L1, ABCG5, and 18srRNA, together with the Taqman Gene Expression Master Mix from Applied Biosystems. The relative quantities of mRNA were determined using the method of comparative changes in threshold cycle (Applied Biosystems), with 18s rRNA as the endogenous control.

## Statistics

Results are expressed as the mean  $\pm$  1 SD. Differences were evaluated with GraphPad Prism using one-way ANOVA followed by the Tukey multiple comparison test.

## RESULTS

### Cholesterol absorption with a basal diet

**Table 1** summarizes the results of experiments using the fecal dual isotope ratio method to examine the contributions of SR-BI and CD36 to intestinal cholesterol absorption. It is apparent that for WT mice fed a basal diet, the 24 h cholesterol absorption efficiency is  $\sim$ 70%, a value that is consistent with prior reports (11, 44); no significant differences between male and female mice were observed (data not shown). The results also confirm that single deletion of either SR-BI (31, 34, 35) or CD36 (36) has no significant effect on the cholesterol absorption efficiency. Furthermore, the SR-BI<sup>-/-</sup>/CD36<sup>-/-</sup> mice behave similarly, indicating that the double deletion of both SR-BI and CD36 does not reduce cholesterol absorption. Twenty-four hours after the gavage of radiolabeled cholesterol, only  $\sim$ 5% of the administered dose is still present in the wall of the small intestine (data not shown), indicating that the digestion of the bolus of cholesterol is essentially complete after this time. The amount of labeled cholesterol retained in the intestinal wall 24 h after the gavage is the same for WT mice and mice lacking SR-BI and CD36 (data not shown); this confirms the results of the fecal dual isotope ratio experiments and shows that SR-BI and CD36 do not contribute to overall cholesterol absorption efficiency.

While SR-BI and CD36 do not affect the net absorption of cholesterol from a meal, it is possible that they modulate the rate of cholesterol absorption. To address this point, the dietary [<sup>14</sup>C]cholesterol content of the small intestine was measured 4 h after the gavage when chylomicron production is occurring. The fraction of the initial dose of radiolabeled cholesterol in the wall of the total small intestine at 4 h is  $40 \pm 7\%$  in WT mice (Table 1). This value is unaltered in SR-BI<sup>-/-</sup> mice, indicating that this

receptor does not affect the rate of cholesterol movement into the intestine in WT mice on a basal diet. The values for the CD36<sup>-/-</sup> and SR-BI<sup>-/-</sup>/CD36<sup>-/-</sup> mice are somewhat lower (Table 1), although the differences to the WT value are not statistically significant; a reduction of dietary cholesterol transport into the lymph has been observed in CD36<sup>-/-</sup> mice (36). As expected, 4 h after the gavage of a bolus containing radiolabeled cholesterol and sitostanol, much more cholesterol than sitostanol is present in the intestinal wall. The selectivity ratio of cholesterol to sitostanol ranges from 20–25/1 in the proximal small intestine to about 15/1 in the medial and distal sections of the small intestine (data not shown); these ratios, reflecting the preferential movement into the intestine of cholesterol over sitostanol, are the same for all four genotypes of mice listed in Table 1. The fractions of the administered dose of labeled cholesterol present in the blood and liver 4 h after the gavage are also not altered significantly by the deletion of SR-BI and/or CD36 (Table 1).

The above results show that the net intestinal absorption of dietary cholesterol 4 and 24 h after the meal is unaffected by the presence of either SR-BI or CD36 in the BBM. The experiments summarized in **Fig. 1** were performed to see if the presence of these receptors affects the location of cholesterol absorption along the gastro-colic axis of the small intestine. The results in **Fig. 1A** for WT mice show that, of the 40% of the [<sup>14</sup>C]cholesterol dose present in the wall of the small intestine at 4 h, most of it is present in the proximal and medial sections A and B. Our results are consistent with a prior report (45) and confirm that most cholesterol absorption occurs in the proximal small intestine. Thus, the ratio of the percentage of cholesterol dose present in section C to that in sections (A + B) is  $\sim$ 0.25 for WT mice (**Fig. 1A**). This ratio does not increase in a systematic fashion for the mice in which SR-BI and/or CD36 are deleted (**Fig. 1B–D**). Comparison of the data in panels A–D shows that the fractions of the initial cholesterol dose present in intestinal sections A–C are not significantly different between the four types of mice. It should be noted that the results for intestinal section D were not included in this analysis because of variability in total small intestine length, with mice lacking CD36 having a longer small intestine (38).

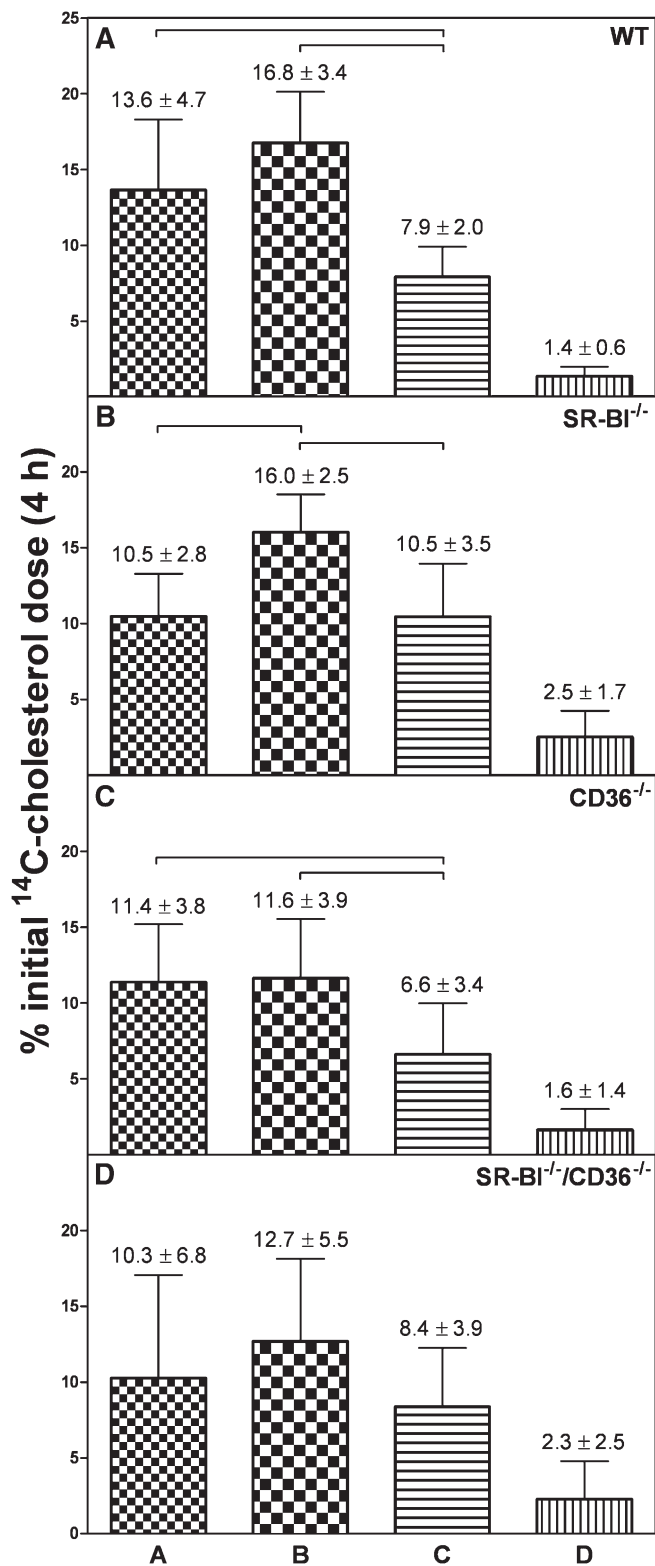
It should be noted that the mRNA levels listed in **Table 2** indicate that, regardless of diet, SR-BI expression is concentrated in the proximal small intestine (cf. Refs. 31, 46, and 47). In contrast, the drop-off in CD36 expression level from the proximal to distal intestinal segments is less

TABLE 1. Influence of scavenger receptors on intestinal cholesterol absorption in mice fed a basal diet<sup>a</sup>

Mouse Genotype	24 h Absorption Efficiency (%) (n = 7/group)	4 h Intestinal Content (% Initial Dose) (n = 8/group)	% Initial Dose in 100 $\mu$ l Plasma at 4 h (n = 8/group)	% Initial Dose in 100 mg Liver at 4 h (n = 8/group)
WT	70 $\pm$ 11	40 $\pm$ 7	0.13 $\pm$ 0.06	0.92 $\pm$ 0.14
SR-BI <sup>-/-</sup>	66 $\pm$ 14	40 $\pm$ 6	0.18 $\pm$ 0.09	0.88 $\pm$ 0.53
CD36 <sup>-/-</sup>	65 $\pm$ 8	31 $\pm$ 11	0.08 $\pm$ 0.04	0.43 $\pm$ 0.22
SR-BI <sup>-/-</sup> /CD36 <sup>-/-</sup>	67 $\pm$ 13	34 $\pm$ 12	0.18 $\pm$ 0.10	0.67 $\pm$ 0.48

All values are mean  $\pm$  SD. No significant differences between mouse genotypes were evident by ANOVA.

<sup>a</sup>The mice weighed  $26.4 \pm 2.8$  g and were aged 3–5 months.



**Fig. 1.** Distribution of [<sup>14</sup>C]cholesterol absorption along the small intestine of mice fed a basal diet. As described in Experimental Procedures, a bolus of [<sup>14</sup>C]cholesterol in corn oil was administered to the mice by gastric gavage. Four hours later, the animals were euthanized and the small intestines isolated for determination of the [<sup>14</sup>C]cholesterol associated with each section (section A = proximal 10 cm, B = medial 10 cm, C = distal 10 cm, D = remainder of small intestine). A: WT mice. B: SR-BI<sup>-/-</sup> mice. C: CD36<sup>-/-</sup> mice. D: SR-BI<sup>-/-</sup>/CD36<sup>-/-</sup> mice. The percentages of [<sup>14</sup>C]choles-

marked so that CD36 is expressed in all regions of the small intestine (cf. Refs. 29, 31, 38, and 48). It is apparent that deletion of SR-BI does not induce a compensatory increase in the expression of CD36 and vice versa; in fact, the deletion of one receptor mostly reduces the expression of the other in intestinal segment A (Table 2). **Table 3** demonstrates that deletion of SR-BI and/or CD36 reduces the expression of NPC1L1 and ABCG5 [and ABCG8, which is coordinately regulated (13)] in all intestinal segments except B; these transporters are expressed in all sections of the small intestine (10, 49).

In summary, for mice on a basal diet, where cholesterol levels are low, SR-BI and CD36 do not significantly affect the position along the gastro-colic axis where absorption occurs. The observation that the cholesterol absorption efficiency in mice with either SR-BI or CD36 deleted is the same as in WT mice cannot be attributed to a compensatory increase in the expression of the remaining receptor.

### Cholesterol absorption with a HFHC diet

Although the contributions of SR-BI and CD36 to cholesterol absorption are minimal for mice fed a basal diet, it is possible that the situation is different when the gastrointestinal system is subjected to a high-cholesterol load (i.e., large pools of dietary and biliary cholesterol). To address this issue, we examined the effects of a HFHC diet on mice lacking SR-BI and/or CD36. As expected, feeding the HFHC diet for 3 weeks increases the serum cholesterol levels in all four mouse genotypes (**Table 4**); the 1.5- to 2-fold increase in serum cholesterol in WT mice fed this diet has been reported before (40). The elevated serum cholesterol levels seen with mice lacking SR-BI are also consistent with prior work (39). Feeding the HFHC diet to the mice for 3 weeks leads to only minor changes in body weight (cf. Tables 1 and 5). The HFHC diet seems to slightly increase the expression of SR-BI and CD36 in certain intestinal segments (Table 2). Consistent with prior reports (6, 50), the HFHC diet induced a 2- to 3-fold decrease in intestinal NPC1L1 expression (data not shown). However, this diet did not change the level of expression of ABCG5 (data not shown).

Table 5 shows that the 24 h cholesterol absorption efficiency for WT mice fed the HFHC diet is about 25% (cf. Ref. 40) compared with the equivalent value of 70% for mice on the basal diet (Table 1). This reduction in cholesterol absorption efficiency is an expected consequence of the increase in dietary cholesterol (51). As was seen with the animals fed the basal diet, the deletion of SR-BI and/or CD36 has no effect on 24 h cholesterol absorption efficiency for mice on the HFHC diet. The [<sup>14</sup>C]cholesterol content of the small intestine 4 h after the gavage is also

terol dose are presented as mean ± SD, n = 8/group. The horizontal bars above the histograms show statistically significant differences, as determined by ANOVA and the Tukey multiple comparison test. The results for section D were not included in the statistical analysis because of variability in the length of this section (see text).



TABLE 2. Expression of SR-BI and CD36 in mouse small intestine

Mouse Genotype	Intestinal Decton <sup>a</sup>	SR-BI/18s <sup>b,c</sup>		CD36/18s <sup>b,c</sup>	
		Basal Diet	HFHC Diet	Basal Diet	HFHC Diet
WT	A	1.0	1.49 ± 0.09	1.0	1.05 ± 0.05
	B	0.07 ± 0.01	0.05 ± 0.01	0.69 ± 0.08	0.88 ± 0.07
	C	0.14 ± 0.02	0.13 ± 0.02	0.39 ± 0.03	0.63 ± 0.06
SR-BI <sup>-/-</sup>	A			0.66 ± 0.07	1.02 ± 0.06
	B			0.56 ± 0.06	0.84 ± 0.05
	C			0.29 ± 0.07	0.59 ± 0.05
CD36 <sup>-/-</sup>	A	0.28 ± 0.03	1.07 ± 0.02		
	B	0.17 ± 0.02	0.29 ± 0.04		
	C	0.10 ± 0.01	0.13 ± 0.01		

<sup>a</sup> mRNA was extracted from pooled intestinal segments (A = proximal 10 cm, B = medial 10 cm, and C = distal 10 cm; see Methods) of mice (n = 3 per group) fed either a basal diet or HFHC diet for 3 weeks and analyzed by real-time PCR.

<sup>b</sup> The relative mRNA levels from at least two real time PCR experiments are normalized to the value for intestinal segment A from WT mice fed a basal diet.

<sup>c</sup> Mean ± SD, n = 6–12. ANOVA followed by the Dunnett multiple comparison test indicates that all SR-BI relative mRNA levels are significantly different (*P* < 0.05) to the control value for intestinal segment A from WT mice fed a basal diet. The same applies to the CD36 relative mRNA levels except that the values for intestinal segment A from WT and SR-BI<sup>-/-</sup> mice fed a HFHC diet are not significantly different to control.

reduced for animals on the HFHC diet compared with the basal diet (cf. Tables 1 and 5). Furthermore, no genotype effect is evident, indicating that SR-BI and CD36 do not affect the rate of cholesterol absorption in mice fed a HFHC diet. The levels of radiolabeled cholesterol present in the liver 4 h after the gavage also indicate that SR-BI and CD36 do not affect the rate of cholesterol absorption. The higher levels of [<sup>14</sup>C]cholesterol dose seen after 4 h in the plasma of mice lacking SR-BI, relative to plasma from WT and CD36<sup>-/-</sup> mice (Table 5), presumably reflect retention in the larger pool of plasma cholesterol in these animals (cf. Table 4).

**Figure 2** summarizes the results of experiments designed to explore the effects of deletion of SR-BI and/or CD36 on

the location of cholesterol absorption in mice fed the HFHC diet. As seen with the WT mice on a basal diet (Fig. 1A), the absorption in mice on the HFHC diet occurs predominantly in proximal regions of the small intestine (Fig. 2A). This finding is demonstrated by the fact that the ratio of the percentage of [<sup>14</sup>C]cholesterol dose present in intestine section C to that in sections (A + B) is ~0.25 for WT mice on the HFHC diet (Fig. 2A) which is the same as the value for the equivalent data in Fig. 1A (basal diet). Inspection of the data in panels B and C of Fig. 2 reveals that the ratio of [<sup>14</sup>C]cholesterol dose C/(A + B) increases by ~50% relative to the ratio from panel A for WT mice; this result indicates that deletion of either SR-BI or CD36 retards absorption to more distal regions of the small intestine. The increase in [<sup>14</sup>C]cholesterol found in section D of the intestine in the SR-BI<sup>-/-</sup> and CD36<sup>-/-</sup> mice (panels B and C) compared with section D of WT mice (panel A) supports this conclusion. The ratio of cholesterol absorbed in intestine section C/(A + B) and the absorption in section D are increased even more in SR-BI<sup>-/-</sup>/CD36<sup>-/-</sup> mice (panel D), indicating that removal of both receptors further retards absorption to more distal regions of the small intestine.

In summary, for mice fed a HFHC diet, the loss of SR-BI and/or CD36 delays intestinal cholesterol absorption to more distal regions, but the overall absorptive capacity of the small intestine is such that there is no alteration in the net level of cholesterol absorption (Table 5).

### Cholesterol uptake into the BBM

Given that SR-BI and CD36 can facilitate the uptake of cholesterol into the BBM (21, 30, 31, 42), removal of either receptor from the BBM is expected to decrease the cholesterol uptake specific activity of intestinal segments. The in vitro BBMV uptake experiments using a defined cholesterol donor particle (Table 6) were performed to investigate this issue. It should be noted that the cholesterol uptake activities listed in Table 6 account for the back flux (efflux) of cholesterol from the BBM to the SUV and

TABLE 3. Effect of SR-BI and CD36 deletion on expression of NPC1L1 and ABCG5 in mouse small intestine

Mouse Genotype	Intestinal Section	NPC1L1/18s <sup>a,b</sup>	ABCG5/18s <sup>a,b</sup>
WT	A	1.0	1.0
	B	0.94 ± 0.06	0.59 ± 0.04
	C	2.02 ± 0.1	0.75 ± 0.07
SR-BI <sup>-/-</sup>	A	0.50 ± 0.03	0.32 ± 0.01
	B	0.92 ± 0.06	0.46 ± 0.04
	C	0.84 ± 0.05	0.33 ± 0.02
CD36 <sup>-/-</sup>	A	0.60 ± 0.04	0.51 ± 0.03
	B	1.12 ± 0.01	0.68 ± 0.08
	C	0.86 ± 0.06	0.35 ± 0.03
SR-BI <sup>-/-</sup> /CD36 <sup>-/-</sup>	A	0.46 ± 0.06	0.37 ± 0.03
	B	0.94 ± 0.05	0.58 ± 0.06
	C	1.44 ± 0.19	0.51 ± 0.03

<sup>a</sup> mRNA was extracted from the pooled small intestines of mice (n = 3 per group) fed a basal diet for 3 weeks and analyzed by real-time PCR. The relative mRNA levels (mean ± SD, n = 9–12) for the small intestine segments (cf. Table 2) are from at least two real-time PCR experiments and are normalized to the values for intestinal segment A from WT mice.

<sup>b</sup> ANOVA followed by the Dunnett multiple comparison test indicates that all NPC1L1 relative mRNA levels, except those for intestinal segment B from WT, SR-BI<sup>-/-</sup>, and SR-BI<sup>-/-</sup>/CD36<sup>-/-</sup> mice, are significantly different (*P* < 0.05) to the control value for intestinal segment A from WT mice fed a basal diet. All ABCG5 relative mRNA levels are significantly different to the control value for intestinal segment A of WT mice fed a basal diet.

TABLE 4. Contributions of scavenger receptors to serum cholesterol levels in mice fed a basal or HFHC diet for 3 weeks<sup>a</sup>

Mouse Genotype	Serum Cholesterol (mg/dl)	
	Basal Diet <sup>b</sup>	HFHC Diet
WT	74 ± 14 (n = 14)	130 ± 34 (n = 8)
SR-BI <sup>-/-</sup>	194 ± 39 <sup>c</sup> (n = 13)	335 ± 83 <sup>d</sup> (n = 8)
CD36 <sup>-/-</sup>	92 ± 37 (n = 13)	213 ± 50 (n = 8)
SR-BI <sup>-/-</sup> /CD36 <sup>-/-</sup>	245 ± 40 <sup>c</sup> (n = 11)	491 ± 94 <sup>d</sup> (n = 8)

All values are mean ± SD.

<sup>a</sup> The mice were fasted overnight.

<sup>b</sup> Data are from (33).

<sup>c</sup> Significantly different from WT on basal diet.

<sup>d</sup> Significantly different from WT on HFHC diet.

for the effects of SR-BI and CD36 on the equilibration of cholesterol between the donor and acceptor particles. The cholesterol uptake specific activity of  $10 \pm 2$   $\mu$ IU/mg protein for BBMV prepared from the proximal one-third of the small intestine (section A) of WT mice fed a HFHC diet is in good agreement with a prior report (31). It is apparent from the data in Table 6 that both SR-BI and CD36 can facilitate cholesterol uptake and that removal of either of both of the receptors reduces the uptake specific activity to 7  $\mu$ IU/mg protein; the value for SR-BI<sup>-/-</sup> mice is consistent with an earlier report (31). The reduction in cholesterol uptake specific activity of the proximal BBM in mice lacking SR-BI and/or CD36 presumably underlies the shift in cholesterol absorption to more distal regions of the small intestine in these mice (Fig. 2). The cholesterol uptake specific activities listed in Table 6 for BBMV from intestinal sections B and C of WT and SR-BI<sup>-/-</sup> mice are generally in good agreement with values reported previously (31); before, we measured a value  $11.5 \pm 2$   $\mu$ IU/mg protein for section B of WT mice, whereas in the current experiments, for some unknown reason, the lower value of  $8.1 \pm 0.1$   $\mu$ IU/mg protein is obtained. Overall, the results in Table 6 for intestinal sections B and C suggest that removal of SR-BI and/or CD36 has essentially no effect on the cholesterol uptake specific activity (perhaps due to the upregulation of an additional transporter; see below). Treatment of the BBMV with proteinase K to digest proteins reduces the cholesterol uptake specific activity to a value of 1–2  $\mu$ IU/mg protein for BBMV prepared from the intestinal segments listed in Table 6; this value is the specific activity expected when cholesterol uptake occurs only by

simple diffusion without any protein facilitation (22). Significantly, BBMV prepared from SR-BI<sup>-/-</sup>/CD36<sup>-/-</sup> mice exhibit a reduced cholesterol uptake specific activity after treatment with proteinase K. This observation indicates that there is a protein facilitation component to cholesterol uptake into BBMV lacking both SR-BI and CD36. It follows that there is an additional protein(s) besides these class B scavenger receptors that can mediate facilitated cholesterol uptake into the BBM (22).

## DISCUSSION

Although SR-BI and CD36 facilitate the intestinal absorption of hydrophobic minor dietary components, such as  $\beta$ -carotene (31) and very long chain fatty acids (33), they do not affect net absorption of dietary cholesterol in mice at 4 and 24 h (Tables 1 and 5). The same conclusion applies to the 4 h absorption data except that with a basal diet there is a trend toward lower values with the CD36<sup>-/-</sup> mice (Table 1). This lack of effect of SR-BI and CD36 is surprising given that, consistent with their known abilities to enhance cholesterol flux into cells (31, 37, 38), deletion of SR-BI and CD36 reduces cholesterol uptake into the proximal BBM (Table 6). To understand the reasons for this apparent discrepancy, it is helpful to consider the factors controlling the flux of cholesterol across the BBM when the levels of dietary cholesterol are low and high.

### Role of scavenger receptors with a basal diet

Since the 24 h absorption of a bolus of cholesterol is unaffected by deletion of SR-BI and/or CD36, which have the ability to modulate the rate of cholesterol equilibration between donor particles in the lumen of the small intestine and the BBM, it follows that the uptake step is not rate-limiting for the overall process. The factors controlling the partitioning of cholesterol across the BBM under basal diet conditions are summarized in Fig. 3A. Under this low cholesterol condition, the net flux of cholesterol across the BBM is controlled by the scavenger receptor-mediated bidirectional flux (37, 52) between BSM and the BBM and the NPC1L1-mediated flux from the BBM into the cell interior (15, 24–28). The local absorptive capacity is never overcome; therefore, removal of the scavenger receptors does not delay or reduce absorption (Table 1; Fig.

TABLE 5. Influence of scavenger receptors on intestinal cholesterol absorption in mice fed a HFHC diet for 3 weeks<sup>a</sup>

Mouse Genotype	24 h Absorption Efficiency (%)	4 h Intestinal Content (% Initial Dose)	% Initial Dose in 100 $\mu$ l Plasma at 4 h	% Initial Dose in 100 mg Liver at 4 h
	(n = 12/group)	(n = 8/group)	(n = 8/group)	(n = 8/group)
WT	25 ± 7	19 ± 5	0.05 ± 0.02	0.38 ± 0.12
SR-BI <sup>-/-</sup>	— <sup>b</sup>	25 ± 6	0.17 ± 0.07 <sup>c</sup>	0.47 ± 0.21
CD36 <sup>-/-</sup>	23 ± 9	17 ± 6	0.04 ± 0.02	0.34 ± 0.17
SR-BI <sup>-/-</sup> /CD36 <sup>-/-</sup>	26 ± 8	20 ± 4	0.26 ± 0.09 <sup>c</sup>	0.64 ± 0.31

All values are mean ± SD.

<sup>a</sup> The mice weighed  $25.8 \pm 3.7$  g and were aged 3–5 months.

<sup>b</sup> The cholesterol absorption in SR-BI<sup>-/-</sup> mice has been reported previously as being the same as WT mice on a HFHC diet (31).

<sup>c</sup> Significantly different from WT.

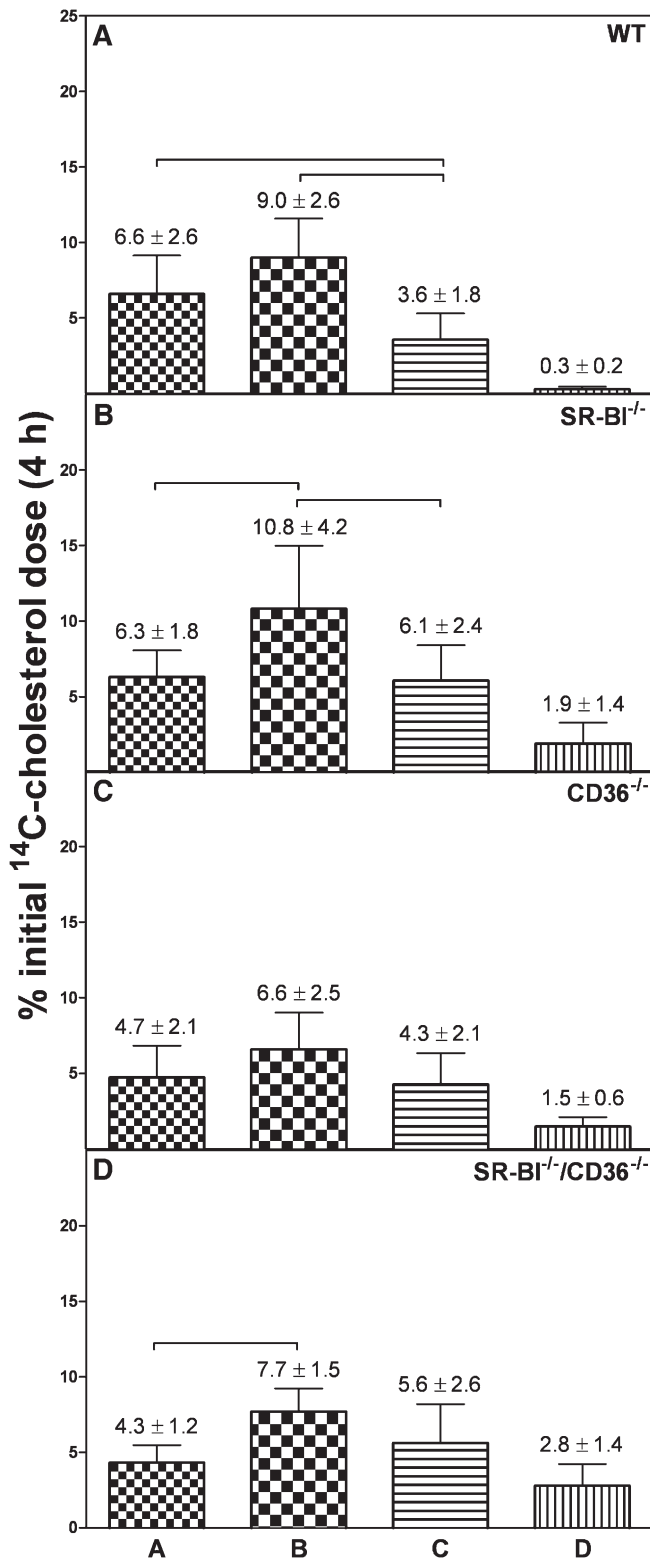


Fig. 2. Distribution of [<sup>14</sup>C-cholesterol] absorption along the small intestine of mice fed a HFHC diet. The presentation of the data in this figure is the same as that described in the legend to Figure 1.

1). Apparently, the ABCG5/G8-mediated efflux of cholesterol from the BBM to BSM is not significant under basal diet conditions because deletion of these two half-transporters has no effect on cholesterol absorption efficiency

TABLE 6. Influence of scavenger receptors on the cholesterol uptake activity of the BBM<sup>a</sup>

Mouse Genotype	Cholesterol Uptake Activity of Intestinal Section (μIU/mg Protein)		
	A	B	C
WT	10 ± 2	8.1 ± 0.1	12.0 ± 0.2
SR-BI <sup>-/-</sup>	7 ± 2 <sup>b</sup>	10.9 ± 0.3	11.5 ± 0.1
CD36 <sup>-/-</sup>	7 ± 2 <sup>b</sup>	11.2 ± 0.8	11.9 ± 0.2
SR-BI <sup>-/-</sup> /CD36 <sup>-/-</sup>	7.0 ± 0.5 <sup>b</sup>	13 ± 2	12.0 ± 0.1

<sup>a</sup>The small intestines of four mice of each genotype (fed an HFHC diet) were divided into three sections (A = proximal 10 cm, B = medial 10 cm, and C = distal 10 cm), the corresponding sections were pooled, and BBMV were prepared. The cholesterol uptake activity at 25 ± 1°C was measured with these BBMVs (0.9 mg protein/ml) and with egg phosphatidylcholine SUVs containing 1 mol% [<sup>14</sup>C] cholesterol (0.05 mg of total lipid/ml) as the donor. The values for the cholesterol uptake specific activity (expressed as μIU = pmol cholesterol transferred per minute and mg BBMV protein) represent the mean ± SD of six measurements.

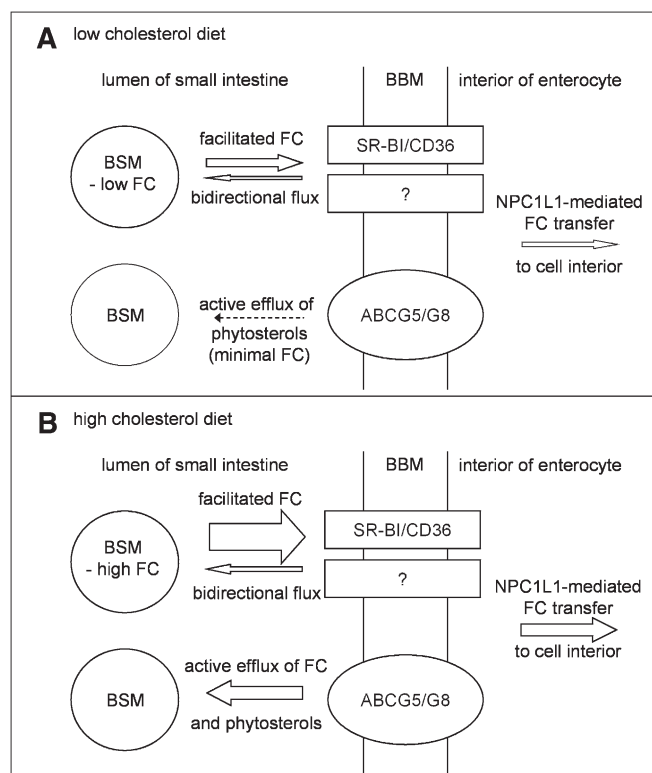
<sup>b</sup>Significantly different from WT.

(11). The limit of ~70% absorption efficiency that keeps the cholesterol secretory pathway into feces open, is presumably influenced by the activity of NPC1L1 (6, 28) and perhaps other as yet unidentified BBM proteins (Fig. 3). The excretion of dietary and biliary cholesterol into feces is a key pathway for eliminating cholesterol and is critical for whole-body cholesterol homeostasis. There is also a contribution to this fecal loss from a pathway involving direct secretion of intestinally derived cholesterol (53, 54). The absorption of less than ~70% of dietary cholesterol in 24 h is seen only when 1) upstream (pre-enterocyte) events are slowed by factors, such as insufficient lipase activity and bile salt availability (14, 16), and 2) when downstream (post BBM) events, such as intra-enterocyte cholesterol trafficking, are slowed by deletion of NPC1L1 (5, 6) and cholesterol esterification is prevented by elimination of ACAT-2 (7–9).

While the uptake step is not rate-limiting for net 24 h cholesterol absorption, the scavenger receptor facilitation of cholesterol uptake into BBM might be expected to affect the 4 h rate. However, this is not the case because the net 4 h intestinal cholesterol content (Table 1) and location of absorption (Fig. 1) are unaltered. If the ~30% reduction in cholesterol uptake into the proximal BBM caused by removal of SR-BI and/or CD36 (Table 6) occurs in vivo, it seems that such a reduction is insufficient to affect the net partitioning of cholesterol between the BSM and the BBM. Interestingly, an ~25-fold increase in jejunal SR-BI expression is sufficient to increase 4 h cholesterol absorption in mice (47).

### Role of scavenger receptors with a HFHC diet

It is well known that the cholesterol absorption efficiency is reduced on a HFHC diet (51), and our results (Table 5) are in agreement with this effect. It is important to note that, although the cholesterol absorption efficiency is only 25% compared with 70% on the basal diet (which contains no cholesterol), the mass of dietary cholesterol absorbed each day by the mice on the HFHC diet is ~50 mg higher (based on typical food intakes). As mentioned above, the



**Fig. 3.** Factors controlling the partitioning of cholesterol across the BBM. In this model, the bidirectional flux of unesterified (free) cholesterol (FC) between mixed BSMs in the lumen of the proximal small intestine and the BBM is facilitated by the class B scavenger receptors SR-BI and CD36. The active efflux of FC from the BBM to BSM is mediated by the ATP binding cassette transporter complex ABCG5/G8. The transport of cholesterol from the BBM to the interior of the enterocytes is mediated by the protein NPC1L1. The thicknesses of the arrows in the diagrams indicate the relative magnitudes of the FC fluxes. **A:** FC fluxes in a mouse on a basal, low-cholesterol diet. In this situation, the BSM donor particles contain low amounts of FC, and the net influx of FC to the BBM is modest; this passive influx comprises facilitated diffusion components involving SR-BI and CD36 and a simple diffusion component. There is another unidentified BBM protein(s) that can facilitate cholesterol uptake (indicated by the box containing the question mark), and this pathway presumably plays a role in mice lacking SR-BI and CD36. ABCG5/G8 is involved primarily in effluxing dietary phytosterols from the BBM to BSM and has minimal influence on FC flux. The rate-limiting step for the transfer of FC from the BSM across the BBM to the cell interior is presumed to be the efflux mediated by NPC1L1. In this situation, about 30% of dietary cholesterol is excreted in the feces. **B:** FC fluxes in a mouse on a HFHC (Western) diet. In this situation, the BSMs contain high amounts of cholesterol, and this concentration gradient drives net influx into the BBM. However, NPC1L1 is downregulated so that the fractional release of cholesterol from the BBM into the cell interior is decreased (although the net cholesterol mass movement is elevated because the pool of cholesterol in the BBM is elevated), and ABCG5/G8-mediated efflux of FC from the BBM back to the BSM is enhanced. In this condition, the local rate-limiting step for transfer of FC into the enterocytes becomes the uptake facilitated by SR-BI/CD36. About 75% of dietary cholesterol is excreted in the feces in this situation. It should be noted that the reaction scheme depicted here is simplified compared with the situation *in vivo* where (1) BSMs are not the only FC donor particles and the FC fluxes alter as the compositions of the particles change along the gastrocolic axis, and (2) mixing of dietary and biliary cholesterol occurs.

HFHC diet decreases expression of NPC1L1 presumably because of its high fat content (50, 55). The resultant increase in BBM cholesterol content and ABCG5/G8 cholesterol efflux activity under HFHC diet conditions leads to a back flux of cholesterol to BSM in the lumen of the small intestine so that the net level of cholesterol flux into the BBM becomes sensitive to the scavenger receptor-mediated influx contribution (Fig. 3B). As a consequence, the local absorptive capacity in the proximal small intestine becomes overwhelmed, and the 4 h absorption is delayed when SR-BI and CD36 are eliminated (Fig. 2). However, the absorptive capacity in distal regions of the small intestine is sufficient so that the total 4 h absorption of dietary cholesterol is unaffected (Table 5). The 24 h cholesterol absorption efficiency is insensitive to removal of the scavenger receptors (Table 5) for the same reasons.

The above discussion is focused on the roles of SR-BI and CD36 in the uptake of cholesterol into the BBM. However, the results in Table 6 indicate that protein-facilitated cholesterol uptake occurs with BBM lacking both SR-BI and CD36. More work is required to identify the transporter(s) responsible for this effect. Since the flux of dietary cholesterol across the BBM is limited by the activities of NPC1L1 and ABCG5/G8 (Fig. 3), other uptake transporters are probably not critical for cholesterol absorption. It is likely that any such transporter is important for the absorption of some hydrophobic minor dietary component(s) and incidentally facilitates cholesterol uptake into the BBM. Such a phenomenon would be consistent with the existence of multiple complementary pathways of lipid transport across enterocytes (56, 57).

In summary, SR-BI and CD36, which promote the absorption of minor hydrophobic components of the diet, also facilitate the uptake of cholesterol from BSM in the lumen of the proximal small intestine into the BBM. However, at least in mice, this uptake step is not normally rate-limiting for cholesterol absorption so that deletion of either or both of the receptors has no effect on the net cholesterol absorption efficiency. Since facilitated uptake of cholesterol occurs in the BBM of mice lacking SR-BI and CD36, apparently there is another functional protein(s) that remains to be identified. **■**

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