Hepatic cholesterol and bile acid metabolism and intestinal cholesterol absorption in scavenger receptor class B type I-deficient mice

Pablo Mardones,* Verónica Quiñones,* Ludwig Amigo,* Mauricio Moreno,* Juan Francisco Miquel,* Margrit Schwarz,† Helena E. Miettinen,§ Bernardo Trigatti,§ Monty Krieger,§ Sonya VanPatten, David E. Cohen,** and Attilio Rigotti[1],***

Departamento de Gastroenterología,* Facultad de Medicina, Pontificia Universidad Católica, Santiago, Chile; Department of Molecular Genetics,† University of Texas Southwestern Medical Center, Dallas, TX 75390; Biology Department,§ Massachusetts Institute of Technology, Cambridge, MA 02139; Departments of Medicine and Biochemistry,** Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, NY 10461

Abstract The scavenger receptor class B type I (SR-BI), which is expressed in the liver and intestine, plays a critical role in cholesterol metabolism in rodents. While hepatic SR-BI expression controls high density lipoprotein (HDL) cholesterol metabolism, intestinal SR-BI has been proposed to facilitate cholesterol absorption. To evaluate further the relevance of SR-BI in the enterohepatic circulation of cholesterol and bile salts, we studied biliary lipid secretion, hepatic sterol content and synthesis, bile acid metabolism, fecal neutral sterol excretion, and intestinal cholesterol absorption in SR-BI knockout mice. SR-BI deficiency selectively impaired biliary cholesterol secretion, without concomitant changes in either biliary bile acid or phospholipid secretion. Hepatic total and unesterified cholesterol contents were slightly increased in SR-BI-deficient mice, while sterol synthesis was not significantly changed. Bile acid pool size and composition, as well as fecal bile acid excretion, were not altered in SR-BI knockout mice. Intestinal cholesterol absorption was somewhat increased and fecal sterol excretion was slightly decreased in SR-BI knockout mice relative to controls. These findings establish the critical role of hepatic SR-BI expression in selectively controlling the utilization of HDL cholesterol for biliary secretion. In contrast, SR-BI expression is not essential for intestinal cholesterol absorption.—Mardones, P., V. Quiñones, L. Amigo, M. Moreno, J. F. Miquel, M. Schwarz, H. E. Miettinen, B. Trigatti, M. Krieger, S. VanPatten, D. E. Cohen, and A. Rigotti. **Hepatic cholesterol and bile acid metabolism and intestinal cholesterol absorption in scavenger receptor class B type I-deficient mice.** ***J. Lipid Res.*** **2001.** 42: **170–180.**

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Whole body cholesterol homeostasis is maintained by a complex network of biosynthetic, trafficking, secretory, and regulatory mechanisms (1). Among these processes, the enterohepatic circulation of cholesterol and bile salts plays a critical role (2). For the maintenance of cholesterol homeostasis in the adult organism, intestinal cholesterol absorption and endogenous cholesterol synthesis must be matched by biliary cholesterol and bile acid secretion. Whereas transporters necessary for enterohepatic cycling of bile salts have been identified (3), many of the genes that control biliary cholesterol secretion and intestinal cholesterol absorption have not yet been described.

A series of in vitro and in vivo experiments in mice has established that the scavenger receptor class B type I (SR-BI) is a physiologically and pathophysiologically relevant lipoprotein receptor that plays an important role in cholesterol metabolism [reviewed in refs. (4–7)]. SR-BI was initially identified as a member of the CD36 family of proteins (8, 9) and as a receptor for native and modified low density lipoproteins (LDL) (9) and anionic phospholipids (10). Subsequently, SR-BI was shown to be the first molecularly well-defined cell surface high density lipoprotein (HDL) receptor (11). Interestingly, SR-BI mediates selective cholesterol uptake (11), a process in which lipoprotein cholesteryl esters and unesterified cholesterol are directly transferred to the plasma membrane of the cells without internalization and hydrolytic disassembly of the lipoprotein particle (11–14). Furthermore, in vitro studies have shown that SR-BI can facilitate both cellular uptake of nonlipoprotein unesterified cholesterol (15, 16) and cellular efflux of unesterified cholesterol (15, 17). However, the physiologic importance of these additional activities of SR-BI remains to be established.

Abbreviations: apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; apoE, apolipoprotein E; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high density lipoproteins; HL, hepatic lipase; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins; SR-BI, scavenger receptor class B, type I; SR-BI KO mice, SR-BI-deficient mice.

[1] To whom correspondence should be addressed.
e-mail: arigotti@med.puc.cl

In rodents, SR-BI is most abundantly expressed in the liver and steroid hormone-producing tissues (11, 18). This distribution is consistent with the important role of these tissues in lipoprotein metabolism. The high level of hepatic SR-BI expression is consistent with the major role of the liver in selective HDL cholesterol uptake and HDL metabolism in vivo (19, 20). In fact, the liver accounts for a significant fraction of total HDL cholesterol clearance by the selective HDL cholesteryl ester uptake pathway both in species that do and do not express the cholesteryl ester transfer protein (19–22). In addition, HDL is the preferential lipoprotein source of plasma cholesterol for biliary lipid secretion [reviewed by Botham and Bravo (23)], the last step of reverse cholesterol transport. As a consequence of its key role in HDL metabolism through the selective lipid uptake pathway, the expression of SR-BI in the liver is expected to be critical in controlling two key features of cholesterol metabolism. These are plasma HDL cholesterol levels and utilization of this cholesterol source for biliary secretion, the major route for body sterol disposal and thus for the maintenance of cholesterol homeostasis in vivo.

Direct evidence supporting the role of hepatic SR-BI expression in reverse cholesterol transport has come from studies of mice in which levels of SR-BI were genetically manipulated in vivo [reviewed in refs. (4–7)]. Overexpression of SR-BI in the mouse liver dramatically decreased plasma HDL (24–26) and increased hepatic and gallbladder biliary cholesterol concentration (24, 27). In contrast, SR-BI deficiency in mice was shown to be associated with increased plasma HDL cholesterol (28, 29) and significantly lowered gallbladder bile cholesterol content (30). Metabolic kinetic analyses of HDL labeled in its cholesterol moiety have suggested that SR-BI expression in the liver is correlated with hepatic HDL cholesterol uptake and secretion into bile (14). In addition, the reduction in biliary cholesterol secretion resulting from estrogen treatment and cholesterol feeding in rats (31) can be correlated with decreased levels of hepatic SR-BI protein induced by these pharmacological and dietary manipulations (18, 32). Taken together, these results suggest that SR-BI influences biliary cholesterol content in vivo; however, the mechanism by which this is accomplished remains unclear. Of particular interest is the potential role of SR-BI-mediated hepatic uptake of HDL cholesterol in the control of hepatic cholesterol content, bile acid metabolism, and biliary lipid secretion.

SR-BI is also expressed in the intestine (18), but its relevance in gastrointestinal physiology has not been defined. Hauser and colleagues have proposed that SR-BI is involved in intestinal cholesterol absorption (16). They demonstrated that *1*) SR-BI was expressed on the apical surface of rabbit enterocytes, and *2*) SR-BI ligands and anti-SR-BI antibodies inhibited the uptake of unesterified and esterified cholesterol from bile salt mixed micelles and phospholipid vesicles into intestinal brush border membrane preparations and Caco-2 cells in vitro (16). Consistent with this hypothesis is the observation that intestinal SR-BI expression is suppressed when the efficiency of intestinal cholesterol absorption is decreased in rodents by impairing biliary lipid delivery into the intestinal lumen (P. J. Voshol, M. Schwarz, A. Rigotti, M. Krieger, A. K. Groen, and F. Kuipers, unpublished data). Nevertheless, a role for SR-BI in the absorption of dietary cholesterol in vivo has not been established.

The availability of SR-BI knockout mice provides a powerful experimental model with which to address the role of SR-BI in a variety of cholesterol-related enterohepatic metabolic processes in whole animals. Therefore, the current study was undertaken to directly evaluate the in vivo effects of murine SR-BI deficiency on hepatic cholesterol balance, cholesterol and lipoprotein metabolism-related gene expression, bile acid metabolism, biliary lipid secretion, fecal neutral sterol excretion, and intestinal cholesterol absorption.

MATERIALS AND METHODS

Animals and diets

Mice with a targeted mutation in the *srbi* locus were obtained by gene disruption in a 129/Sv-derived ES cell line as described (28). The mutation in the *srbi* gene was maintained in a mixed genetic background (C57BL/6 × 129/Sv) by crossing heterozygous *srbi* mutant females with homozygous *srbi* mutant (SR-BI KO) males. Homozygous SR-BI KO mice were screened by polymerase chain reaction (PCR) as previously described (28). Control wild-type mice were also maintained in a mixed (C57BL/6 × 129/Sv) strain background. Male SR-BI KO mice (2–3 months old) as well as sex- and age-matched control (C57BL/6 × 129/Sv) mice were used for experiments. Wild-type C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME), bred, and used when they were 2–3 months old.

Animals were housed in a temperature- and humidity-controlled room with reverse light cycling and fed ad libitum with a low cholesterol-containing diet (<0.02% cholesterol, 4.5% fat, 22.5% protein) (Prolab RMH3000; PMI Feeds, St. Louis, MO) with free access to water. For biliary lipid secretion and hepatic cholesterol and bile acid metabolism experiments, control and SR-BI KO mice were kept on a Prolab RMH3000 chow diet and studied after overnight fasting toward the middle/end of the dark phase of the light cycle. For the cholesterol-dependent hepatic SR-BI regulation studies, C57BL/6J mice were fed the Prolab RMH3000 chow diet, the same diet supplemented with 2% cholesterol, or a high cholesterol/high fat/bile acid-containing diet (1.25% cholesterol, 15% total fat, and 0.5% cholic acid) (TD90221; Harlan Teklad, Madison, WI) for 4 weeks. For intestinal cholesterol absorption and fecal neutral sterol excretion studies, control and SR-BI KO mice were kept on a chow diet (<0.02% cholesterol, 4% total fat, 24% protein) (Teklad 7001; Harlan Teklad) or were switched as indicated to the same diet supplemented with 0.2% or 1% cholesterol for 3–6 weeks. Protocols were carried out according to accepted criteria for humane care of experimental animals and approved by the review boards for animal studies of the Pontificia Universidad Católica and the Massachusetts Institute of Technology.

Hepatic and gallbladder bile, blood, and liver sampling

Mice were anesthetized with pentobarbital (4.5 mg/100 g body weight). The abdomen was opened, the cystic duct was ligated, and a common bile duct fistula was performed with a polyethylene catheter. Hepatic bile was collected for 30–60 min while mice

were kept under anesthesia at 37°C with a heating lamp. After hepatic bile sampling, blood was removed by puncture of the inferior vena cava with a heparinized syringe, mice were euthanized, and livers were removed. Plasma was separated by centrifugation at 10,000 rpm for 10 min at 4°C. Hepatic bile flow, calculated by dividing the volume of bile (determined gravimetrically assuming a specific density = 1.0) by the collection time and the liver weight, was expressed as μl/min/g of liver. Bile and plasma were kept at −20°C while liver was stored at −70°C until they were processed for biochemical analyses.

Hepatic Northern blot analysis

Total RNA was prepared from mouse liver by the acid guanidinium thiocyanate-phenol-chloroform method (33). cDNA probes for mouse apolipoprotein A-I (apoA-I), apoA-II, apoE, LDL receptor, hepatic lipase, lecithin:cholesterol acyltransferase (LCAT), 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, cholesterol-7α-hydroxylase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared from total liver RNA by a standard reverse transcriptase (Life Technologies, Gaithersburg, MD)-PCR procedure using primers based on mouse cDNA sequences available through GenBank databases. PCR-generated probes were subcloned into the pGEM-T vector (Promega, Madison, WI), sequenced, released from the cloning vector by restriction enzymes, and purified by agarose gel electrophoresis before radiolabeling by the random primer method (Promega), using [α-^{32}P]dCTP. Equal amounts (10–30 μg per individual mouse from each experimental group) of total liver RNA were size fractionated by agarose-formaldehyde gel electrophoresis and transferred to a nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ). The filters were hybridized with the indicated ^{32}P-labeled probes in 0.5 M phosphate buffer (pH 7.0), 1 mM ethylenediaminetetraacetic acid, 7% sodium dodecyl sulfate (SDS), and 0.5% bovine serum albumin for 18 h at 65°C, washed once with 2× sodium chloride-sodium citrate (SSC) buffer (pH 7.0), 0.1% SDS for 10 min at 65°C, and twice with 1× SSC buffer (pH 7.0), 0.1% SDS for 10 min at 65°C, and exposed to Kodak (Rochester, NY) film with intensifying screens for 10 min to 16 h at −80°C. The resulting bands were quantified by densitometric analysis, using a Macintosh Color One scanner and NIH Image software. The mRNA expression levels for each gene were normalized to the signal generated from hybridization of a ^{32}P-labeled mouse GAPDH cDNA probe on the same filter.

Hepatic immunoblotting analysis

Total membrane extracts (postnuclear 100,000 *g* membrane pellets) from mouse liver were prepared (34), size fractionated (30 μg of protein/sample) by 10% SDS-polyacrylamide gel electrophoresis (PAGE), and immunoblotted on nitrocellulose with a rabbit polyclonal antipeptide antibody against mouse SR-BI protein (11). Anti-ε-COP antibody was used as a membrane protein loading control (35). Antibody binding to protein samples was visualized by the enhanced chemiluminiscence procedure (Amersham Pharmacia Biotech) and measured with the GS-525 Molecular Image system (Bio-Rad, Hercules, CA). SR-BI protein expression levels were normalized to the signal of the ε-COP protein on the same nitrocellulose membrane.

Hepatic cholesterol synthesis

Sterol synthesis in the liver was measured as previously described (36). During the middark phase of the light cycle, mice received an intraperitoneal dose of 50 mCi of tritiated water (New England Nuclear, Boston, MA). After 1 h, mice were anesthetized with pentobarbital, the abdomen was opened, and blood and liver were removed. Plasma was separated by centrifugation and stored at 4°C for determination of tritiated water-specific activity. Liver was briefly rinsed in a normal saline solution, weighed, and stored at −70°C until processed for measurement of the incorporation of tritiated water into digitonin-precipitable sterols as previously described (36). The rate of hepatic sterol synthesis is expressed as nanomoles of tritiated water incorporated into digitonin-precipitable sterols per hour per gram of liver tissue.

Bile acid pool size and composition

Bile acid pool size was determined as the sum of total bile acids measured in gallbladder bile, hepatic bile, and ethanolic extracts of liver and small intestine prepared as described (37). Bile acid content was measured with an enzymatic assay (see below) and corrected for recovery of [^{14}C]taurocholate internal standard added to each sample before extraction. The data are expressed as micromoles of bile acids per 100 g of body weight. Bile acid pool composition was determined in selected aliquots of the ethanolic bile acid extracts by high pressure liquid chromatography as previously described (38, 39). The data are expressed as molar percentage of each bile acid molecular species.

Fecal bile acid and neutral sterol excretion

Individually housed animals were subjected to a 72-h period of stool collection, after which stools were dried, weighed, and ground into a powder. Fecal bile acid excretion was measured as previously described (40). Stool aliquots (1 g each) were treated with sodium borohydride and subjected to alkaline hydrolysis. The final extracts were dried under nitrogen and resuspended in sodium hydroxide, and bile salts were separated in C_{18} Sep-Pack column as described elsewhere (41). Aliquots of eluted samples were used for enzymatic bile acid measurement (see below) and for recovery determination of [^{14}C]taurocholate internal standard added to each stool sample before hydrolysis. For the measurement of neutral sterol excretion, a second 1-g aliquot was subjected to alkaline hydrolysis at 120°C for 12 h and dried, and 10 ml of water and 10 ml of ethanol were added. Neutral sterols were extracted by the addition of 15 ml of petroleum ether containing 1.0 mg of 5-cholestene (Sigma, St. Louis, MO) as an internal standard. The amounts of cholesterol, coprostanol, epicoprostanol, and cholestenone were quantitated by gas chromatography (42). The excretion rates of both bile acid and neutral sterol are expressed as micromoles per day per 100 g of body weight.

Intestinal cholesterol absorption

Cholesterol absorption was measured by a fecal dual-isotope ratio method (43). Toward the end of the dark phase of the light cycle, mice were subjected to a light methoxyfluorane anesthesia. Per 25 g of body weight, a mixture of 1 μCi of [5,6-^{3}H]sitostanol (American Radiolabeled Chemicals, St. Louis, MO) and 2 μCi of [4-^{14}C]cholesterol (New England Nuclear) in 100 μl of MCT oil was delivered by intragastric gavage. Animals were housed individually in cages and stools were collected after 72 h. Separate 0.5-g stool aliquots were used for measurement of total neutral sterol excretion as described above, and for extraction of radiolabeled sterols. The percentage of cholesterol absorption was calculated from the ^{14}C/^{3}H ratio in the extracted sterol mixture as previously described (43).

Plasma, hepatic, and biliary lipid analyses

Plasma total cholesterol was measured by an enzymatic assay (28). Hepatic total, unesterified, and esterified cholesterol content and biliary cholesterol concentration were determined after lipid extraction as described (44, 45). Bile acid content in bile, stools, and bile acid pool extracts was measured by the 3α-hydroxysteroid dehydrogenase assay (46). Biliary phospholipid concen-

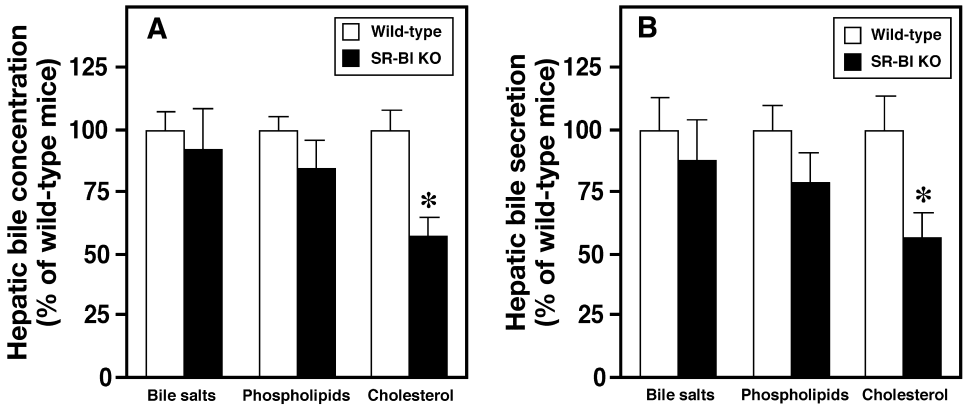

Fig. 1. Biliary lipid concentration (A) and secretion (B) in wild-type and SR-BI KO mice. Wild-type and SR-BI KO mice were fed a standard chow diet and were fasted overnight prior to bile sampling for biliary lipid concentration (A) and secretion (B) determinations. Analyses were performed in 6–13 animals from each experimental group. Data are shown as the percentage of values for control wild-type mice. A: Hepatic biliary lipid concentration; the 100% control values for biliary bile salt, phospholipid, and cholesterol concentrations in wild-type mice were 38 ± 3, 6.7 ± 0.4, and 1.3 ± 0.1 mM, respectively. The absolute values of the same parameters in SR-BI KO mice were 35 ± 6, 5.6 ± 0.7, and 0.7 ± 0.1 mM, respectively. B: Hepatic biliary lipid secretion; the 100% control values for biliary bile salt, phospholipid, and cholesterol secretion in wild-type mice were 80 ± 10, 16 ± 2, and 2.7 ± 0.4 nmol/min/g of liver, respectively. The absolute values of the biliary lipid secretion rates in SR-BI KO mice were 70 ± 14, 13 ± 2, and 1.5 ± 0.3 nmol/min/g of liver, respectively. Values are expressed as means ± standard error. * Value is significantly different from wild-type mice.

tration was measured as inorganic phosphorus released by acid hydrolysis (47). Biliary lipid output was derived from biliary lipid concentration and measured hepatic bile flow.

Statistical analysis

Results are presented as means ± standard error. The statistical significance of the differences between the means of the experimental groups was tested by the Student's *t*-test for unpaired data. A difference was considered statistically significant when $P < 0.05$.

RESULTS

Previous studies have shown that SR-BI plays a key role in delivering HDL cholesterol to the liver [reviewed in refs. (4–7)] and therefore should have a significant influence on hepatic cholesterol metabolism and the enterohepatic circulation of cholesterol. Indeed, we reported that the absence of SR-BI expression in mice results in decreased gallbladder biliary cholesterol (30). To explore further the mechanism by which SR-BI influences biliary cholesterol content in particular, and enterohepatic circulation and hepatic sterol metabolism in general, we have performed a series of studies using wild-type and SR-BI KO mice. Animals were 2- to 3-month-old males and were maintained in a mixed (C57BL/6 × 129/Sv) genetic background. At the time of the study the mean body weights of the wild-type and SR-BI-deficient mice were similar (~22–24 g), as were the average liver weights (~0.9 g). In contrast, plasma total cholesterol concentrations in SR-BI KO mice were significantly higher (213 ± 13 mg/dl) than those found in wild-type mice (71 ± 5 mg/dl). This finding was similar to that reported previously (28), in which the higher plasma total cholesterol was shown to be due mostly to increases in cholesterol transported in abnormally large and heterogeneous apoE-rich HDL particles of SR-BI KO mice, with only minor increases in non-HDL cholesterol.

Figure 1 shows the effects of ablation of SR-BI expression on the hepatic biliary concentrations (Fig. 1A) and hepatic secretion rates (Fig. 1B) of the three major lipids in bile: bile salts, phospholipids, and cholesterol. Neither the biliary concentrations nor secretion rates for bile salts and phospholipids in SR-BI KO mice were significantly different from those for the wild-type animals (100% control values; see Fig. 1 legend for absolute values of the parameters). Furthermore, bile flow in SR-BI KO mice (2.0 ± 0.2 μl/min/g liver; n = 12) was similar to that in wild-type mice (2.0 ± 0.2 μl/min/g liver; n = 13). Thus, the livers of SR-BI KO mice exhibited no apparent defects in the ability

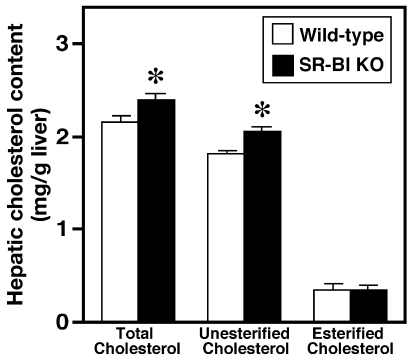

Fig. 2. Hepatic cholesterol content in wild-type and SR-BI KO mice. Liver samples from each mouse strain (n = 11 for wild-type mice, n = 9 for SR-BI KO mice) were removed, extracted with chloroform-methanol, and processed for total, unesterified, and esterified cholesterol analyses, using an enzymatic method. Values are expressed as means ± standard error. * Value is significantly different from wild-type mice.

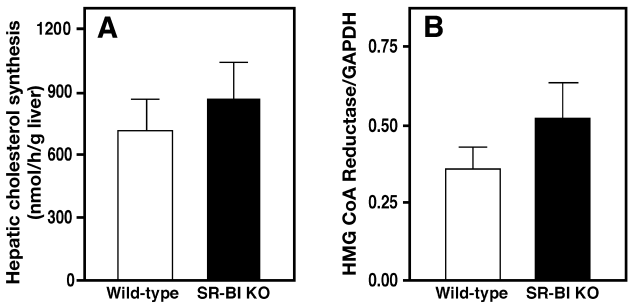

Fig. 3. Hepatic cholesterol synthesis (A) and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase mRNA levels (B) in wild-type and SR-BI KO mice. A: One hour after injection of tritiated water, mice of each strain (n = 12 for wild-type mice, n = 8 for SR-BI KO mice) were killed and plasma and liver samples were processed for measurements of incorporation of tritium-labeled water into digitonin-precipitable sterols. B: Liver samples (n = 3 animals for each mouse strain) were removed and total RNA was isolated. RNA samples (30 μg per lane per single mouse) were fractionated by electrophoresis, transferred to nylon membrane, and hybridized with ^{32}P-labeled HMG-CoA reductase, and GAPDH cDNA probes and bands were visualized by autoradiography. Bands were quantified by densitometric analysis and HMG-CoA reductase mRNA expression levels were normalized to the signal generated by the GAPDH probe. Values are expressed as means ± standard error.

to generate or secrete essentially normal amounts of bile. Nevertheless, the secreted hepatic bile was abnormal, because both the biliary cholesterol concentration and secretion rate were significantly lower (~55%) than those of the controls. These results are consistent with the previous report of a 30% lower than control level of gallbladder biliary cholesterol concentration in SR-BI KO mice (30). These data strongly suggest that the abnormally low level of biliary cholesterol in SR-BI KO mice was specifically due to a reduction in the rate of cholesterol secretion into bile.

We then explored whether the reduced biliary cholesterol secretion was a consequence of SR-BI-dependent changes in hepatic sterol metabolism (cholesterol content, cholesterol synthesis rate). SR-BI-mediated selective uptake is an important pathway for HDL cholesterol uptake by the liver and loss of SR-BI might be expected to be accompanied by reduced hepatic cholesterol levels. However, total cholesterol concentration in the livers of SR-BI KO mice was slightly, but significantly, higher than in control mice (**Fig. 2**). The small difference was due to an increase in the unesterified cholesterol concentration. There were no differences in the hepatic esterified cholesterol concentrations. The small increase in total hepatic cholesterol content in SR-BI KO mice was accompanied by minor increases in hepatic cholesterol synthesis (**Fig. 3A**) and GAPDH-normalized mRNA levels of hepatic HMG-CoA reductase, the rate-controlling enzyme in cholesterol biosynthesis (Fig. 3B). However, the increases were not significant. Thus, SR-BI-mediated HDL cholesterol uptake either does not normally contribute substantially to the maintenance of steady-state hepatic cholesterol levels, or hepatic homeostatic mechanisms in SR-BI KO mice were able to adequately compensate for the loss of SR-BI. Indeed, we also observed no significant differences in the bile acid metabolism of SR-BI KO and control mice, as evaluated by measuring bile acid pool size (**Fig. 4A**) and the rate of fecal bile acid excretion (Fig. 4B). The level of hepatic expression (steady-state mRNA concentration) of the major rate-controlling enzyme in bile acid synthesis, cholesterol 7α-hydroxylase (Fig. 4C), was somewhat reduced, but the difference observed was not significant. There also appeared to be no difference in the overall bile acid pool composition in SR-BI KO mice, which exhibited a normal cholic-to-muricholic acid ratio (results not shown). Furthermore, we did not observe significant differences in the levels of hepatic expression (mRNA) of

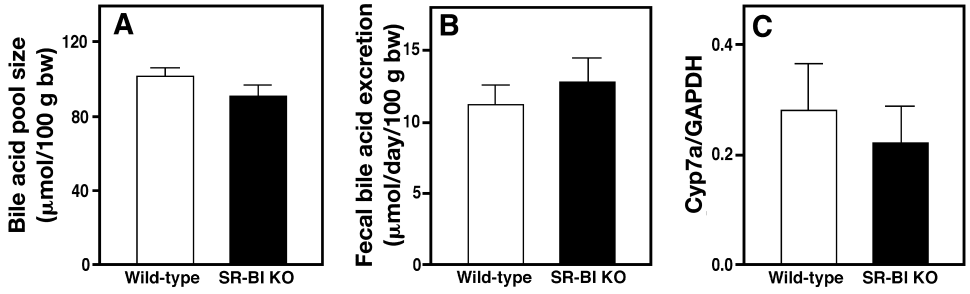

Fig. 4. Bile acid pool size (A), fecal bile acid excretion (B), and hepatic cholesterol 7α-hydroxylase mRNA levels (C) in wild-type and SR-BI KO mice. A: Mice of each genotype (n = 9 for wild-type mice, n = 8 for SR-BI KO mice) were killed and bile acid pool size, which represents the total bile acid content in the small intestine, gallbladder, and liver combined, was determined enzymatically after ethanol extraction. B: Mice of each strain (n = 7 for wild-type mice, n = 6 for SR-BI KO mice) were individually housed and fed chow diet; stools were collected daily for 72 h, and bile acids were extracted and measured enzymatically. C: Liver samples (n = 4 for wild-type mice, n = 3 for SR-BI KO mice) were removed and total RNA was isolated. RNA samples (30 μg per lane per single mouse) were fractionated by electrophoresis, transferred to nylon membrane, and hybridized with ^{32}P-labeled cholesterol 7α-hydroxylase (Cyp7a), and GAPDH cDNA probes and bands were visualized by autoradiography. Bands were quantified by densitometry and Cyp7a mRNA expression levels were normalized to the signal generated by the GAPDH probe. Values are expressed as means ± standard error.

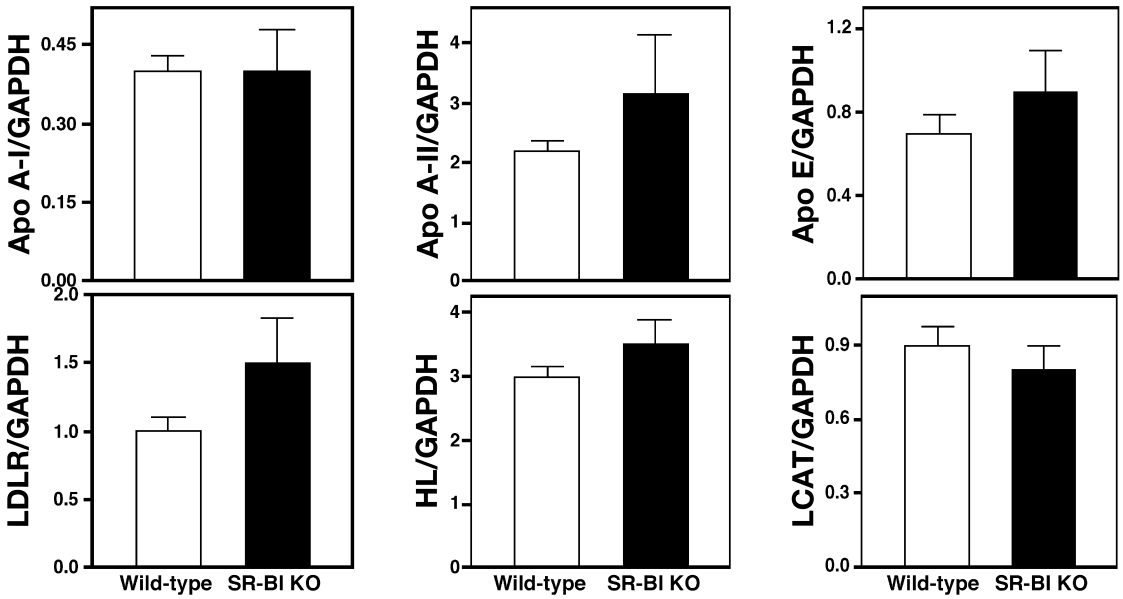

Fig. 5. Hepatic apolipoprotein, low density lipoprotein receptor (LDLR), hepatic lipase, and LCAT mRNA levels in wild-type and SR-BI KO mice. Liver samples (n = 3 or 4 animals for each strain) were removed and total RNA was isolated. RNA samples (10–30 μg per lane per each mouse) were fractionated by electrophoresis, transferred to nylon membrane, hybridized with ^{32}P-labeled apoA-I, apoA-II, apoE, LDLR, hepatic lipase, LCAT, and GAPDH cDNA probes, and bands were visualized by autoradiography. The resulting bands were quantified by densitometric analysis and mRNA expression data for each gene were normalized to the signal generated by the GAPDH probe on the same filter. The results of this figure are representative of two or three independent RNA expression analyses for each gene. Values are expressed as means ± standard error.

several other genes related to lipoprotein metabolism (apoA-I, apoA-II, and apoE, three major apolipoprotein constituents of HDL), or the HDL-remodeling enzymes LCAT and hepatic lipase (HL) (**Fig. 5**). There appeared to be an increase in hepatic LDL receptor (LDLR) expression in SR-BI KO mice (Fig. 5); however, this difference was not significant, presumably because of the relatively small sample size (n = 3 or 4) and large interanimal variation. Taken together, these findings indicate that neither a reduction in hepatic cholesterol content nor substantial alterations in hepatic bile acid metabolism were responsible for the decreased biliary secretion of cholesterol in SR-BI KO mice.

The previous experiments suggested that SR-BI expression is not critical for the control of major hepatic cholesterol metabolism parameters in mice. The next experiment was aimed at evaluating whether changes in hepatic cholesterol content induced by dietary cholesterol could affect hepatic SR-BI protein levels in C57BL/6 mice. When animals were fed for 4 weeks with either a 2% cholesterol diet or a cholesterol/fat/cholic acid-containing diet, both of which increase hepatic cholesterol content and suppress LDL receptor mRNA levels (results not shown), SR-BI expression was not changed when evaluated by immunoblotting of total liver membranes (**Fig. 6**). This finding indicates that SR-BI expression in the mouse liver is not sensitive to cholesterol-dependent feedback regulation.

The decreased level of biliary cholesterol in the SR-BI KO animals might influence dietary cholesterol absorption because of a potential reduction in the intestinal intralumenal cholesterol pool (27). Under these circumstances, the rate of absorption of labeled cholesterol administered as a bolus by gastric gavage might be higher in the SR-BI KO mice relative to the controls because of the reduced amount of biliary cholesterol delivered into the intestine, even if there was no change in the absolute rate of transport. However, Hauser and colleagues have suggested that intestinal SR-BI may directly mediate absorption of intestinal cholesterol (16). If SR-BI were essential for intestinal cholesterol absorption, then the rate of absorption of labeled cholesterol administered by gastric gavage should be decreased in the SR-BI KO relative to control mice. **Figure 7** shows the relative amounts of cholesterol absorption by SR-BI KO and control mice measured by the fecal dual-isotope ratio method (43). In these experiments, the animals were fed either a low fat, chow diet (Fig. 7A), or diets supplemented with either 0.2% (Fig. 7B) or 1% (Fig. 7C) cholesterol. For the chow-fed animals, there was a small, but reproducible increase in intestinal cholesterol absorption in SR-BI KO mice (66% of administered dose, n = 15) relative to the controls (55% of administered dose, n = 15, $P = 0.0097$). As expected (27), there was a dietary cholesterol dose-dependent decrease in the percentage of cholesterol absorption for both genotypes. In each case, however, absorption was greater in the SR-BI KO mice than in the controls.

Changes in the rates of intestinal cholesterol absorption in mice are inversely correlated with changes in the rates of fecal neutral sterol excretion (43). Accordingly, if cholesterol absorption is indeed increased in the absence of

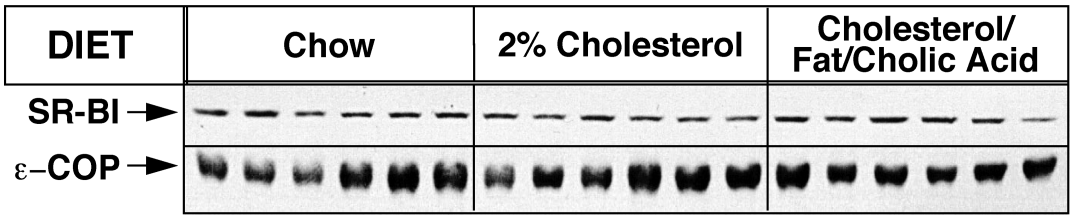

Fig. 6. Hepatic SR-BI protein levels in mice fed standard chow, a 2% cholesterol-containing diet, or a 1.25% cholesterol/15% fat/0.05% cholic acid-containing diet. C57BL/6 mice were fed the indicated diets for 4 weeks. Liver samples were removed and total membranes were isolated. Membrane proteins (30 μg per lane) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-SR-BI and anti-ε-COP antibodies. Antibody binding was detected by chemiluminescence, using phosphor screen technology. SR-BI protein expression was normalized to the signal generated by the anti-ε-COP antibody on the same membrane. Results are representative of three independent experiments.

SR-BI, then the SR-BI KO mice would be expected to show lower rates of fecal neutral sterol excretion. To test this hypothesis, we measured the rates of fecal neutral sterol excretion in mice of both genotypes. SR-BI KO mice excreted less neutral sterols than control mice (7.9 ± 2.2 vs. 9.5 ± 1.2 μmol per day per 100 g body weight, n = 10, $P = 0.035$). These results support the conclusion from the direct absorption experiments. Together, these studies establish unequivocally that SR-BI is not required for intestinal cholesterol absorption. If SR-BI normally participates directly in the cholesterol absorption process, SR-BI KO animals were apparently able to compensate for this loss of intestinal absorptive activity.

DISCUSSION

HDL is thought to play a critical role in reverse cholesterol transport by removing cholesterol from peripheral tissues and delivering it to the liver for recycling to plasma or for removal from the body through biliary secretion. In rodents and humans, both unesterified cholesterol and cholesteryl esters from plasma HDL are sources of cholesterol for biliary secretion, either as unesterified cholesterol or as bile acids [reviewed by Botham and Bravo (23)].

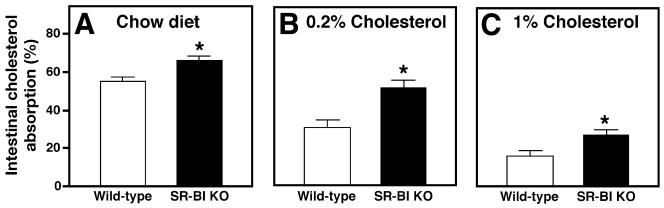

Fig. 7. Intestinal cholesterol absorption in wild-type and SR-BI KO mice fed standard chow (A) and cholesterol-enriched (B, C) diets. Mice of each genotype (n = 6–15 for wild-type mice and SR-BI KO mice) were fed a standard chow diet or cholesterol-supplemented diets. Intestinal cholesterol absorption was measured in individually housed mice by a fecal sterol dual-isotope ratio method. The cholesterol absorption values of chow-fed mice shown were obtained after combining data from two independent experiments. Values are expressed as means ± standard error. * Value is significantly different from wild-type mice.

HDL cholesterol has been reported to be utilized preferentially over non-HDL cholesterol for bile acid synthesis and biliary cholesterol secretion (23), emphasizing the importance of hepatic HDL metabolism for body sterol homeostasis. The current work and previous investigations have shown that the HDL receptor SR-BI can play an important role in hepatic HDL cholesterol metabolism. For example, hepatic overexpression of SR-BI in mice lowers plasma HDL (24–26) and increases hepatic and gallbladder bile cholesterol concentrations (24, 27), while SR-BI deficiency in mice increases HDL cholesterol (28, 29) and reduces gallbladder bile cholesterol content (30). This study, which compared several cholesterol-related metabolic parameters in SR-BI KO and control mice, provides additional characterization of the role of SR-BI in hepatic cholesterol and bile acid homeostasis as well as in intestinal cholesterol absorption.

In the absence of SR-BI, mice exhibited a selective reduction in hepatic biliary cholesterol concentration and biliary cholesterol secretion rate, whereas the biliary secretion of bile acids or phospholipids was unaffected. This was presumably due to the essential role that SR-BI plays in the normal transfer of HDL cholesterol from plasma through the liver into the bile. Under physiological conditions, SR-BI might facilitate biliary cholesterol secretion by directly mediating hepatic uptake of plasma lipoprotein cholesterol (influx) or by directly participating in biliary cholesterol secretion from the canalicular membranes (efflux), or both. The evidence of the role of SR-BI in the delivery of lipoprotein cholesterol to the liver is compelling [reviewed in refs. (4–7)]. In addition, experiments with cultured cells have shown that SR-BI can mediate cellular cholesterol efflux (15, 17) and SR-BI has been detected in the canalicular membranes of mice overexpressing hepatic SR-BI (24). However, the presence of SR-BI in the biliary canaliculus of wild-type animals has not been clearly established; thus, a direct role for SR-BI in biliary cholesterol secretion remains uncertain. The reduced biliary cholesterol secretion in SR-BI KO mice may have contributed to the small, but significant, increase in hepatic unesterified cholesterol content in SR-BI KO relative to control mice, but other explanations are possible. For example, cholesterol influx may have been increased via SR-BI-independent pathways (e.g., LDLR pathway),

perhaps as a consequence of the altered structure of the HDL in the mutant mice (28). It is possible that small increases in endogenous cholesterol production contributed to the minor increase in hepatic cholesterol content, because slight increases in both hepatic sterol synthesis and in hepatic expression of key biosynthetic enzyme HMG-CoA reductase mRNA were noted. These observed increases, however, were not significant. Clearly, the reduced biliary cholesterol secretion was not due to reduced hepatic cholesterol pools.

The detailed pathway by which HDL cholesterol is transported from the blood through the liver into canalicular bile is not well understood [reviewed in refs. (48–50)], but it is clear that many proteins in addition to SR-BI participate in the process. For example, a role for HL in facilitating hepatic cholesteryl ester uptake from HDL was proposed, on the basis of studies using isolated perfused liver (51, 52) and cultured cells (53). Experiments using HL knockout mice have established that this enzyme can contribute to the hepatic selective clearance of HDL cholesterol (54). In contrast, the Niemann-Pick type C (NPC) gene product, which is critical for intracellular cholesterol trafficking of endocytosed LDL cholesterol into lysosomes, is not essential for the intracellular transport of HDL cholesterol to the canalicular membrane for biliary secretion. NPC patients exhibit normal cholesterol flux from HDL cholesteryl esters into the bile (55). It is not known if the ability of SR-BI to bind LDL (9) and mediate selective uptake of cholesterol from LDL (12, 13) contributes to biliary cholesterol secretion in humans. It is possible that transport mechanisms that bypass the classic endosome/lysosome endocytic pathway are normally important for the transcellular transport of lipoprotein cholesterol to the canalicular bile [reviewed in refs. (48–50)]. Neutral cholesteryl ester hydrolase (56, 57), cholesterol transport proteins {e.g., sterol carrier protein-2 (45, 58, 59) and caveolins [reviewed in ref. (60)]}, and cholesterol-rich carrier vesicles [reviewed in refs. (48–50)] may be involved in such mechanisms. Another interesting hypothesis is that unesterified cholesterol, which can also be taken up by hepatocytes through the activity of SR-BI (14), could be directly transferred from HDL particles to the hepatic sinusoidal plasma membrane, translocated to the inner leaflet, and moved by lateral passive diffusion to the canalicular plasma membrane to contribute to biliary secretion (61–63).

As was the case for hepatic cholesterol synthesis, the absence of SR-BI did not appear to substantially alter hepatic bile acid metabolism. When fed a chow diet without added cholesterol, SR-BI KO mice exhibited an essentially normal biliary bile acid secretion rate, bile acid pool size and composition, and fecal bile acid excretion. Under steady-state conditions, the amount of fecal bile acid excretion reflects the rate of bile acid synthesis (37), suggesting that bile acid synthesis was normal in the SR-BI KO mice. Indeed, the expression of cholesterol 7α-hydroxylase, the rate-limiting enzyme in the classic pathway of bile acid synthesis, in the mutant mice was not dramatically different than that of the controls. It is difficult to reconcile these findings with the earlier report that hepatic selective cholesterol uptake was efficiently coupled to bile acid synthesis (64). Perhaps stimulation of alternative pathways for bile acid synthesis (65) and/or as yet unrecognized compensatory mechanisms related to bile acid production were operating in SR-BI KO mice.

In the course of measuring the levels of hepatic mRNA expression for the key enzymes in cholesterol and bile acid synthesis, we also examined the hepatic expression (mRNA) of several other genes related to lipoprotein metabolism. These included apoA-I, apoA-II, and apoE (three major apolipoprotein constituents of HDL), the LDL receptor, and the HDL-remodeling enzymes LCAT and HL. In no case was there a significant difference in expression between SR-BI KO and wild-type control mice. Thus, changes in the steady-state mRNA levels for these proteins could not have been responsible for the changes in biliary cholesterol secretion. These data also suggest that alterations in hepatic transcription of the apoA-II and apoE genes were not responsible for the altered plasma levels of these apolipoproteins that are observed in the abnormally large HDL particles in the SR-BI KO mice (28).

The finding that murine hepatic SR-BI expression was not decreased as a consequence of feeding cholesterol-enriched diets suggests that intrahepatic cholesterol content does not play a dominant role in controlling hepatic SR-BI expression in mice. This result differs from an earlier study in rats that demonstrated that cholesterol feeding reduced SR-BI expression in hepatocytes, whereas SR-BI levels were increased in Kupffer cells (32). This difference may be due to species (rat vs. mouse) differences in the regulation of hepatic SR-BI expression. For example, high dose estrogen treatment dramatically suppresses hepatic SR-BI expression in rats, but not in mice (18). Additional studies that have evaluated the cholesterol-dependent regulation of hepatic SR-BI expression are consistent with the results reported here. Hepatic SR-BI levels and HDL cholesteryl ester transport in the hamster were not regulated as a consequence of changes in hepatic cholesterol content induced by dietary cholesterol (66). In addition, the deficiency of plasma HDL in apoA-I knockout mice did not increase SR-BI expression in the liver (67). Taken together, these studies indicate that, at least in some species, hepatic levels of SR-BI are not under sensitive feedback control by hepatic cholesterol content and/or plasma HDL cholesterol concentration in vivo.

Anatomical localization of intestinal SR-BI (16) (P. J. Voshol, M. Schwarz, A. Rigotti, M. Krieger, A. K. Groen, and F. Kuipers, unpublished data), the coordinate regulation of its in vivo expression with cholesterol absorption (P. J. Voshol, M. Schwarz, A. Rigotti, M. Krieger, A. K. Groen, and F. Kuipers, unpublished data), and in vitro transport studies using brush border membranes (16) have all provided indirect or correlative support for the proposal that SR-BI might be involved in intestinal cholesterol absorption. Because intestinal cholesterol absorption in mice has been shown to be inversely correlated with fecal neutral sterol excretion (43) (M. Schwarz, S. Turley, and J. Dietschy, unpublished data), one might also have ex-

pected SR-BI to influence sterol excretion. Surprisingly, SR-BI KO mice exhibited an increase in intestinal absorption of labeled cholesterol compared with wild-type mice when fed either a standard chow diet or cholesterol-supplemented diets. In addition, there was decreased fecal neutral sterol excretion by SR-BI KO mice relative to wild-type controls. Thus, both direct cholesterol absorption experiments and analysis of fecal neutral sterol excretion unequivocally establish that SR-BI does not play an essential role in intestinal cholesterol absorption. These findings do not rule out the possibility that SR-BI may normally participate directly in intestinal cholesterol absorption. However, if it does, SR-BI-independent mechanisms must have been able to efficiently compensate for the loss of SR-BI expression in the mutant mice. It is conceivable that the main influence of the SR-BI deficiency on cholesterol absorption may have been the reduction of the biliary cholesterol concentration and a subsequent decrease in lumenal cholesterol content. The absorption process may be saturable (27) or subject to negative feedback regulation. If so, a reduction in size of the lumenal pool of endogenous cholesterol derived from the liver in the SR-BI-deficient relative to control mice might have contributed to the apparent increase in intestinal cholesterol absorption measured using a bolus of radiolabeled cholesterol administered by gastric gavage.

In addition, the slight but apparent increase in cholesterol absorption rates in the SR-BI KO mice compared with controls may have been caused, at least in part, by the impaired secretion into the bile of labeled cholesterol after its intestinal absorption and delivery to the liver. During the 72-h stool collection used for cholesterol absorption measurements, it is possible that some of the labeled cholesterol administered by gastric gavage was absorbed by the intestines and secreted by the liver back into the intestinal lumen, thus re-entering the pool of lumenal-labeled cholesterol that could be excreted or reabsorbed. In wild-type mice, this recirculation causes a decrease in apparent absorption rates when fecal samples are collected over a 3-day period compared with a 24-h collection period (S. Turley and M. Schwarz, unpublished observations). The reduced rate of hepatic cholesterol secretion exhibited by SR-BI-deficient mice might have lowered the amount of labeled cholesterol that, after absorption, could be secreted back into the intestines and subsequently excreted. This would result in an apparent increase in cholesterol absorption.

Alternatively, SR-BI might mediate cholesterol efflux from the apical surface of the enterocytes into the lumen. If so, SR-BI deficiency could have reduced cholesterol efflux into the lumen and thus reduced the lumenal pool of cholesterol independently of reduced biliary secretion. Regardless of the mechanism, the reduction in the lumenal intestinal cholesterol pool may have resulted in the observed apparent increase in absorption of the labeled cholesterol administered as a bolus by gastric gavage (27). Reduced efflux of newly absorbed radiolabeled cholesterol would also have resulted in an apparent increase in net cholesterol absorption. McNeish and colleagues (68) reported increased intestinal cholesterol absorption in ATP-binding cassette transporter-1 (ABC1) KO mice relative to controls. They suggested that ABC1 might normally serve as a unidirectional transporter mediating the efflux of cholesterol to the intestinal lumen and that apparent absorption of exogenous cholesterol would increase in ABC1 KO mice due to reduced efflux. Repa et al. (69) have further extended these previous findings, showing that activation of nuclear retinoid X receptor heterodimers by small molecule agonists modulates intestinal cholesterol absorption in mice. They suggested that this effect was due to the regulation of intestinal ABC1-mediated cholesterol efflux. Additional studies will be required to define the function of the intestinal expression of SR-BI and any potential relationships between the roles of ABC1 and SR-BI in intestinal cholesterol absorption and secretion.

In summary, this and other studies have established that murine SR-BI plays an important role in controlling plasma HDL cholesterol levels, the structure of HDL, delivery of HDL cholesterol to the liver, and excretion of biliary and fecal sterols. In contrast, SR-BI is not essential for the absorption of cholesterol in the small intestine. These metabolic effects are likely to have an influence on reverse cholesterol transport. As a consequence, the expression of SR-BI in the liver is expected to be critical for its antiatherogenic activity, as shown in mice [(30, 70–72) and reviewed in (7, 73, 74)]. Therefore, hepatic SR-BI expression might be important for the pathophysiology and management of two highly prevalent cholesterol metabolism-related diseases, atherosclerosis and cholesterol gallstone disease.

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