Down-regulation of intestinal scavenger receptor class B, type I (SR-BI) expression in rodents under conditions of deficient bile delivery to the intestine

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Scavenger receptor class B, type I (SR-BI) is expressed in the intestines of rodents and has been suggested to be involved in the absorption of dietary cholesterol. The aim of this study was to determine whether intestinal SR-BI expression is affected in animal models with altered bile delivery to the intestine and impaired cholesterol absorption. SR-BI protein and mRNA levels were determined in proximal and distal small intestine from control, bile-duct-ligated and bile-diverted rats and from control and bile-duct-ligated mice. Two genetically altered mouse models were studied: multidrug resistance-2 P-glycoprotein-deficient [*Mdr2*$^{(-/-)}$] mice that produce phospholipid/cholesterol-free bile, and cholesterol 7α-hydroxylase-deficient [*Cyp7a*$^{(-/-)}$] mice, which exhibit qualitative and quantitative changes in the bile-salt pool. Cholesterol-absorption efficiency was quantified using a dual-isotope ratio method. SR-BI was present at the apical membrane of enterocytes in control rats and mice and was more abundant in proximal than in distal segments of the intestine. In bile-duct-ligated animals, levels of SR-BI protein were virtually absent and mRNA levels were decreased by $\approx 50\,\%$. Bile-diverted rats, *Mdr2*$^{(-/-)}$ mice and *Cyp7a*$^{(-/-)}$ mice showed decreased levels of intestinal SR-BI protein while mRNA levels were unaffected. Cholesterol absorption was reduced by $> 90\,\%$ in bile-duct-ligated and bile-diverted animals and in *Cyp7a*$^{(-/-)}$ mice, whereas *Mdr2*$^{(-/-)}$ mice showed an $\approx 50\,\%$ reduction. This study shows that SR-BI is expressed at the apical membrane of enterocytes of rats and mice, mainly in the upper intestine where cholesterol absorption is greatest, and indicates that bile components play a role in post-transcriptional regulation of SR-BI expression. Factors associated with cholestasis appear to be involved in transcriptional control of intestinal SR-BI expression. The role of SR-BI in the cholesterol-absorption process remains to be defined.

Key words: absorption, bile salts, cholesterol, knockout mouse, phospholipid.

INTRODUCTION

Cholesterol absorption from the intestine is a key process in the regulation of whole-body cholesterol homoeostasis [1]. Luminal intestinal cholesterol can be of endogenous (bile, intestinal cells) or of exogenous (dietary) origin. The sterol is taken up by enterocytes in its free (unesterified) form, esterified, packaged into chylomicrons and secreted into the lymph [2,3]. After partial hydrolysis of their triacylglycerol contents, the resulting cholesterol-rich chylomicron remnants are rapidly cleared by the liver [4].

Bile plays a crucial role in the cholesterol-absorption process [5,6]. Bile components provide the solubilizing vehicles for cholesterol by formation of mixed micelles, consisting of bile salts, phospholipids and cholesterol [7,8]. Several studies (e.g. [2,9]) have shown that bile salts are essential for lipid absorption and available data indicate that the individual micellar components are taken up independently [10–12]. More recently, Sehayek et al. [13] proposed that biliary cholesterol secretion (co-) regulates dietary cholesterol absorption in mice, but the underlying mechanisms have remained unclear. In mice fed standard laboratory chow, the amounts of dietary and biliary cholesterol entering the intestine are similar [14]. However, it is important to note that in humans on a Western-type diet, bile delivers 2–3 times more cholesterol to the intestinal lumen on a daily basis than does the diet [1,3].

The sequential steps of intestinal cholesterol absorption have been studied extensively, but the actual mechanism(s) responsible for cholesterol uptake by the intestinal cells from the lumen is not known. It has been suggested that cholesterol uptake is energy-independent and reflects passive diffusion down a concentration gradient [15]. Other studies indicate that cholesterol uptake is a protein-mediated process. Thurnhofer et al. [16] provided evidence to suggest that uptake of cholesterol may be catalysed by an intrinsic membrane protein. Landschulz et al. [17] have reported that a member of the scavenger-receptor family, scavenger receptor class B, type I (SR-BI) is expressed in the intestines of rodents. Furthermore, Hauser et al. [18] have shown that SR-BI is present in the small-intestinal brush border of rabbits and, in experiments *in vitro*, it appears to facilitate uptake of free cholesterol from bile-salt micelles and phospholipid vesicles into brush-border membrane (BBM) vesicles. SR-BI is a high-density lipoprotein receptor [19,20] involved in selective uptake of cholesteryl esters from high-density lipoprotein by various

Abbreviations used: HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; mdr2/*Mdr2*, multidrug resistance-2 protein/gene; *Cyp7a*, cholesterol 7α-hydroxylase gene; SR-BI, scavenger receptor class B, type I; BBM, brush-border membrane; RT-PCR, reverse transcriptase PCR.

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organs, including liver and steroidogenic tissues [17,19,21,22]. In addition, SR-BI facilitates non-lipoprotein cholesterol uptake [23] as well as free-cholesterol efflux from cultured cells [24]. To date, however, there has been no description of the regulation of intestinal SR-BI expression, especially in relation to (patho)-physiological states associated with changes in cholesterol-absorption efficiency.

In view of the role of bile constituents in cholesterol absorption and the proposed involvement of SR-BI in this process, we have investigated the influence of bile components on intestinal SR-BI expression in rats and mice. Bile delivery into the intestine is completely blocked in bile-duct-ligated rats and mice, leading to accumulation of bile components in liver and plasma and to elevated plasma cholesterol levels [25]. Long-term bile-diverted rats also exhibit a complete absence of bile in the intestinal lumen, but without the potentially interfering consequences of cholestasis [26]. In an attempt to differentiate between actions of biliary bile salts and phospholipids/cholesterol, we used multidrug resistance-2 (mdr2) P-glycoprotein-deficient [*Mdr2*$^{(-/-)}$] mice [27]. mdr2-P-glycoprotein is a member of the ATP-binding-cassette (ABC) transporter superfamily (code abcb4) that is localized to the canalicular membrane of hepatocytes, where it functions as an ATP-dependent phospholipid translocator. Consequently, biliary phospholipid secretion is virtually absent in *Mdr2*$^{(-/-)}$ mice [27,28]. Cholesterol secretion is also dramatically reduced ($\approx 2\,\%$ of control values), whereas bile-salt secretion is unaffected [28]. We also studied cholesterol 7α-hydroxylase-deficient [*Cyp7a*$^{(-/-)}$] mice, in which bile-salt synthesis is severely affected [29,30]. As a result, the circulating bile-salt pool is dramatically reduced ($\approx 20\,\%$ of control values) in *Cyp7a*$^{(-/-)}$ mice [31]. As the molar ratio of biliary lipids, i.e. bile salts, phospholipids and cholesterol, is not affected in *Cyp7a*$^{(-/-)}$ mice [31], the amounts of these lipids that enter the intestine will be reduced to a similar extent.

MATERIALS AND METHODS

Animals

Male Wistar rats (≈ 300 g) from the breeding colony of the University of Groningen (Groningen, The Netherlands) were used. Bile-diverted rats were prepared as described previously [26] by providing the animals with a permanent silastic bile-duct catheter. Bile was diverted for 7 days prior to performing the analyses described below. Bile-duct ligation was performed in rats and FVB mice under halothane anaesthesia. These animals were used 5 days after surgery. Mice homozygous for disruption of the Mdr2 gene [*Mdr2*$^{(-/-)}$] and control mice [*Mdr2*$^{(+/+)}$] of the same FVB background were obtained from the breeding colony at the Animal Facility of the Academic Medical Center, Amsterdam, The Netherlands. All mice were ≈ 3 months of age. Animals were housed in a temperature- and light- (12 h/12 h light/dark cycle) controlled environment and were fed a standard laboratory chow (RMH-B, Hope Farms BV, Woerden, The Netherlands), which contained 6.2 % (w/w) fat and $\approx 0.01\,\%$ (w/w) cholesterol.

Mice homozygous for disruption of the cholesterol 7α-hydroxylase gene [*Cyp7a*$^{(-/-)}$] and control mice [*Cyp7a*$^{(+/+)}$] were maintained on a mixed C57BL/6/129 background in the breeding colony at the The University of Texas Southwestern Medical Center, Dallas, TX, U.S.A. Mice were housed individually in a humidity- and temperature-controlled room with a 12 h/12 h light/dark cycle and were fed a cereal-based rodent diet (7001, Harlan Teklad, Madison, WI, U.S.A.), which contained 0.02 % (w/w) cholesterol, 4 % (w/w) total fat, 24 % (w/w) protein and 5 % (w/w) fibre. All mice were male and 2–3 months of age.

The experimental protocols were approved by the Ethical Committee on Animal Testing, University of Groningen, and by the Institutional Animal Care and Research Advisory Committee, The University of Texas Southwestern Medical Center.

Intestinal SR-BI protein levels

Except for *Cyp7a*$^{(-/-)}$ mice (see below), animals were anaesthesized with halothane and the small intestines were rapidly removed, divided into two equal parts and flushed with PBS containing protease inhibitors (Complete®, Boehringer Mannheim, Mannheim, Germany). Intestinal mucosa was scraped into buffer containing 250 mM sucrose, 10 mM Tris-base (pH 7.4) and protease inhibitors (Complete®) and homogenized by eight strokes in a Potter–Elvejhem homogenizer. From these homogenates, a crude total-membrane fraction was isolated by centrifugation for 1 h at 100000 ***g***. When indicated, BBM fractions were isolated by calcium precipitation as described by Schmitz et al. [32]. In short, homogenates were mixed with buffer (50 mM sucrose/2 mM Tris/HCl, pH 7.4) containing $CaCl_2$ (final concentration, 10 mM). The mixture was incubated for 15 min on ice and centrifuged at 2000 ***g*** (Optima^{TLX} Tabletop Ultracentrifuge, Beckman, Fullerton, CA, U.S.A.) for 15 min at 4 °C. The supernatant was centrifuged at 20000 ***g*** for another 15 min at 4 °C. The remaining pellet (BBM) was resuspended in homogenization buffer. Alkaline phosphatase activity, used as a marker for enrichment of the BBM [32], was determined using the method described by Keeffe et al. [33].

After determination of total protein concentrations, equal amounts of protein, i.e. 5 μg for BBM and 30 μg for total-membrane fractions, were loaded on a 4–15 % gradient SDS/PAGE gel (Bio-Rad, Hercules, CA, U.S.A.) and electophoresed at 100 V. Proteins were blotted on to nitrocellulose membranes by tank-blotting (300 mA, 2 h). Nitrocellulose membranes were blocked overnight in 5 % skimmed milk powder solution in Tris-buffered saline (TBS) containing 0.1 % Tween-20 (Sigma, St. Louis, MO, U.S.A.) and subsequently incubated with the primary antibody (rabbit polyclonal anti-murine SR-BI, 495 [19]) diluted 1:10000 in 5 % skimmed milk powder in TBS/Tween-20 for 2 h at room temperature. After washing, a secondary antibody, anti-rabbit Ig linked to horseradish peroxidase (Amersham, Little Chalfont, Bucks, U.K.), diluted 1:1000 in 5 % skimmed milk powder in TBS/Tween-20, was added for another hour. Detection was performed using ECL (Amersham) according to the manufacturer's instructions. Liver total-membrane fractions used for comparison were prepared as described in [14]. For Western-blot analysis of liver homogenates, ≈ 1 μg of total membrane proteins were separated by SDS/PAGE. Constitutive expression levels of the β-subunit of Na^+/K^+-ATPase were used as a reference signal, detected with antibodies provided kindly by Dr Wilbert Peters (University of Nijmegen, Nijmegen, The Netherlands).

Cyp7a$^{(-/-)}$ and control mice were killed by cervical dislocation. Small intestines were removed, divided into five parts of equal length and rinsed with ice-cold PBS. BBMs were prepared from intestinal homogenates by a calcium-precipitation method [34]. Proteins were quantified using the BCA assay (Pierce) and analysed by SDS/PAGE and immunoblotting [35]. The SR-BI polyclonal antibody [21] was used at 20 μg/ml.

Deglycosylation of BBM and liver-homogenate proteins was performed by incubation with N-deglycosylase (PNGase kit, New England Biolabs, Beverly, MA, U.S.A.) following the manufacturer's instructions.

Imunohistochemistry and confocal laser scanning microscopy

Liver and small-intestinal sections were collected as described in [36], frozen immediately in liquid isopentane and 4 μm slices cut and fixed with acetone. SR-BI was visualized by incubating the sections first with anti-SR-BI in 1% BSA in PBS followed by washing with PBS. Endogenous peroxidase in the samples was inhibited using 30% methanol/0.3% H_2O_2, and the primary antibody was detected with peroxidase-linked rabbit anti-guinea-pig Ig (Dako A/S, Glostrup, Denmark) with an amplification step using goat anti-rabbit Ig (Dako A/S). 3-Amino-9-ethylcarbozole (Sigma) was used as a substrate and tissue was counterstained with haematoxylin. For confocal laser scanning microscopy, detection was performed using FITC-linked anti-rabbit Ig.

Intestinal SR-BI mRNA levels

Total RNA was isolated from intestinal tissue by a combination of the TRIzol reagent (Gibco-BRL, Grand Island, NY, U.S.A.) and the SV Total RNA isolation system (Promega, Madison, WI, U.S.A.) according to the manufacturers' instructions. Single-stranded cDNA was synthesized from 4.5 μg of RNA and subsequently subjected to PCRs using specific primer sets for rat and mouse 3-hydroxy-3-methylglutaryl CoA reductase (HMGR; sense primer, 5′-GAC ACT TAC TAT CTG TAT GAT G-3′; antisense primer, 5′-CTT GGA GAG GTA AAA CTG CCA-3′), SR-BI (sense primer, 5′-CTC ATC AAG CAG CAG GTG CTC A-3′; antisense primer, 5′-GAG GAT TCG GGT GTC ATG AA-3′) and β-actin (sense primer, 5′-AAC ACC CCA GCC ATG TAC G-3′; antisense primer, 5′-ATG TCA CGC ACG ATT TCC C-3′). For each primer set, an increasing number of PCR cycles with otherwise fixed conditions was performed to determine the optimal number of cycles, which was chosen as the number half way through the exponential phase. The PCR products were fractionated on 2.5% agarose gels and stained with ethidium bromide. Images were taken using a CCD video camera of the ImageMaster VDS system (Pharmacia, Uppsala, Sweden). Where indicated, intensities of bands, corrected for background, were quantified using the program ImageMaster 1D Elite, version 3.0.

Intestinal cholesterol absorption

Intestinal cholesterol absorption was determined using the dual-isotope ratio method of Zilversmit and Hughes [37], as modified for use in rodents by Turley et al. [38]. In short, animals were given an intravenous dose of [1,2-^{3}H]cholesterol (0.54 mg, 5.0 μCi for rats and 0.27 mg, 2.5 μCi for mice) dissolved in Lipofundin® S (20%, B. Braun Melsungen AG, Melsungen, Germany) and, at the same time, a dose of [4-^{14}C]cholesterol (0.18 mg, 2.5 μCi for rats and 0.07 mg, 1.0 μCi for mice) dissolved in medium-chain triacylglycerol oil by gastric gavage. After 48 h, a blood sample was drawn by tail bleeding and the ratio between ^{14}C-labelled and ^{3}H-labelled cholesterol in plasma was determined by scintillation counting. Intestinal cholesterol absorption was calculated as described previously [14]. Cholesterol-absorption studies performed in *Cyp7a*$^{(-/-)}$ mice are described in [31].

Biochemical analysis

Protein contents of tissue total-membrane fractions and BBM fractions were determined using the method of Lowry et al. [39]. Plasma cholesterol levels were determined using a commercially available kit (Boehringer Mannheim). Plasma alanine transaminase, aspartate aminotransferase, alkaline phosphatase and total bilirubin were determined by standard clinical chemical procedures. Contents of cholesterol in intestinal homogenates were determined after lipid extraction as described previously [14].

Statistical analysis

Results are presented as means ± S.D. for the number of animals indicated. Differences between three experimental groups were determined by one-way ANOVA, with *post-hoc* comparisons by Newmann–Keuls *t* test. Differences between two experimental groups were determined using the Mann–Whitney U test. The level of statistical significance was set at $P < 0.05$. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL, U.S.A.).

RESULTS

Plasma biochemical analyses

To evaluate the role of bile components on SR-BI expression, we compared bile-diverted rats, bile-duct-ligated rats and mice, *Mdr2*$^{(-/-)}$ mice and *Cyp7a*$^{(-/-)}$ mice with their respective controls. Plasma bilirubin, aspartate aminotransferase, alanine transaminase and cholesterol levels were increased markedly in bile-duct-ligated rats when compared with control rats, whereas no changes were noted in bile-diverted rats (Table 1). Body weight and food intake were similar for all three groups (results not shown). Transaminases and bilirubin were also elevated in *Mdr2*$^{(-/-)}$ mice as compared with wild-type mice, as described previously [28,40]. Bile-duct ligation in wild-type mice provoked a more pronounced increase in these parameters. Plasma cholesterol was reduced in *Mdr2*$^{(-/-)}$ mice [14] and increased in cholestatic mice. Plasma bilirubin, aspartate aminotransferase, alanine transaminase and cholesterol levels were unaffected in *Cyp7a*$^{(-/-)}$ mice when compared with controls. There were no genotype-dependent differences with respect to food intake, body weight, or length and weight of the small intestine.

Intestinal cholesterol absorption

Cholesterol absorption decreased greatly in bile-duct-ligated and bile-diverted animals (Table 1). As reported previously [14], the absence of biliary phospholipid and cholesterol in *Mdr2*$^{(-/-)}$ mice was associated with an $\approx$ 40% reduction in cholesterol-absorption efficiency and cholesterol absorption was virtually absent in the *Cyp7a*$^{(-/-)}$ mice [31]. Total cholesterol content of the intestinal mucosa did not differ between control, bile-diverted and bile-duct-ligated rats, with values ranging from 8.5 to 10.5 nmol/mg of protein and from 11 to 12 nmol/mg of protein for proximal and distal parts of the intestine, respectively. Similar results were observed in *Mdr2*$^{(-/-)}$, *Cyp7a*$^{(-/-)}$ and bile-duct-ligated mice, with values ranging from 14 to 18 nmol/mg of protein and from 19 to 23 nmol/mg of protein for proximal and distal parts of the intestine, respectively.

Intestinal SR-BI protein expression

Protein levels of SR-BI were assessed by Western-blot analysis in BBM fractions of proximal and distal segments of rat intestine. The protein band detected in proximal BBM was $\approx$ 78 kDa, i.e. $\approx$ 4 kDa less than in hepatic total-membrane fractions ($\approx$ 82 kDa; Figure 1). After N-deglycosylase treatment, SR-BI migrated to $\approx$ 60 kDa in both hepatic and intestinal membrane fractions (Figure 1), indicating the presence of a similar intact SR-BI amino acid chain in both tissues. The abundance of SR-

Table 1 Plasma parameters and cholesterol-absorption efficiency in bile-diverted rats, bile-duct-ligated rats and mice, *Mdr2*$^{(-/-)}$ mice and *Cyp7a*$^{(-/-)}$ mice

All results are given as means ± S.D. ($n = 3$–5). Plasma aspartate aminotransferase (AST), alanine transaminase (ALT), bilirubin and cholesterol were determined by standard clinical chemical procedures. Cholesterol absorption was determined via a dual-isotope ratio method [14,31]. BDL, bile-duct ligation; BD, bile diversion. *Significantly different from control rats, $P < 0.05$. †Significantly different from control mice, $P < 0.05$.

Species	Experimental conditions	AST (units/l)	ALT (units/l)	Bilirubin (μM)	Cholesterol (mM)	Cholesterol absorption (%)
Rat	Control	59 ± 9	34 ± 6	7.3 ± 2.3	1.9 ± 0.2	57 ± 10
	BDL	475 ± 261*	131 ± 80*	181 ± 42*	6.7 ± 4.3*	2 ± 1*
	BD	66 ± 4	48 ± 7	10.0 ± 5.3	1.6 ± 0.1	5 ± 2*
Mouse	Control FVB	78 ± 24	29 ± 6	3.7 ± 0.5	3.5 ± 0.8	70 ± 13[1]
	Mdr2$^{(-/-)}$	203 ± 64†	217 ± 68†	5.7 ± 0.7†	1.5 ± 0.9†	42 ± 8†
	BDL	409 ± 32†	372 ± 124†	138 ± 31†	5.6 ± 2.1†	2 ± 1†
Mouse	Control C57BL/6/129	46 ± 3	28 ± 7	1.7 ± 1.7	2.8 ± 0.3	37 ± 4[2]
	Cyp7a$^{(-/-)}$	65 ± 7	54 ± 3	3.4 ± 1.7	2.8 ± 0.2	Undetectable†

[1]Published previously in [14].
[2]Published previously in [31].

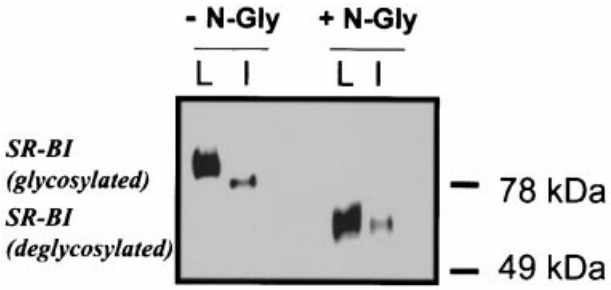

Figure 1 Western blot of SR-BI in hepatic plasma membranes and intestinal BBMs isolated from rats

Deglycosylation was performed as described in the Materials and methods section. N-Gly, N-deglycosylase; L, liver; I, intestine. The amount of protein loaded per lane was 1 μg for hepatic and 5 μg for intestinal preparations. The molecular-mass standards are indicated.

BI (band intensity) was clearly lower in rat intestine than in rat liver, as reported previously [17]. Similar results were obtained for mouse intestine (not shown).

Immunohistochemistry showed specific SR-BI staining of the apical membranes of rat and mouse enterocytes (Figures 2A and 2B). Only enterocytes were stained: the abundant goblet cells were all negative. Confocal laser scanning microscopy confirmed the immunohistochemistry, showing a strong apical staining of rat enterocytes (Figures 2C and 2D). Some intracellular staining was also evident.

SR-BI protein expression was also examined using immunoblot analysis of total membranes and BBMs isolated from the proximal or distal halves of rat small intestine. Na^+/K^+-ATPase content in the same BBM samples was analysed to ascertain that observed differences in the amounts of SR-BI were not due to loading artifacts. Figure 3 shows that SR-BI protein expression was greater in the proximal (Figure 3, lane 2) than in the distal (Figure 3, lane 3) portion of the intestine from control animals. The intensities of the bands from the total membranes (Figure 3, top panel, lanes 2 and 3) were similar to those of the BBMs (Figure 3, middle panel), even though 6-fold greater amounts were loaded for the total-membrane samples. This is consistent with the immunochemical and confocal localization studies indicating that SR-BI is located at the apical membrane of enterocytes. Figure 4 shows that BBMs isolated from mouse intestine also had higher abundance of SR-BI protein in the proximal compared with the distal region (Figure 4, compare lanes 1 and 2). A more detailed analysis of the distribution of SR-BI protein along the length of the murine small intestine is shown in Figure 5 (left-hand panel). The intestine was divided into five portions of equal length: SR-BI protein was present almost exclusively in the most proximal section (Figure 5, left-hand panel, lane 1).

Steady-state levels of SR-BI mRNA detected using reverse transcriptase PCR (RT-PCR) followed a similar pattern. In both rats (Figure 6, middle panel, lanes 1 and 2) and mice (Figure 7, top panel, lanes 1 and 2) the level of SR-BI mRNA was higher in the proximal than in the distal region of the intestine. The proximal intestine appears to be more involved in cholesterol absorption than the distal region [3]. Accordingly, the mRNA level of HMGR, a key enzyme in cholesterol synthesis whose expression is regulated by feedback suppression [41], was more abundant in distal than proximal portions of the small intestine (Figure 6, top panel, lanes 1 and 2).

Regulation of intestinal SR-BI expression by disruption of enterohepatic circulation

Immunoblot analysis of SR-BI protein in proximal and distal parts of the intestine showed substantially reduced SR-BI contents in bile-duct-ligated rats (Figure 3, lanes 4 and 5) when compared with control rats (Figure 3, lanes 2 and 3). Steady-state SR-BI mRNA levels were also lower in the bile-duct-ligated rats than in controls (e.g. Figure 6, middle panel, compare lanes 1 and 3), although the differences in mRNA levels were less dramatic than those of the protein. SR-BI protein was also reduced in bile-diverted rats (Figure 3, lanes 6 and 7), although this decrease was less pronounced than that in the bile-duct-ligated samples. The decrease in protein was more dramatic in the total-membrane fractions of bile-diverted rats than in the BBM fractions, which may indicate altered intracellular distribution of SR-BI, but more studies will be required to examine this issue. There was no statistically significant difference between control and bile diversion in terms of SR-BI mRNA levels (Figure 6, middle panel, lanes 1 and 2 compared with lanes 5 and 6), although, as expected, bile-diverted rats showed a strong increase in HMGR mRNA levels, which was particularly evident in the proximal intestine (Figure 6, top panel, lanes 5 and 6).

Figures 4 and 7 show that the effects of bile-duct ligation on proximal intestinal SR-BI expression in mice were similar to those in the rat. Bile-duct ligation substantially reduced immunodetectable SR-BI in the BBM of murine proximal intestine

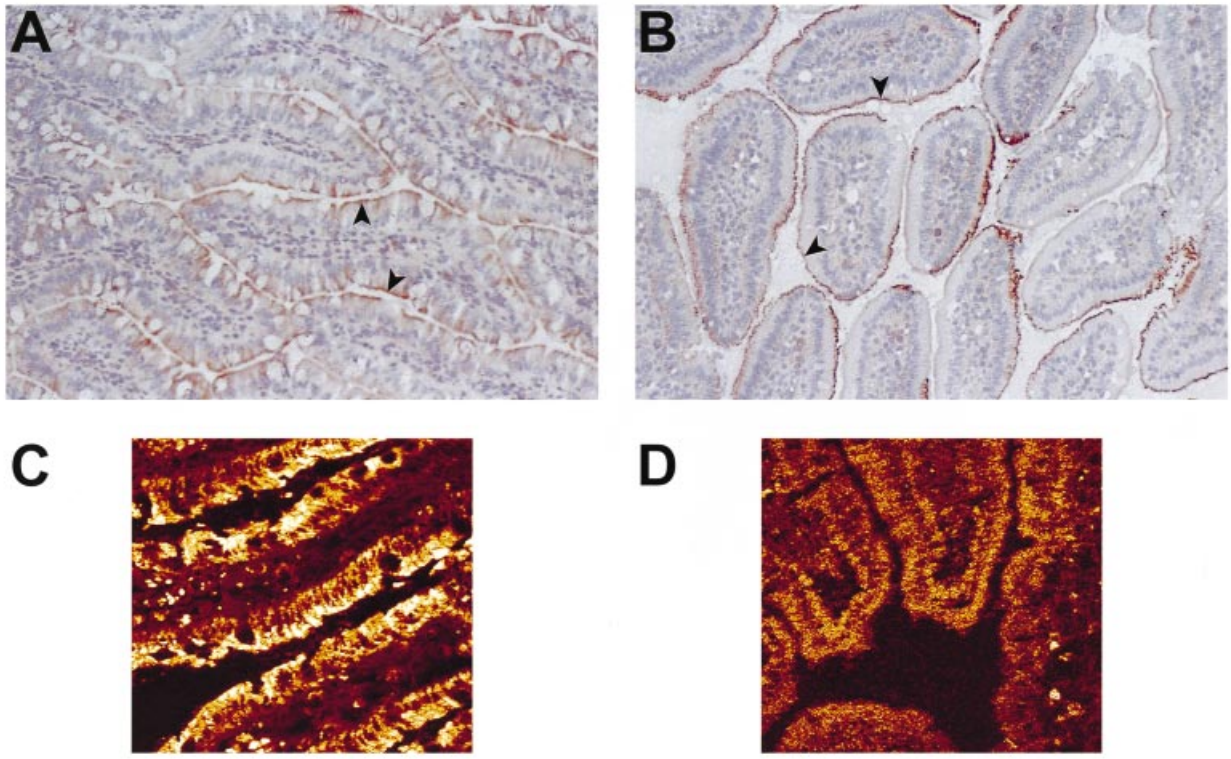

Figure 2 Immunohistochemical (A, B) and confocal microscopy (C, D) of SR-BI in frozen small-intestinal sections from rodents

(**A**, **C** and **D**) Control rat; (**B**) control mouse. The small-intestinal sections shown are taken ≈ 15 cm distal from the stomach. Arrowheads indicate apical-membrane staining of the enterocytes. (**D**) Negative control (no anti-SR-BI added). Magnification, 20× (**A** and **B**) and 40× (**C** and **D**). Similar SR-BI localization was observed for bile-diverted and bile-duct-ligated animals.

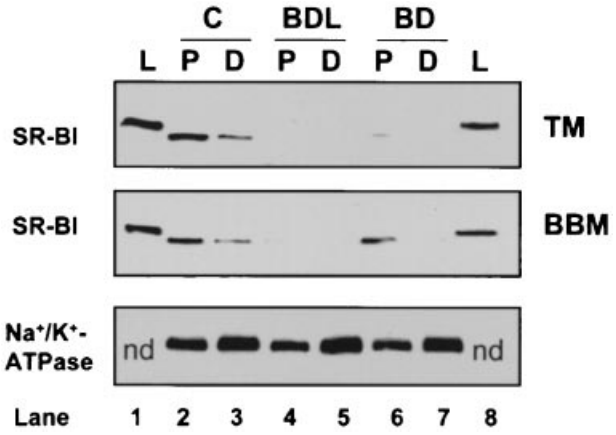

Figure 3 Representative Western blot of SR-BI in small-intestinal total membrane (TM) and BBM fractions of control (C), bile-duct-ligated (BDL) and bile-diverted (BD) rats

Proximal (P) and distal (D) parts of the intestine were used. Hepatic plasma membranes (L) were included as a positive control. The amounts of protein loaded were 1 μg for hepatic BBM, 5 μg for intestinal BBM and 30 μg for intestinal total-membrane preparations. Na^+/K^+-ATPase content in the same BBM preparations was determined to ascertain that differences in SR-BI content were not due to loading artifacts. Comparison of SR-BI band intensities by densitometric analysis of proximal BBM samples of three individual rats/group yielded values of 100 ± 11.2% (controls), 15.8 ± 12.8% (bile-duct-ligated, $P < 0.05$) and 79.7 ± 10.4% (bile-diverted). nd, not determined.

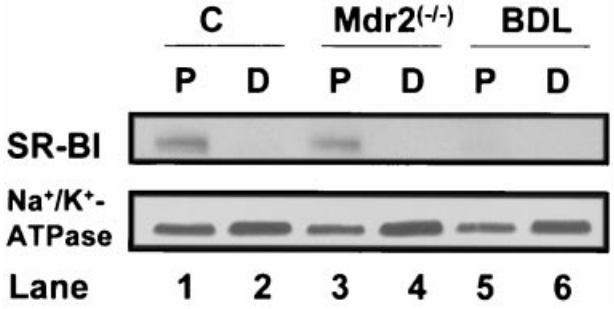

Figure 4 Representative Western blot of SR-BI in intestinal BBMs of control (C), *Mdr2*$^{(-/-)}$ and bile-duct-ligated (BDL) mice

Proximal (P) and distal (D) parts of the intestine were used for analysis. The amount of protein loaded was 10 μg/lane. Na^+/K^+-ATPase content in the same BBM preparations was determined to ascertain that differences in SR-BI content were not due to loading artifacts. Comparison of SR-BI band intensities by densitometric analysis of proximal BBM samples of three individual mice/group yielded values of 100 ± 12.4% (controls), 61.6 ± 2.5% [*Mdr2*$^{(-/-)}$ mice, $P < 0.05$] and 12.6 ± 2.3% (bile-duct-ligated, $P < 0.05$).

(Figure 4, lane 5) relative to control (Figure 4, lane 1) and reduced the mRNA levels (Figure 7, top panel, compare lanes 1 and 2 with lanes 5 and 6). The very weak immunoblot signal in the distal portion of the murine intestine made it difficult to assess the effects of manipulation on SR-BI protein expression in this region of the intestines (see below). In contrast, *Mdr2*$^{(-/-)}$

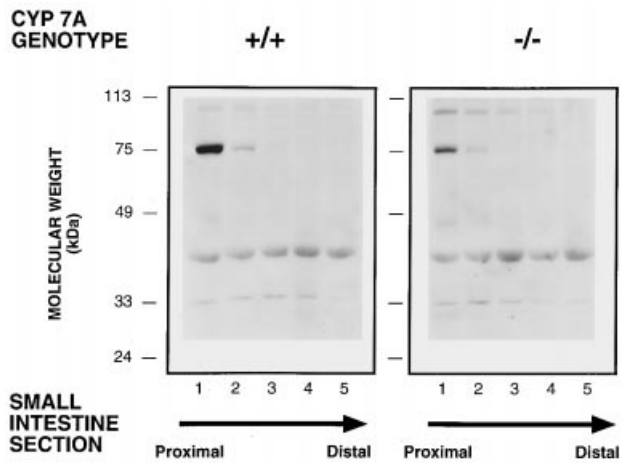

Figure 5 Expression of SR-BI protein in the small intestine of control C57BL/6/129 mice$^{(+/+)}$ and *Cyp7a*$^{(-/-)}$ mice of the same strain

Small intestines were cut into five sections of equal length and homogenized. BBMs were prepared by a calcium-precipitation method. Membrane proteins were separated on a denaturating 10% polyacrylamide gel, transferred on to a PVDF membrane and incubated with anti-SR-BI polyclonal antibody [21]. The lower band at $\approx$ 40 kDa is a background band; similar levels in subsequent sections indicate equal loading of the lanes.

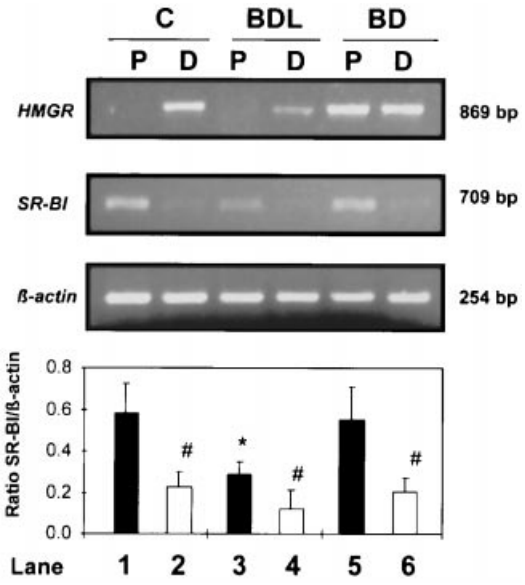

Figure 6 RT-PCR analysis of intestinal HMGR and SR-BI mRNA levels in control (C), bile-duct-ligated (BDL) and bile-diverted (BD) rats

The mRNA levels of HMGR, SR-BI and β-actin shown are from individual animals of each group. The graph shows amounts of SR-BI relative to β-actin mRNA. The ratio between SR-BI and β-actin mRNA was determined in three individual animals of either group (means $\pm$ S.D.). *Significantly different from control rats, $P < 0.05$; #significantly different from the proximal part of the intestine, $P < 0.05$, as determined by Mann–Whitney U test. P, proximal; D, distal.

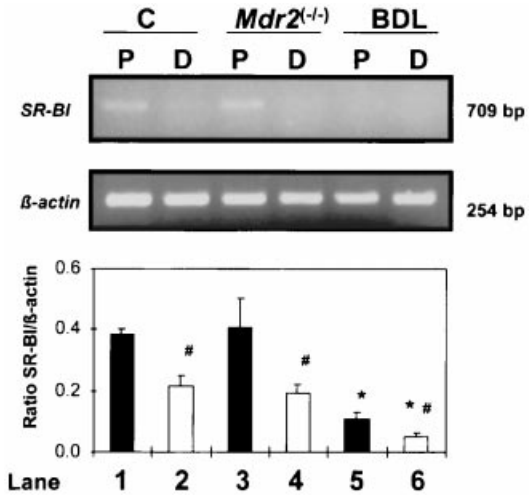

Figure 7 RT-PCR analysis of intestinal SR-BI mRNA levels in control (C), *Mdr2*$^{(-/-)}$ and bile-duct-ligated (BDL) mice

The mRNA levels of SR-BI and β-actin shown are from individual animals of each group. The graph shows amounts of SR-BI relative to β-actin mRNA. The ratio between SR-BI and β-actin mRNA was determined in three individual animals of each group (means $\pm$ S.D.). *Significantly different from control mice, $P < 0.05$; #significantly different from the proximal part of the intestine, $P < 0.05$, as determined by Mann–Whitney U test. P, proximal; D, distal.

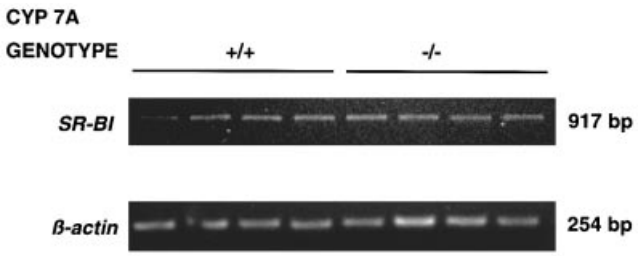

Figure 8 RT-PCR analysis of SR-BI mRNA levels in the proximal intestine of C57BL/6/129 mice$^{(+/+)}$ and *Cyp7a*$^{(-/-)}$ mice of the same strain

The mRNA levels of SR-BI and β-actin shown are from four individual mice of each strain. Total RNA was isolated from segments representing the most proximal 20% of the small intestine. The ratios of β-actin/SR-BI mRNA band intensities were 0.09 ± 0.2 and 0.09 ± 0.02 in control and knockout mice, respectively.

mice exhibited a relatively small ($\approx 40\%$, mean of three separate isolations/strain, quantified by densitometry) decrease in SR-BI protein in the proximal part of the intestine (Figure 4, lane 3 versus lane 1) and we observed no significant differences in mRNA levels (Figure 7, lanes 3 and 4 versus lanes 1 and 2). We also compared the levels of SR-BI protein expression in *Cyp7a*$^{(+/+)}$ and *Cyp7a*$^{(-/-)}$ mice by immunoblotting BBMs isolated from segments taken along the length of the small intestine. Figure 5 shows that the distribution pattern (high in proximal segments, low in distal segments) in the *Cyp7a*$^{(-/-)}$ mice (right-hand panel) was unchanged relative to *Cyp7a*$^{(+/+)}$ mice (left-hand panel). Most notably, the presence of a greatly reduced bile-salt pool in the *Cyp7a*$^{(-/-)}$ animals was associated with an $\approx$ 5-fold reduction in SR-BI protein levels (Figure 5, compare lanes 1 of the left- and right-hand panels). Yet, no differences in SR-BI mRNA levels were evident upon RT-PCR analysis of samples obtained from the proximal 20% of the small intestines of *Cyp7a*$^{(-/-)}$ and control mice (Figure 8).

DISCUSSION

This study confirms the presence of SR-BI in rat and murine intestine. The molecular mass of SR-BI protein in intestinal BBMs of rats and mice was $\approx$ 4 kDa lower than that of hepatic

SR-BI, as described previously [17], probably due to differences in oligosaccharide modification. It is not known whether this reflects differences in SR-BI structure in these tissues *in vivo* or if they arose during sample preparation (e.g. due to hydrolytic activity in intestinal preparations). Immunohistochemical, immunofluorescence and tissue-fractionation experiments demonstrated clearly that SR-BI protein is expressed at the apical membrane of enterocytes, consistent with the situation in rabbit intestine [18]. Under normal conditions, expression of SR-BI was much higher in the proximal relative to the distal parts of the small intestine. A similar proximal-to-distal gradient of SR-BI mRNA levels in mouse small intestine has recently been reported by Repa et al. [41]. In fact, the protein appeared to be localized almost exclusively to the proximal 20 % of the murine intestine, i.e. in the duodenum and upper jejunum, where absorption of biliary and dietary cholesterol occurs [3]. Interestingly, the mRNA-expression pattern through the small intestines of rats for HMGR, the rate-controlling enzyme of cholesterol biosynthesis, was the opposite to that of SR-BI. Diversion of bile led to disappearance of this zonal distribution pattern for HMGR. Since HMGR expression is subjected to negative-feedback suppression [42], these observations are consistent with primarily proximal uptake of cholesterol in rats under normal conditions. These findings raised the possibility that intestinal SR-BI expression might be functionally related to cholesterol absorption, as suggested previously [18]. Thus we examined the regulation of intestinal SR-BI expression under physiological conditions in which cholesterol absorption was altered by manipulating one or more lipid components (bile salt, cholesterol, phospholipid) of the bile.

Both surgical and genetic methods were used to alter biliary lipids in rats and/or mice. Previous studies have established that these interventions significantly reduce intestinal absorption of cholesterol ([14,31] and Table 1): the current study shows that this was accompanied by reduced intestinal SR-BI protein expression in these models. The greatest reduction in the expression of SR-BI protein as well as of its mRNA was observed after bile-duct ligation, in both rats and mice. It may be that accumulation of bile components in the blood, including cholesterol and phospholipids in the form of lipoprotein-X [25], hepatic damage and/or inflammatory processes associated with obstructive cholestasis contributed to suppression of SR-BI expression. Deficiency of mdr2-P-glycoprotein in mice is also associated with increased transaminase activities and bile-salt levels in plasma [27,40], albeit to a lesser extent than observed after bile-duct ligation (see Table 1). Intestinal SR-BI protein levels in *Mdr2*$^{(-/-)}$ mice were reduced to a similar or slightly lesser extent to that observed in bile-diverted rats and in *Cyp7a*$^{(-/-)}$ mice, without clear changes in steady-state mRNA levels. As neither bile-diverted rats nor *Cyp7a*$^{(-/-)}$ mice showed signs of liver damage, the combination of data indicates strongly that disturbances in normal delivery of bile components into the intestinal lumen *per se* leads to reduced SR-BI protein expression in the upper intestine. Why complete absence of bile (in bile-diverted rats) yielded less effect on SR-BI expression than partial depletion of bile components [*Cyp7a*$^{(-/-)}$ mice] is not clear, but may be related to the time period during which the intestine experienced deficient delivery of bile, i.e. 7 days in the rat model versus 2–3 months in the mouse model. In addition, species-specific factors may have been involved. Cholestasis appears to provide an additional trigger in rats as well as in mice, leading to a rapid transcriptional down-regulation of expression of the scavenger receptor.

All models of abnormal enterohepatic circulation examined here have in common an apparent impairment of micellar cholesterol solubilization in the intestinal lumen. In bile-duct-ligated and bile-diverted animals, bile is completely absent from the intestine. In *Mdr2*$^{(-/-)}$ mice, biliary phospholipids are absent but bile salts are present, probably enabling micellization of some cholesterol. A substantial reduction in bile-salt pool size in *Cyp7a*$^{(-/-)}$ mice is apparently responsible for the reduced cholesterol absorption, since restoration of the bile-salt pool by feeding essentially normalizes cholesterol absorption [31]. Further research, including intestinal infusion of bile components into bile-diverted rats, will give more insight into the specific role of bile components in the regulation of SR-BI in the intestine.

To the best of our knowledge, no prior studies of the regulation of intestinal SR-BI expression have been reported. In fact, understanding of the molecular mechanisms underlying the regulation of SR-BI expression is still limited (reviewed in [43]). Consensus binding-site sequences for several transcription factors have been identified in the putative promoter of the human SR-BI gene [44], including CCAAT-enhancer-binding protein (C/EBP), sterol regulatory-element binding-protein-1 (SREBP1) and steroidogenic factor-1 (SF-1). *In vivo* and *in vitro* regulation of SR-BI expression has been examined in the adrenal gland, ovary, testis and liver (see [43]). The observations that hormonal (high-dose oestrogen) and dietary (high cholesterol diet) treatments can induce SR-BI expression in some tissues and cell types and suppress it in other cell types, and that these effects, in part, are species-dependent ([17], summarized in [43]), clearly indicate that there are species- and cell-type-dependent differences in the systems that regulate SR-BI expression. It has been reported that SR-BI expression might be sensitive to cellular cholesterol content [43,45]. In our experiments, we did not detect differences in total cholesterol contents of intestinal scrapings (rats and FVB mice) or intestinal homogenates (C57BL/6/129 mice) in the models in which SR-BI protein expression was clearly affected, although differences in the sizes of putative regulatory cholesterol pools obviously cannot be excluded on the basis of these studies.

In addition to proposing that SR-BI might be involved in cholesterol absorption, Hauser et al. [18] also raised the possibility that this receptor might participate directly in non-cholesterol lipid (fatty acids, monoacylglycerol) absorption. Interestingly, there is a marked difference in the absorption of dietary cholesterol and non-cholesterol fat in the models employed here. Both bile-duct-ligated [46] and bile-diverted rats [47] absorb up to 60 % of dietary non-cholesterol fats, whereas control rats absorb $\approx 92\,\%$. *Mdr2*$^{(-/-)}$ mice are able to absorb $>95\,\%$ of non-cholesterol dietary fat [36,48]. A similar dissociation between absorption efficiency of cholesterol and non-cholesterol fat was observed in the *Cyp7a*$^{(-/-)}$ mice [31]. Thus there does not seem to be a strong relation between SR-BI expression and non-cholesterol fat absorption. As a consequence, it seems unlikely that SR-BI plays a major quantitatively important role in controlling non-cholesterol fat absorption.

In conclusion, we have demonstrated that SR-BI is present at the apical membrane of enterocytes of rats and mice, almost exclusively in the proximal part of the small intestine. SR-BI protein levels are reduced in surgical and genetic models of cholesterol malabsorption characterized by reductions of bile components in the intestinal lumen. Cholestatic models (bile-duct-ligated rats and mice) were associated with a transcriptional down-regulation of intestinal SR-BI expression. Thus bile components directly or indirectly play a role in the regulation of intestinal SR-BI expression in rodents. The observed reduced SR-BI expression in murine models of cholesterol malabsorption and the similar anatomical distributions of sites of SR-BI expression and cholesterol absorption suggest a possible functional role of SR-BI in cholesterol absorption. *In vitro* studies

have suggested that SR-BI has the capacity to mediate cellular uptake of non-lipoprotein cholesterol and to facilitate cellular efflux of cholesterol [23,24,49]. Thus SR-BI could mediate cholesterol absorption directly or influence net cholesterol absorption by facilitating secretion of cholesterol from enterocytes into the intestinal lumen. Recent studies using SR-BI-deficient mice have shown that intestinal cholesterol absorption does not require the expression of SR-BI [50]. Indeed, there is a small but significant increase in intestinal cholesterol-absorption efficiency in SR-BI-knockout mice relative to controls, consistent with a potential role of SR-BI in secretion. Thus down-regulation of SR-BI protein in situations with impaired cholesterol absorption may reflect a reaction of the body aimed at keeping cholesterol inside enterocytes and thereby preventing faecal loss of the sterol. While all of the data available indicate that intestinal SR-BI is likely to influence cholesterol absorption, additional studies are required to define this critically important lipid-transport process.

We thank Vincent Bloks and Torsten Plösch for primer development and Rick Havinga and Juul Baller for technical assistance. This work was supported by grant 902-23-097 from the Netherlands Organization for Scientific Research (NWO) and National Institutes of Health (NIH; Bethesda, MD, U.S.A.), grants HL41484, HL52212 and HL64737 to M. K. M. S. was supported by training grant no. 5-T32-DK07745 from the NIH. We thank Dr David Russell for encouragement and support from NIH grant HL20948.

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Received 8 January 2001; accepted 9 March 2001