Novel insights in cholesterol excretion

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Regulation of transintestinal cholesterol efflux in mice

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ABSTRACT
Background: biliary secretion is generally considered to be an obligate step in the pathway of excess cholesterol excretion from the body. We have recently shown that an alternative route exists. Direct transintestinal cholesterol efflux (TICE) contributes significantly to cholesterol removal in mice. Aim: to investigate whether the activity of this novel pathway can be influenced by dietary factors. In addition, we studied the role of cholesterol acceptors at the luminal side of the enterocyte. Methods: mice were fed Western-type diet {0.25 % (w/w) cholesterol; 16 % (w/w) fat}, a high fat diet {no cholesterol; 24 % (w/w) fat}, or high cholesterol diet {2 % (w/w)} and TICE was measured by isolated intestinal perfusion. Bile salt/phospholipids mixtures served as cholesterol acceptor. Results: Western-type and high fat diet increased TICE by 50 % and 100 %, respectively. In contrast, the high cholesterol diet did not influence TICE. Intestinal scavenger receptor class B type 1 (Sr-B1) mRNA and protein levels correlated with the rate of TICE. Unexpectedly, TICE was significantly increased in Sr-B1 deficient mice. Apart from the long term effect of diets on TICE, acute effects by luminal cholesterol acceptors were also investigated. The phospholipid content of perfusate was the most important regulator of TICE, bile salt concentration or hydrophobicity of bile salts had little effect. In conclusion, TICE can be manipulated by dietary intervention. Specific dietary modifications might provide means to stimulate TICE and, thereby to enhance total cholesterol turnover.

INTRODUCTION
Fecal excretion is the final step in the reverse cholesterol transport (RCT) pathway. This term was coined in 1973 by Glomset and Norum (1) to define the pathway responsible for excretion of excess cholesterol from the body. Since activation of this pathway may lead to regression of atherosclerosis many research programs aim at identification of the critical steps in the pathway. Hepatobiliary cholesterol secretion is generally considered to be one of these critical steps. However, we have recently shown that in addition to the hepatobiliary route there also is a direct pathway of intestinal cholesterol secretion. In mice this pathway is more important than the hepatobiliary route (2). This finding originated from in vivo cholesterol balance studies in wild-type and relevant knockout mouse models. In particular balance studies in Abcg5/abcg8 double knockout mice (3) and Abcb4-/- mice (4) which have low or even abrogated biliary cholesterol secretion did not show the
expected low levels of fecal neutral sterols. Furthermore, Kruit et al. (5) showed that in Abcb4-/- mice, intravenously injected radiolabeled cholesterol can be recovered in feces. All these findings indicated that biliary cholesterol secretion cannot be the only route to excrete cholesterol. We confirmed the activity of a direct transintestinal pathway for cholesterol excretion (TICE) in experiments in which intestinal segments were perfused (2). In mice, the amount of cholesterol secreted via the intestine is approximately 2-fold higher than the amount of biliary secreted cholesterol. The existence of this pathway has been suggested as early as 1927 (6), but it has never been validated until recently.

TICE involves transport of cholesterol directly from blood across the enterocytes into the intestinal lumen. We could demonstrate that the rate of TICE strongly depends on the presence of a cholesterol acceptor. Bile salts in the intestinal lumen, particularly when combined with phospholipid, strongly stimulate the pathway. It was also shown that Western-type diet fed mice secreted significantly more cholesterol via the intestine (2).

To delineate the effect of dietary manipulation on intestinal cholesterol secretion and to identify factors involved in control of this alternative cholesterol secretion pathway, we studied the effects of dietary fat in the absence and presence of cholesterol on intestinal secretion by performing intestine perfusions in mice fed these diets. Intestinal gene expression analysis was performed to establish whether a correlation in expression of genes known to be involved in sterol transport and the rate of TICE could be established. To investigate specific effects of luminal modifications on TICE, proximal small intestine perfusions were performed with different bile salt-phospholipid combinations added as cholesterol acceptors to the perfusion fluid. Data indicate that dietary fat content and modifications of luminal lipid content affect transintestinal cholesterol efflux in mice.

MATERIAL AND METHODS

Animals and Diets
Male FVB mice (2 - 4 months) were purchased from Harlan (Horst, the Netherlands). The mice were fed a semi-purified reference diet (no 4068.02, Abdiets, Woerden, The Netherlands; 20 % (w/w) casein, 5 % (w/w) fat (soy oil), no cholesterol added), a high cholesterol diet (2 % (w/w) cholesterol added to the reference diet), a Western-type diet (Diet W, no 4021.06, Abdiets; 20 % (w/w) casein, 16 % (w/w) fat (15 % cacao butter, 1 % corn oil), 0.25 % (w/w) cholesterol),
a high fat diet {High Fat, no 4031.00, Abdiets; 24 % casein, 24 % fat (corn oil), no cholesterol added}. The amount of energy delivered by fat in reference diet, Western-type diet, and the high fat diet are 17 %, 33 % and 46 %, respectively. Although there was no cholesterol added to the reference diet, it contained trace amounts of cholesterol: 0.006 % (w/w) (determined by gas chromatographic analysis). There was no difference in energy intake between these diets. Intestinal perfusions were also performed on chow (RM3, Special Diet Services, Witham, UK, 4 % (w/w) fat, no cholesterol added) fed Sr-B1-/- mice (7) and wild-type littermates. To study the effect of luminal modifications on TICE, mice were used that received chow diet {CRM (E); Special Diets Services, 3 % (w/w) fat, no cholesterol added}. Food and water were supplied ad libitum. Mice were maintained on a 24 h light-dark cycle. All experiments were performed with the approval of the local Ethical Committee for Animal Experiments.

**Cholesterol intake and output measurements**

Mice were housed in normal mouse cages (3 mice per cage), to mimic their natural situation as much as possible. Every day, mice and remaining chow pellets were weighed and feces was collected. Fecal neutral sterols were determined as described below.

**Intestine perfusion procedures**

Mice were anaesthetized by intraperitoneal injection with 0.1 ml FFD {Hypnorm (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg)} / 5 g body weight and placed on a heat pad to maintain body temperature. The bile duct was cannulated via the gallbladder and bile was collected in 15 min portions. Bile flow was determined gravimetrically assuming a density of 1 g/ml. The first fraction was used to measure biliary cholesterol secretion. Proximal small intestines (first 10 cm) were perfused, because here the majority of TICE takes place. Perfusions were performed as previously described (2). At the end of the perfusion period, blood was collected by cardiac puncture. The perfused intestinal segments were isolated for gene expression analysis.
Perfusion fluid composition

Perfusions were carried out with a modified Krebs solution (119.95 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 15 mM HEPES, 1.3 mM CaCl₂·2H₂O and 10 mM L-glutamine; final pH 7.4) supplemented with bile salts and phospholipids. Bile salt-phospholipid mixtures were made as follows: taurocholate (Sigma, Zwijndrecht, The Netherlands), tauroursodeoxycholate (TUDC; Calbiochem, Amsterdam, The Netherlands) or taurodeoxycholate (TDC; Sigma, Zwijndrecht, The Netherlands) dissolved in methanol and egg yolk L-α-phosphatidylcholine (Sigma, Zwijndrecht, The Netherlands) dissolved in chloroform were mixed and solvents were evaporated under a mild stream of nitrogen at 45°C. After evaporation, films were lyophilized overnight. Lyophilized samples were stored under nitrogen gas at -20°C until the day of the intestine perfusions. Before the start of the intestine perfusions, the films were dissolved in perfusion buffer.

Determination of mRNA Levels

Total RNA was isolated using the Trizol reagent according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). Purified RNA was treated with RQ1 RNase-free DNase (1 units / 2 μg of total RNA, Promega, Leiden, The Netherlands) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen, Breda, The Netherlands) according to the protocols supplied by the manufacturer. Gene expression analysis was performed on a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System by using the Bio-Rad iQ SYBR Green Supermix (Bio-Rad). PCR primers were designed on the basis of Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and validated for identical efficiencies. Hypoxanthine-guanine phosphoribosyl transferase (HPRT), cyclophilin, and acidic ribosomal phosphoprotein P0 (36B4) were used as standard housekeeping genes.

Western blotting

Lysis buffer containing phosphate buffered saline, 1 % Triton X-114, 0.5 % sodium deoxycholate, 0.1 % SDS and CompleteTM protease inhibitor was added to the intestinal tissue. Homogenates were made by sonication. The protein concentrations of the lysates were determined using the BCA assay (8). Equal amounts of protein (40 μg) were separated on SDS/PAGE (8 % gels) and transferred onto nitrocellulose membranes (Schleicher and Schuell, ‘s-
Membranes were probed using a mouse anti-Sr-B1 (pAb anti-SRB1 NB400-104, (Novus Biologicals, Littleton, CO, USA), diluted 1:1000, followed by detection by Lumi-Light Western blotting substrate (Roche, Woerden, The Netherlands). In selected cases, the membranes were stripped for 30 min by incubation in buffer containing 100 mM 2-mercaptoethanol, 2 % (w/v) SDS and 62.5 mM Tris/HCl, pH 6.7, at 55°C, followed by washing and probing with a rabbit anti-Na⁺/K⁺-ATPase antibody (1:1000) (9). Protein abundance was calculated by densitometry using LumiAnalyst 3.1 software (Roche, Woerden, The Netherlands).

**Analytical procedures**

Perfusate and biliary lipids were extracted using the Bligh & Dyer method (10). Biliary and perfusate cholesterol concentrations were measured by a fluorescent method as described previously (11). To verify whether this method indeed measures only cholesterol in the perfusate samples, in some experiments cholesterol in the perfusate was also measured by GC as described below. Good agreement (CV<6 %) was observed. Total cholesterol concentrations of serum samples were determined using the Cholesterol RTU assay (Biomerieux, Marcy l’Etoile, France). For fecal neutral sterol analysis, 1-day fecal samples were collected, lyophilized, weighed and grinded. Fecal neutral sterols were measured as follows. 50 mg dried feces was used for the analysis of cholesterol and coprostanol after addition of 500 nmol 5α-cholestane as internal standard. A second portion of 50 mg dried feces was used for the analysis of epi-cholesterol and dehydrocholesterol after addition of 20 nmol 5α-cholestane as internal standard. Release of neutral sterols was achieved by addition of 1 ml methanol: 1M NaOH 3:1 (v/v), and heating at 80°C for 2 hours. After cooling to room temperature sterols were extracted 3 times with 3 ml petroleumether (60-80) and the combined petroleumether layers were evaporated to dryness under nitrogen. Thereafter the sterols were derivatized to the Trimethylsilyl derivative (TMS) after adding 100 μL of a mixture of N,O-bis(Trimethylsilyl)Trifluoroacetamide (BSTFA), pyridine and trimethylchlorosilane (TMCS) in a ratio of 5:5:1 and incubated for 1 h at room temperature. After evaporation to dryness the residue was taken up in 1 ml hexane and the sample was shaken, sonicated and centrifuged. The supernatant was transferred to a 2 ml GC vial. Calibration samples were prepared containing
0 - 5 μmoles cholesterol and coprostanol or 0 - 50 nmoles epicholesterol and 0 - 250 nmoles dihydrocholesterol. The samples were measured in an HP 5890 gas chromatograph, splitless injection at 290°C and a Flame Ionization Detector at 275°C. A 25 m x 0.25 mm CP WAX 52, 12.5 mtr*0.25mm GC column (film thickness 0.2 μm) was used (Varian, Middelburg, the Netherlands) applying temperature programming (85°C for 1,5 min, 20°C/min up to 270°C, for 15 min) at a constant helium flow of 0.8 ml/min.

Statistics
All results are presented as mean ± SD. Group means for TICE, as depicted in the figures, were calculated by averaging the outcomes of all mice that got the same treatment. The values for the individual mice, used for the calculation of the group mean, were obtained by averaging the data of the last three perfusion time points. Differences between different groups were determined by one way ANOVA. Outcomes of p < 0.05 were considered to be significant. Analysis was performed using SPSS.

RESULTS
Presence of luminal phospholipid stimulates TICE
To study whether luminal mixed micelles have a different cholesterol acceptor capacity than vesicles, intestine perfusions were performed with different combinations of TC (2 mM, 5 mM or 10 mM) and 2 mM PC in perfusate. By combining 10 mM TC with 2 mM PC, mixed and simple micelles are being formed whereas mainly vesicles and TC monomers are present with lower concentrations of TC (12). TICE rates initiated by the different TC/PC combinations are depicted in figure 1. All rates ranged from 1.5 to 2 nmol/min.100 g body wt and were not significantly different from each other. Intestine perfusions with bile salts more hydrophilic (TUDC) and more hydrophobic (TDC) than TC were performed to assess whether bile salt hydrophobicity influenced the acceptor capacity for intestinally secreted cholesterol. Figure 2A shows that TUDC in combination with PC evoked a comparable TICE rate as was observed with TC/PC. Both TUDC/PC and TC/PC significantly stimulated TICE compared to the rate obtained without use of any acceptor. The concentration of free cholesterol in the perfustae was maximally 30μM which is about 10 times below saturation at these bile salt and PC concentrations (13). Figure 2B shows that TDC in combination with PC did not
result in a different TICE rate as compared to TC/PC as acceptor. To avoid potential toxic effects of TDC, only 2 mM of this bile salt was used. Lowering PC did affect TICE, as shown in figure 3, 1 mM instead of 2 mM PC decreased TICE by 50%. During the perfusion procedure some of the PC may be degraded by intestinal phospholipases. We therefore measured the PC recovery and the release of free fatty acids. The FFA concentration in the perfusate was about 0.3 mM, choline recovery was 100% indicating that about 10% of the PC was converted to lyso-PC.

Figure 1. Effect of different TC/PC mixtures on intestinal cholesterol secretion. The proximal small intestines of FVB mice (n=6, per group) were perfused with Krebs supplemented with different TC/PC combinations (2, 5, or 10 mM TC with 2 mM PC). Values are depicted as mean ± SD.

Figure 2. Effect of bile salt hydrophobicity on intestinal cholesterol secretion. (A) The proximal small intestine of FVB mice (n=6, per group) were perfused with Krebs, Krebs supplemented with 10 mM TUDC / 2 mM PC or 10 mM TC / 2 mM PC. (B) The proximal small intestine of FVB mice (n=6, per group) were perfused with Krebs supplemented with 2 mM TDC / 2 mM PC or 2 mM TC / 2 mM PC. Values are depicted as mean ± SD. * indicates a significant difference in TICE between mice receiving a cholesterol acceptor and mice receiving no cholesterol acceptor.
High fat containing diets affect TICE

Previously, we have shown that feeding mice a Western-type diet increased TICE (2). To establish whether it was the high cholesterol or high fat content of the diet which induced this effect, we fed mice diets in which the various components were selectively adapted. After a week run-in-time, food intake and output were determined and after 2 weeks intestine perfusions were performed. As shown previously, Western-type diet increased TICE by more than 50% (fig. 4A). The high fat diet, increased TICE even more (100%) (fig. 4B). Interestingly a high cholesterol diet without additional fat had no effect on TICE (fig. 4C).

The data obtained from intestine perfusions were confirmed by cholesterol balance data. Reference diet fed mice ingested 1.5 ± 0.1 μmol cholesterol/day.100 g body wt and 1.7 ± 0.6 μmol entered the intestinal lumen via bile. These animals excreted 6.0 ± 0.6 μmol/day.100 g body wt via feces. The mice that were fed a high fat diet received 1.3 ± 0.2 μmol cholesterol/day.100 g body wt from diet and 1.0 ± 0.3 μmol from bile in the intestinal lumen and excreted 13.2 ± 0.6 μmol/day.100 g body wt. We could not perform accurate balance studies in the mice fed Western-type diet or high cholesterol only diet due to high levels of cholesterol in these diets. Confirming data from others (14; 15), we did observe a 3 - 4 fold increased biliary cholesterol secretion in animals fed the Western-type of high cholesterol diet (data not shown).
Figure 4. Transintestinal cholesterol efflux is affected by dietary manipulation. (A) The proximal small intestine of FVB mice receiving reference diet (Ref. Diet; n=6) or Western-type diet (Diet W; n=6) for 3 weeks, were perfused with Krebs supplemented with TC/PC (10:2 mM). (B) The proximal small intestine of FVB mice receiving reference diet (Ref. Diet; n=6) or high fat diet (High fat; n=6) for 3 weeks, were perfused with Krebs supplemented with TC/PC (10:2 mM). (C) The proximal small intestine of FVB mice receiving reference diet (Ref. Diet; n=6) or high cholesterol diet (High cholesterol; n=6) for 3 weeks, were perfused with Krebs supplemented with TC/PC (10:2 mM). Values are depicted as mean ± SD. * indicates a significant difference in cholesterol secretion between mice receiving different diets.
Regulation of TICE

Figure 5. Intestinal gene expression analysis. Mice received reference diet (n=6), Western-type diet (Diet W; n=6) or high fat diet (High fat; n=6) for 3 weeks. After the intestine perfusion, the perfused intestines were sampled for RNA isolation and gene expression analysis was performed. Intestinal Abca1, Abcg5, Sr-B1, Npc111, and Hmg-CoA red expression levels were measured. As control genes HPRT, cyclophilin, and 36B4 were used. * indicates a significant difference between mice receiving reference diet (Ref. Diet) and Western-type diet (Diet W) or high fat diet (High fat).
**TICE-inducing diets showed differential expression pattern of cholesterol related genes**

To investigate the effect of the different dietary regimens on gene expression, perfused intestinal segments were harvested. The mRNA expression levels of several genes known to be involved in cholesterol metabolism were quantified (fig. 5). In intestines of Western-type diet fed mice, the observed upregulation of Abca1, Abcg5 and the downregulation of Hmg-CoA reductase and Npc1l1 gene expression are in agreement with earlier findings (2; 16). In mice fed high fat diet both intestinal Npc11l and Abca1 gene expression were reduced.

Interestingly, feeding of both Western-type and high fat diets led to an increased expression of intestinal Sr-B1 (fig. 5). This increase of Sr-B1 with the TICE-inducing diets was not only seen on the mRNA level, but also the amount of protein increased (fig. 6). To directly assess the role of Sr-B1 in TICE, intestine perfusions were performed in Sr-B1-/- mice. Surprisingly, TICE was increased in Sr-B1-/- compared to their wild-type littermates (fig. 7).

**Figure 6.** Sr-B1 protein levels. Mice received reference diet (Ref. Diet; n=3), Western-type diet (Diet W; n=3) or high fat diet (High fat; n=3) for 3 weeks. After the intestine perfusion, the perfused intestines were sampled for determination of intestinal Sr-B1 protein levels. As control for protein loading Na+/K+-ATPase levels were determined as well.

**Figure 7.** Transintestinal cholesterol efflux is increased in Sr-B1 deficient mice. Intestinal perfusions were performed on chow fed Sr-B1-/- mice (n=4) and their wild-type littermates (n=5). The proximal small intestines were perfused with cholesterol acceptor containing 10 mM TC and 2 mM PC for 90 minutes and the cholesterol concentration in the perfusate was measured by determining cholesterol in the perfusate. Values are expressed as mean ± SD. * indicates a significant difference between Sr-B1-/- mice and wild-type littersmates.
DISCUSSION

An important finding of the present study is the fact that the rate of TICE in mice is remarkably insensitive to the type of bile salt and intraluminal bile salt concentration. Although rigorous dose-response testing of bile salt phospholipid combinations in this experimental model is too laborious, we feel that the experimental results allow the conclusion that the presence of bile salt micelles does not seem to be very important. In the physiological range neither the luminal bile salt concentration, nor bile salt hydrophobicity affected TICE, indicating that the presence of phospholipids is the dominant factor modulating the amount of cholesterol secreted via TICE. Interestingly, this is in contrast to the effects the bile salts have on biliary cholesterol secretion (17) hence regulation of biliary secretion and TICE is different. As shown previously, bile salts alone also stimulate TICE (2), the presence of phospholipids is therefore not essential. The stimulating effect of phospholipids on TICE may explain in part why fecal sterol excretion is strongly stimulated by phospholipid supplementation to the diet of humans (18). TICE has not yet been quantified in humans and it may be less important than in mice, yet in 1967 Simmonds et al. (19) performed elegant intestinal perfusion studies in humans and observed significant secretion of cholesterol in the small intestine.

Apart from the acute effect of phospholipids, we demonstrate that a high content of lipids in the diet also stimulates TICE in mice. Western-type and high fat-only diet increased TICE about twofold. This increase correlated with the twofold increase in neutral sterol output found in high fat diet fed mice.

A differential effect of both TICE-inducing diets on cholesterol metabolism parameters was seen at the level of gene expression. Western-type diet feeding upregulated intestinal Abca1 and Abcg5 and downregulated Hmg-CoA reductase, as to be expected from a high cholesterol diet (16). In contrast, Abcg5 and Hmg-CoA reductase remained unaffected while Abca1 was strongly downregulated upon feeding the high fat diet. Although a negative correlation between unsaturated fatty acids and ABCA1 expression has been reported recently (20) such a strong downregulation in vivo by increased fatty acid uptake has not been shown before. By shutting down efflux via Abca1, the enterocyte may save cholesterol for chylomicron assembly in order to be able to export fatty acid in the form of triglyceride. The downregulation of Abca1 seems essential because import of cholesterol is also limited due to the decrease in the cholesterol importer Npc1l1 and the increase in TICE. Apparently, for as yet unknown reasons, it is important
for the enterocytes in the small intestine to maintain an adequate amount of cholesterol in the intestinal lumen. Excess cholesterol maybe reabsorbed further down in the intestine (19).

The only gene we investigated that was upregulated under Western-type diet feeding and even more prominently upregulated by high fat feeding was Sr-B1. So, for the genes tested, only intestinal Sr-B1 expression correlated with TICE. Considering the well accepted role of Sr-B1 in reverse cholesterol transport via the hepatobiliary route, direct participation of Sr-B1 in stimulating TICE would be plausible (7). Surprisingly, intestinal perfusions in mice deficient for Sr-B1 showed a significant, twofold increase in TICE compared to WT mice. A change in intestinal cholesterol absorption might explain this seemingly contradicting finding. The group of Hauser has provided considerable evidence supporting a role for this transporter in cholesterol absorption (21). In addition, Bietrix *et al.* (22) demonstrated increased cholesterol and triglyceride uptake in a mouse model with selective overexpression of Sr-B1 in the intestine. The total amount of secreted cholesterol via TICE might, in part, be affected by the absence of Sr-B1 mediated cholesterol absorption. This, however, does not explain the mechanism underlying the specific cholesterol secretion itself. Reduced intestinal absorption in mice fed with high fat diet seems to be compensated by induction of Sr-B1 expression.

In conclusion, TICE can be altered by the fat content of the diet and by modification of the cholesterol acceptor in the intestinal lumen. The stimulating effect of high fat containing diets may serve as an experimental means to identify the key players in TICE. Furthermore, the presence of luminal PC plays a crucial role in the facilitation of TICE. Luminal manipulation might provide an interesting target to stimulate TICE, and, thereby increase total cholesterol excretion.
REFERENCES


13. Carey MC and Ko G. The importance of the total lipid concentration in determining cholesterol solubility in bile and the development of critical tables for calculating percent cholesterol saturation with a correction factor for URSO-rich bile. in Biological effects of bile acids (Paumgartner G Stehl A Gerok W eds ) 299, 1978.


