Transintestinal Cholesterol Excretion Is an Active Metabolic Process Modulated by PCSK9 and Statin Involving ABCB1

Cédric Le May,* Jean Mathieu Berger,* Anne Lespine, Bruno Pillot, Xavier Prieur, Eric Letessier, M. Mahmood Hussain, Xavier Collet, Bertrand Cariou, Philippe Costet

Objective—Transintestinal cholesterol excretion (TICE) is an alternate pathway to hepatobiliary secretion. Our study aimed at identifying molecular mechanisms of TICE.

Approach and Results—We studied TICE ex vivo in mouse and human intestinal explants, and in vivo after bile diversion and intestinal cannulation in mice. We provide the first evidence that both low-density lipoprotein (LDL) and high-density lipoprotein deliver cholesterol for TICE in human and mouse jejunal explants at the basolateral side. Proprotein convertase subtilisin kexin type 9 (PCSK9)−/− mice and intestinal explants show increased LDL-TICE, and acute injection of PCSK9 decreases TICE in vivo, suggesting that PCSK9 is a repressor of TICE. The acute repression was dependent on the LDL receptor (LDLR). Further, TICE was increased when mice were treated with Lovastatin. These data point to an important role for LDLR in TICE. However, LDLR−/− mice showed increased intestinal LDL uptake, contrary to what is observed in the liver, and tended to have higher TICE. We interpret these data to suggest that there might be at least 2 mechanisms contributing to TICE; 1 involving LDL receptors and other unidentified mechanisms. Acute modulation of LDLR affects TICE, but chronic deficiency is compensated for most likely by the upregulation of the unknown mechanisms. Using mice deficient for apical multidrug active transporter ATP-binding cassette transporter B1 a and b, and its inhibitor, we show that these apical transporters contribute significantly to TICE.

Conclusions—TICE is operative in human jejunal explants. It is a metabolically active process that can be acutely regulated, inversely related to cholesterolemia, and pharmacologically activated by statins. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: ATP-binding cassette transporter B1 ■ lipoprotein ■ low-density lipoprotein receptor ■ PCSK9 ■ transintestinal cholesterol excretion

Excess cholesterol must be eliminated from the body to avoid its accumulation in tissues and to prevent or diminish atherosclerosis. The hepatobiliary pathway has been considered as the major cholesterol elimination route. Nevertheless, the existence of a nonbiliary route contributing to fecal sterol loss was revealed decades ago in dogs,1 rats,2 and later in humans3 with impaired biliary secretion. More recently, a normal fecal cholesterol excretion rate was observed in mice deficient for cholesterol biliary excretion attributable to the absence of the canalicular phospholipid transport multidrug resistance 2 or to the overexpression of hepatic Niemann-Pick C1-Like 1.4,5 Normal or lower biliary cholesterol excretion and increased fecal sterol loss were also observed in mice with no or dramatically less liver cholesterol esterification by the enzyme acyl-coenzyme A cholesterol acyltransferase 2.6 Transintestinal cholesterol excretion (TICE) is now recognized as a significant alternative route to the hepatobiliary pathway.7 It has been estimated that TICE represents constitutively 33% of total fecal sterol loss in mice.8 More recently, Temel et al9 showed that TICE is essential to macrophage reverse cholesterol transport in mice suggesting a potential antiatherogenic role for TICE. Intestinal cholesterol excretion is inducible nutritionally by a high-fat diet10 or pharmacologically by ligands of LXR8 or PPARα,11 but the molecular mechanisms remain unknown.

Several receptors mediate hepatobiliary cholesterol elimination. Hepatic low-density lipoprotein receptor (LDLR)
plays a major role in the classic hepatobiliary route and clears plasma cholesterol through the endocytosis of apolipoprotein (apo) B-containing LDL. Plasma proprotein convertase subtilisin kexin type 9 (PCSK9) induces the LDLR degradation because it binds to the extracellular domain of the LDLR and prevents its recycling to the cell surface. Thus, mice deficient in PCSK9 have more LDLR protein in the liver and in the intestine. The functional importance of intestinal LDLR and its circulating regulator PCSK9 in TICE remains to be explored.

Efflux of free cholesterol from the apical side of the enterocytes mainly takes place via ABCG5/G8. TICE is reduced by only 40% in ABCG5-deficient mice, suggesting that TICE-derived cholesterol exit enterocytes via other cholesterol transporters. P-Glycoprotein (P-gp; multidrug resistance-1 [MDR1]) or ATP-binding cassette transporter B1 (ABCBI) belonging to the ATP-binding cassette family is an integral membrane protein expressed in most epithelial cells, notably in the liver and at the apical side of enterocytes. It exports a large variety of structurally unrelated compounds out of cells and out of organisms protecting tissues from xenobiotic toxicity. ABCBI is also involved in the movement of endogenous molecules, such as cholesterol, phospholipids, and sphingolipids, and a variety of steroids. Although its role in cholesterol efflux per se remains controversial, it has been shown that ABCBI contributes to cholesterol distribution from the cytosolic leaftlet to the exoplasmic leaftlet of the plasma membrane by acting as a floppase.

Although the existence of TICE per se is not debated, the nature of the phenomenon remains unclear. The main missing elements are (1) a molecular mechanism for cholesterol entry on the basal side of enterocytes; (2) a direct evidence of TICE in intestinal explants, in particular, in human tissues; (3) data showing an increase of TICE in the context of lower cholesterolemia; (4) the contribution of apical transporters; and (5) acute modulation of TICE. The present study was developed to fill these gaps.

**Material and Methods**

Material and Methods are available in the online-only Data Supplement.

**Results**

**TICE Is an Active Metabolic Process**

To develop an ex vivo assay for TICE, we mounted mouse duodenal explants in Ussing chambers and added LDL (0.7 mg/mL) radiolabeled with 3H-free cholesterol (3H-LDL) to the basolateral side of duodenal mouse explants and incubated at 37°C or 4°C. After 30 minutes and at 37°C, 0.07% of the initial dose of 3H-cholesterol present in the basal chamber was collected into the apical chamber. We determined the passage of cholesterol to the apical chamber >90 minutes (3×30 minutes), and the radioactive content remained in the explants at the end of the experiment. We verified that TICE and 3H-cholesterol intestinal content values were stable over the first, the second, and the last period of 30-minute measurement (data not shown). 3H-LDL TICE (Figure 1A) and uptake (Figure 1B) were repressed by cold temperatures. Next, we measured TICE from 3H-LDL in explants over 3 consecutive periods of 30 minutes in the presence or absence of oxygen in the media (Figure 1C); TICE was observed when media were oxygenated. In group C, oxygenation-induced TICE is stronger when the oxygenated period is consecutive to a 30-minute phase without oxygen. This could be attributable to cells being loaded with cholesterol during the nonoxygenated period. This might mean that oxygen is important to cholesterol efflux. As a control, we verified that bubbling of N2 instead of oxygen had no effect on TICE (data not shown).

TICE was not secondary to cytotoxicity as there was no significant increase in LDH under different conditions (Figure 1D). Next we measured the impact on TICE of apical cholesterol acceptors present in Figure 1A–1D (Figure 1E). Removing cholesterol acceptors from the apical chamber significantly reduced TICE. No cytotoxicity associated with the presence of cholesterol acceptor was detected and, therefore, we systematically used cholesterol acceptors, unless stated. Therefore, transport of 3H-LDL cholesterol is attributable to an active metabolic pathway when explants were provided with oxygenated media at 37°C. To our knowledge, this is the first report of a method to measure TICE ex vivo.

Both HDL and LDL Provide Cholesterol for TICE

To determine whether HDL and LDL contribute to TICE, we added 3H-LDL and 3H-HDL to the basolateral side of duodenal mouse explants. Comparison of TICE showed that both lipoproteins can deliver cholesterol for TICE in explants (Figure 2A). Similar results were observed when LDL and HDL were labeled with 3H-cholesterol oleate (Figure 1A in the online-only Data Supplement). Next we measured TICE in vivo. Previous studies measured TICE with 3H-cholesterol diluted in intralipid. Here, we delivered 3H-cholesterol using lipoproteins. Bile-diverted C57Bl6/J mice were intravenously injected with 3H-cholesterol labeled LDL or HDL (1.8 mg of LDL and HDL cholesterol in a volume of 130 μL), and TICE was measured by quantifying intestinal perfusates. After injection, both plasma 3H-cholesterol and total cholesterol were increased (Figure 1B and 1D in the online-only Data Supplement) because of the accumulation of cholesterol in the LDL or the HDL fraction, as verified by fast protein liquid chromatography (Figure 1C in the online-only Data Supplement). TICE had already reached a plateau 15 minutes after injection of lipoproteins and was stable >2 hours (Figure 1E in the online-only Data Supplement). Both LDL and HDL were found to provide cholesterol for in vivo TICE (Figure 2B). Thus, various lipoproteins can participate in TICE.
Figure 1. Transintestinal cholesterol excretion (TICE) is an active metabolic pathway. C57Bl6J mouse proximal intestinal explants were mounted on Ussing chambers. 1H-cholesterol–labeled low-density lipoprotein (LDL; 1H-LDL) was added to the Krebs/glucose buffer at the basolateral side. A, TICE was determined by measuring the appearance of radioactivity at the apical side and (B) uptake of was measured by quantifying radioactivity in the explants incubated at 37°C or 4°C (n=8–16 explants from 4 to 8 mice per group). Results represent the average of TICE measured over 3 periods of 30 minutes. C, TICE was measured at 37°C in 3 groups of C57Bl6J explants for 3 consecutive phases of 30 minutes (1, 2, 3). In group A, the buffers were not oxygenated. In group B, buffers were constantly oxygenated. In group C, buffers were oxygenated only during the second period of 30 minutes (n=8–10 explants from 8 to 10 mice per group). D, The effect of oxygenation on LDH release was measured in the apical chamber at 37°C in 2 groups of C57Bl6J explants for 2 consecutive phases of 30 minutes (4 explants per condition from 4 mice). In group D, the buffers were not oxygenated during the first 30-minute period (1) and were oxygenated for the second 30-minute period (2). In group E, explants were oxygenated for the first period but not during the second one. E, C57Bl6J mouse proximal intestinal explants were mounted on Ussing chambers. 1H-cholesterol–labeled LDL (1H-LDL) was added to the Krebs/glucose buffer at the basolateral side. TICE was determined by measuring the appearance of radioactivity at the apical side over 30 minutes during 2 consecutive conditions: with a buffer containing cholesterol acceptors (taurocholate/phosphatidylcholine, 10/2 mmol/L; Black bar) or without acceptors. Cell cytotoxicity was assessed in the apical chamber during the 2 periods of 30 minutes by measuring LDH release in the apical chamber. All values represent mean±SEM. **P<0.05, ***P<0.001. For positive cytotoxic effects, explants were exposed to a cell lysis solution containing Triton-X-100. NS indicates nonsignificant.
TICE Is Operative in Human Intestine

There has been no evidence of TICE in humans yet. Using an Ussing Chamber, we measured TICE with 3H-LDL and 3H-HDL in jejunal explants from 3 patients who underwent bariatric surgery (Figure 2C). Explants were collected 50 cm below duodenojejunal flexure for all patients. We also compared TICE in the absence or in the presence of cholesterol acceptors in the apical chamber. TICE was operative in human explants. The presence of cholesterol acceptors (taurocholate/phosphatidylcholine, 10/2 mmol/L) increased TICE by roughly 100% for 3H-LDL and 300% to 400% for 3H-HDL. Thus, human intestine might play a role in TICE.

PCSK9 Deficiency Increases TICE

To determine whether the LDLR can contribute to TICE, we measured TICE derived from LDL in PCSK9−/− mice that present with higher amounts of LDLR in their gut, as we previously showed.17 LDL-TICE was increased by 103% (P<0.05) in duodenal explants from PCSK9−/− mice, compared with explants from PCSK9+/+ mice (Figure 3A). Next, we verified these results in vivo. PCSK9−/− mice have 50% less plasma cholesterol than littermates because of an increased clearance of HDL and LDL, respectively, via the apoE and apoB.16 We measured TICE with 3H-LDL in PCSK9−/− and PCSK9+/+ littermates. Injection of 3H-LDL increased plasma cholesterol in wild type (WT) and PCSK9−/− mice but increases were higher in WT mice (Figure IIA in the online-only Data Supplement). Moreover, increases in 3H-cholesterol were similar in WT and PCSK9−/− mice, but subsequent clearance was faster in PCSK9−/− mice (Figure IIB in the online-only Data Supplement). These data are consistent with observations that PCSK9−/− mice clear LDL-derived 3H-cholesterol more rapidly than control mice.16 In vivo TICE was increased by 62% (P<0.01) in PCSK9−/− mice (Figure 3B), despite their lower plasma cholesterol levels throughout the experiment (Figure IIA in the online-only Data Supplement). Similar results were observed with LDL labeled with 3H-cholesterol oleate (Figure IIC and IID in the online-only Data Supplement). In accordance with increased TICE, PCSK9−/− mice have increased fecal cholesterol loss, despite normal cholesterol absorption (Figure IIC and IID in the online-only Data Supplement). Thus, PCSK9 deficiency increases TICE.

Acute Degradation of LDLR by PCSK9 Decreases TICE

Next we verified whether acute depletion of LDLR decreases TICE. It has been shown that injection of recombinant

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**Figure 2.** Both low-density lipoprotein (LDL) and high-density lipoprotein (HDL) contribute to transintestinal cholesterol excretion (TICE). A, C57Bl6J mouse proximal intestinal explants were mounted on Ussing chambers. 3H-LDL or 3H-HDL (specific activities of 119 CPM/nmol and 116 CPM/nmol, respectively) was added to Krebs/glucose buffer in the basolateral chamber (n=16–19 explants from 10 mice per group). After 30 minutes, TICE was determined by measuring the appearance of radioactivity in the apical chamber filled with Krebs buffer enriched with cholesterol acceptors (taurocholate/phosphatidylcholine; TC/PC: 10/2 mmol/L). B, 3H-LDL or 3H-HDL (specific activities of 1494 CPM/nmol and 1088 CPM/nmol, respectively) was injected in the penile vein of C57Bl6J mice (6–8 mice per group) and transintestinal 3H-cholesterol excretion was measured 2 hours by quantifying radioactivity in perfusates collected from cannulated intestinal segments. C, Jejunal explants from 3 patients were mounted on Ussing chambers. Equal amounts of 3H-cholesterol contained in 3H-LDL (patients 1 and 2) or 3H-HDL (patient 3) were added to the basolateral side. Two explants were used from patients 1 and 2, and 4 explants were used from patient 3. TICE was measured over 30 minutes during 2 consecutive conditions: with a buffer containing cholesterol acceptors (white bars) or with the same buffer without cholesterol acceptor (black bars). The order of the bars from the left to the right corresponds to the order the buffers were used during the experiments. Values represent mean±SEM. NS indicates nonsignificant.
PCSK9 in the circulation rapidly degrades the hepatic and extrahepatic LDLR. We first measured TICE in PCSK9−/− mice, 1 hour after they were intravenously injected with 100 μg recombinant PCSK9. Western blot analysis showed that the LDLR content of the duodenum was reduced within 1 hour (Figure 3E). As expected, increases in cholesterol after the injection of 3H-LDL were more pronounced in mice infused with PCSK9 (Figure 3C; Figure III in the online-only Data Supplement). TICE was decreased by ≈35% in these mice (Figure 3D). As a control, PCSK9 was injected in LDLR−/− mice (Figure 3C and 3D). LDLR−/− mice presented with 40% higher TICE in absolute value than PCSK9−/− mice (data not shown). It is unclear whether this is because of very active TICE in LDLR−/− mice or to the difference in genetic background between strains. Indeed, Van der Velde et al24 showed that mice of different genetic backgrounds have different levels of TICE. Nevertheless as expected, no effect of injected PCSK9 was observed on cholesterolemia nor on TICE in LDLR−/− mice. These studies show that circulating PCSK9 acutely decreases TICE in an LDLR-dependent fashion.

Lovastatin Stimulates TICE

Next we hypothesized that cholesterol synthesis inhibitors, such as statins, that upregulate LDL receptors could stimulate TICE. To test this hypothesis, C57BL6J mice were given a diet containing or not 0.02% Lovastatin for 10 days. This treatment decreased cholesterolemia by ≈18% (P<0.05; Figure 4A) mainly because of a marked decrease in HDL cholesterol (Figure IVB in the online-only Data Supplement). Injection of 3H-LDL increased plasma cholesterol (Figure IVC in the online-only Data Supplement) and 3H-cholesterol (Figure IVB in the online-only Data Supplement). Lovastatin increased TICE in WT mice by 71% (P=0.02; Figure 4). Thus, TICE can be pharmacologically activated by Lovastatin.

TICE Is Increased in the Absence of LDLR

The above studies involving modulation of LDLR by PCSK9 and Lovastatin indicated that LDLR might play an important role in TICE. Therefore, we hypothesized that TICE might be significantly reduced in LDLR−/− mice. Chronic LDLR deficiency results in delayed LDL plasma clearance27 because of less binding of LDL particles in the liver. To evaluate

Figure 3. Proprotein convertase subtilisin kexin type 9 (PCSK9) modulates transintestinal cholesterol excretion (TICE) in an low-density lipoprotein receptor (LDLR)–dependent manner. A, Intestinal explants from PCSK9+/+ and PCSK9−/− mice were mounted in Ussing chambers and TICE measured with 3H-LDL (n=12–13 explants from 8 to 9 mice per group). B, TICE was measured for 2 hours in vivo (n=10–13 mice per group). C, Plasma cholesterol levels of PCSK9−/− and LDLR−/− mice. Solutions of 100 μg recombinant human PCSK9 protein or NaCl were injected at 0 minute and 3H-LDL at 60 minutes (n=6–7 mice per group). D, TICE was measured for 2 hours in these mice in vivo. Figure represents percentage reduction of TICE in PCSK9−/− mice (left) and LDLR−/− mice (right) after NaCl or PCSK9 intravenous injection. For each mouse strain, results are expressed as relative to mice injected with NaCl. E, Representative Western blot analysis of scraped intestinal mucosae harvested 1 hour after injection of recombinant PCSK9. Note the decrease in LDLR content in liver and intestine. Values represent mean±SEM. *P<0.05.
whether TICE is affected by the absence of LDLR, we compared TICE in C57Bl6J and LDLR−/− mice using 3H-LDL. In contrary to what was expected, we observed that TICE is slightly higher in LDLR−/− mice than in C57Bl6 in vivo (P=0.055; Figure 5A; Figure V in the online-only Data Supplement). TICE also tended to be increased in LDLR −/− explants (P=0.28; Figure 5B). In both experiments, the differences were not statistically significant but the trends were reproducible over time. To explain no reduction in TICE in LDLR−/− mice, we studied the uptake of 125I-LDL by the various intestinal segments in C57Bl6J and LDLR−/− mice after intravenous injections. Clearance from plasma was determined at various time points and radioactive content of liver and intestinal segments at 2 hours after injection; a duration equivalent to that we used for in vivo TICE (Figure 5D). We observed a delay in 125I-LDL clearance in LDLR−/− mice and a reduction of 125I-LDL hepatic uptake (~24%; P<0.05) consistent with other studies.27,28 There was no significant difference in radioactive uptake by the medial and distal intestinal segments, but a significant increase in radioactivity was seen in the proximal segment. Thus, contrary to what occurs in the liver, chronic LDLR deficiency results in increased LDL uptake in the proximal intestine. This might contribute to increased TICE.

**ABCB1 Contributes to TICE**

Next we focused on molecules that could efflux cholesterol from the apical side of enterocytes. ABCG5 contributes to TICE,8 probably because of its important role in cholesterol efflux. However, ABCG5−/− mice still have an appreciable level of TICE, suggesting that other apical transporters play a role in TICE. Among the candidates, multidrug transporter ABCB1, which is located at the apical pole of enterocyte, can act as a cholesterol floppase.23 We recently showed that older mice deficient for both isoforms of the apical cholesterol floppase

**Figure 4.** Transintestinal cholesterol excretion (TICE) in C57Bl6J on Lovastatin treatment. C57Bl6J mice were fed for 10 days with a regular chow diet supplemented or not with 0.2% Lovastatin (10–12 mice per group). A, Plasma cholesterol levels at the end of the treatment. B, Effect of Lovastatin on 3H-cholesterol labeled low-density lipoprotein TICE. Values represent mean±SEM. *P<0.05.

**Figure 5.** Transintestinal cholesterol excretion (TICE) and low-density lipoprotein (LDL) uptake in C57Bl6 and LDLR−/− mice. 3H-LDL TICE in C57Bl6 and LDLR−/− mice (A) in vivo (10–12 mice per group) and (B) ex vivo (n=12 mice and 16–17 explants in total for each genotype). Values represent mean±SEM. C, 12-hour–fasted male C57Bl6J or LDLR−/− mice (6–7 mice per group) received intravenous injection of 125I-LDL (10 μg; 1252 cpm/ng) and were euthanized after 120 minutes (cf. Methods in the online-only Data Supplement). Plasma content of 125I-LDL was measured at 2, 15, and 120 minutes. 125I radioactivity content was measured in livers and in 3 equals parts of the intestine: Proximal (P), medial (M), distal (D; histograms). Values are expressed as percentage of the dose injected. NS indicates nonsignificant; *P<0.05; **P<0.01.
ABCB1a and ABCB1b (∆∆∆∆∆∆∆∆∆∆) spontaneously develop hepatic steatosis, obesity, diabetes mellitus, and increased HDL-C.

We performed our studies in 15-week-old male mice that presented with similar cholesterol levels (Figure VIA in the online-only Data Supplement), before metabolic disturbances arise. We observed that ABCB1a/b−/− mice present with 26% less fecal cholesterol excretion (P<0.05; Figure 6A).

Next, we injected intravenously 3H-cholesterol diluted in endolipid to measure TICE in control FVB and ABCB1a/b−/− mice. Increases and subsequent decays of cholesterol were similar in WT and in ABCB1a/b−/− mice (Figure VIB in the online-only Data Supplement). TICE was decreased by 26.5% (P<0.05) in ABCB1a/b−/− mice (Figure 6B). We further verified involvement of ABCB1a/b in TICE using pharmacological inhibitors (Figure 6C). We added ABCB1 reference inhibitor PSC-833 to the apical chamber. Thirty minutes after the addition of 3H-cholesterol/endolipid to the basal chamber, the medium was collected in the apical chamber for determination of 3H-cholesterol level (initial TICE). The apical chamber was rinsed and PSC-833 was added with new medium for 30 minutes. TICE observed with no inhibitor was arbitrarily set at 1. PSC-833 inhibited TICE by 50% to 64% (P<0.05) in control mice but had no effect in ABCB1a/b−/− mice. These studies provide evidence that ABCB1a/b contribute to TICE.

Discussion

These results fill a gap in our understanding of plasma cholesterol excretion by the small intestine. We demonstrate that both LDL and HDL provide cholesterol for TICE in vivo and ex vivo in mice. Further, we provide the first direct evidence that human intestinal explants can excrete plasma-derived LDL or HDL cholesterol. On the basis of these studies, we propose a model (Figure 7) in which both LDL and HDL can contribute to TICE, a metabolically active pathway present in mice and humans. LDL-TICE can be modulated positively by statins and negatively by PCSK9. In addition, the multi-drug transporter ABCB1 contributes to the luminal efflux of cholesterol.

TICE has generally been studied by injecting 3H-cholesterol as part of intralipid emulsions. Our studies demonstrate that cholesterol present as part of plasma lipoproteins is amenable to TICE. How do these lipoproteins contribute to TICE? Studies in mice deficient for hepatic acyl-coenzyme A:cholesterol acyltransferase 2,6 where hepatic cholesterol cannot be esterified and stored, and recent findings in mice with impaired hepatobiliary cholesterol excretion5 suggest that cholesterol is first delivered to the liver before being rerouted to the intestine for excretion. Our findings do not exclude this possibility. However, our ex vivo experiments reveal that LDL and HDL can directly provide cholesterol to the intestine for TICE. While this article was in preparation, Vrins et al31 published a study stating that TICE is not mediated by HDL. Overall, the main point of this article is that TICE derived from radiolabeled HDL is much lower than TICE measured after injection of 3H-cholesterol diluted in a mixture of lipids. In particular, HDL-derived TICE seems to be roughly 10× lower

Figure 6. ABCB1 cholesterol floppase contributes to transintestinal cholesterol excretion (TICE). A, Fecal cholesterol excretion was measured in 15-week-old FVB control and ABCB1a/b male mice (n=7–8 per group). B, TICE was measured in these animals after injecting 3.5 µCi 3H-cholesterol diluted in 20% endolipid. C, TICE was measured in explants from mice of the same age and sex (n=6 explants per condition from 6 mice). 3H-cholesterol diluted in endolipid was added to the basal chamber to determine initial TICE for 30 minutes. Apical media was switched to new media with the same composition or containing ABCB1a/b inhibitor PSC-833, and TICE was measured after 30 minutes. The results represent the mean of TICE for each genotype, relative to control (no PSC-833) arbitrarily set at 1. Values represent mean±SEM. *P<0.05.
than biliary cholesterol excretion. For comparison, the authors determined that lipid emulsions contribute twice as much to TICE compared with biliary excretion. Specific activity in the bile was also 10× lower when using HDL compared with lipid emulsions, but the doses of 3H-CO injected were 5× lower. Further, HDL-TICE was actually measurable and unfortunately no other lipoproteins, such as LDL, were used for comparison. Thus, we believe that under the experimental conditions used by these investigators, HDL is much less efficient than intralipid.

In this study, we used LDL and HDL. The importance of triglyceride-rich lipoproteins or remnants in TICE was not explored here. A study suggested that triglyceride-rich emulsion particles are not cleared directly by the small intestine but are rapidly taken in by the liver. Cholesterol is then secreted into the plasma mainly on HDL. More experiments are required to address the role of larger triglyceride-rich lipoproteins in TICE.

Using a genetic model (PCSK9−/− mice) and pharmacological treatment (Lovastatin), we showed for the first time that TICE and cholesterolemia can be inversely related. Lovastatin-treated and PCSK9−/−-injected mice have low plasma cholesterol and yet show increased TICE. Thus, TICE does not seem to be a concentration-dependent passive process. This observation was further supported by ex vivo studies showing that TICE occurs at physiological temperatures and in the presence of oxygen. Therefore, TICE seems to be an active process.

We observed that Lovastatin increased LDL-derived TICE in C57Bl6J mice. The effect of Lovastatin was also investigated in parallel in LDLR−/− mice and Lovastatin reproducibly failed to increase TICE in the absence of LDLR (data not shown). However, we cannot exclude that TICE reached a plateau in LDLR−/− mice under Lovastatin. Thus, more work is needed to fully understand the relevance of the modulation of LDLR to TICE.

As discussed above, our data show that modulation of LDLR expression by PCSK9 and statins affects TICE. However, we were surprised to see that TICE was not decreased in LDLR−/− mice. To explain these apparently contradictory findings, we propose that uptake of LDL by the intestine that contributes to TICE might involve several mechanisms. Comodulation of LDLR and TICE by PCSK9 and statins suggests that LDLR plays a role in TICE in WT mice. However, in LDLR−/− mice TICE is not reduced suggesting that other unknown mechanism(s) might have been upregulated to compensate for genetic deficiency of LDLR. This is consistent with the observations showing increased gastrointestinal sterol cholesterol excretion in patients with homozygous familial hypercholesterolemia and bile diversion. The nature of this unknown mechanism(s) remains to be elucidated. There are several possible candidates that can be tested in the future, such as LDLR-related protein 1 that is expressed in the intestine, the very low-density lipoprotein receptor which is also degraded by PCSK9, Sortilin 1, and proteoglycans. Of note, although apoE receptor LRP1 does not clear LDL under normal conditions, LDLR−/− mice do accumulate apoE in their plasma and present with apoE on LDL. Thus, it is possible that excess plasma apoE bound LDL after their injection in LDLR−/− mice.

Besides LDL, we also show that HDL-derived cholesterol is also amenable to TICE. It is unclear yet which receptor on

![Diagram](http://atvb.ahajournals.org/)

**Figure 7.** Model transintestinal cholesterol excretion (TICE) is an active metabolic pathway that involves both low-density lipoprotein (LDL) and high-density lipoprotein (HDL). On the basal side of proximal intestine enterocytes, LDL-mediated TICE is induced by statins. TICE is repressed by proprotein convertase subtilisin kexin type 9 (PCSK9) in an LDLR fashion, but LDL-mediated TICE is present in LDLR-deficient mice suggesting that undefined alternative pathway exists. The HDL receptor(s) responsible of the HDL-mediated TICE remain(s) to be determined. On the apical side, efflux of cholesterol is mediated at least by ABCG5/G8 and ABCB1a/b.
the basolateral side mediates HDL-TICE. Selective uptake of cholesterol esters from HDL is made possible by scavenger receptor B1. TICE has been found to be increased or unchanged in SRB-1−/− mice.39–41 Another possible entry gate for HDL cholesterol in TICE could be P2Y13/ectoF1-ATP synthase pathway,39 although it is unclear whether this pathway is functional in the small intestine. Obviously, more work is needed to answer these questions.

Our data show that ABCB1 contributes to TICE. It is unclear why the effect seen in vivo in the absence of transporter was not more pronounced that that seen ex vivo with the inhibitor. Several human studies describe a link between cholesterol levels and ABCB1 gene polymorphisms.39–41 Owing to its apical location in enterocytes, it is tempting to speculate that ABCB1 could work additively or in synergy with ABCG5/G8 transporter to manage the luminal efflux of cholesterol. It would be very interesting to characterize the effect of ABCB1 deletion/ inhibition in ABCG5/G8 knockout mice. Altogether results presented here provide evidence that TICE is an active process in human and in rodent intestine. Further, we identified molecules that could participate in this process.

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Disclosures

None.

References


Atherosclerosis is an inflammatory disease promoted by the accumulation of cholesterol in macrophages of the arterial wall, leading to the complication of coronary heart disease, a major cause of death in westernized countries. The classic pathway of cholesterol elimination is hepatic-biliary cholesterol secretion. There is accumulating evidence that transintestinal cholesterol excretion is an alternative pathway. There is compelling evidence from population-based data and clinical trials that low-density lipoprotein–associated cholesterol reduction is an effective strategy to prevent coronary heart disease. It is unclear whether high-density lipoprotein is antiatherogenic because of their role in reverse cholesterol transport, the main hypothesis, or via other properties, such as their anti-inflammatory, antioxidant actions. Here, we identified low-density lipoprotein and high-density lipoprotein direct contribution to transintestinal cholesterol excretion as well as pharmacological agents, proteins that modulate cholesterol entry at the basal side of enterocytes, and its efflux into the intestinal lumen, at the apical side.
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MATERIAL AND METHODS

Materials
Human PCSK9 recombinant protein was purchased from Cyclex (Nagano, Japan). L-phosphatidylcholine, sodium taurocholate were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). \(^{3}\)H-cholesterol and \(^{3}\)H-cholesteryl oleate were obtained from Perkin Elmer (France). PSC-833 was purchased from R&D System Europe (Lille, France), Endolipid 20% was provided by B. Braun Medical SA (Boulogne, France).

Animals & Diet studies
PCSK9\(^{+/−}\) and LDLR\(^{−/−}\) mice were purchased from Jackson Laboratories (Maine, USA). C57Bl6J mice were from Charles River Laboratory (l’Arbresle, France). ABCB1a/b\(^{−/−}\) and FVB control mice were purchased from TACONIC (Germantown, NY, USA). Mice had free access to food and water under a 12-hour light/12-hour dark cycle in a temperature-controlled environment. All animal studies were conducted on male mice and approved by the Ethic Committee for Animal Experimentation of Pays de la Loire. For one study, C57Bl6J and LDLR\(^{−/−}\) mice were fed \textit{ad libitum} a regular chow diet (Safe A03 diet) supplemented or not with 0.2% (wt/wt) Lovastatin (Sequoia Research, CA, USA) for 10 days.

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Male mice were anesthetized with a ketamine/xylazine mix (90/10 mg/Kg BW IP) and placed onto a heat pad. Perfusion solution composition as well as surgical procedures to realize bile duct diversion and proximal intestinal cannulation was performed as described by Van der Velde et al.\(^2\). The intestinal perfusion was initiated 15 minutes prior to injection of lipoproteins to verify that surgical procedures did not cause luminal bleeding. Bile was diverted and collected by gravimetry. Proximal small intestines (10 cm) were perfused using a stable flux of 5 ml/h. The perfusion solution consisted of a modified solution of KREBS (119,95 mmol/L NaCl, 4,8 mmol/L KCl, 1,2 mmol/L KH\(_2\)PO\(_4\), 1,2 mmol/L MgSO\(_4\).7H\(_2\)O, 15 mmol/L sulfonic acid 4-(2-Hydroxyethyl)-1-piperazineethane, 1,3 mmol/L CaCl\(_2\).2H\(_2\)O and 10 mmol/L L-glutamine ; pH 7,4) supplemented with taurocholate / phosphatidylcholine (10 mmol/L taurocholate : 2 mmol/L phosphatidylcholine) as cholesterol acceptors. \(^{3}\)H-cholesterol, \(^{3}\)H-cholesteryl oleate labeled
lipoproteins (6.10^6 and 1.10^6 CPM/mouse respectively) or endolipid containing ^3H-cholesterol (6.10^6 CPM/mouse) were injected via the penile vein. Blood samples were harvested at 0, 2, 30, 60 and 120 minutes after injection to determine plasma cholesterol and ^3H-cholesterol levels. Intestinal perfusates were collected every 15 minutes over 120 minutes. The results correspond to the average of radioactivity measured in these collections.

**TICE measurement in Ussing chambers**

Proximal intestinal explants from C57Bl/6 mice and human jejunum were fixed on sliders (P2405 - physiologic instruments) and then placed in Ussing chambers (P2400 - physiologic instruments). Unless specified in the legend, intestinal explants were incubated at 37°C in presence of an oxygenated Krebs solution (NaCl 115 mM; NaHCO_3 25 mM; K_2HPO_4 2,4 mM; MgCl_2 1,2 mM; CaCl_2 1,2 mM; KH_2PO_4 0,4 mM) containing 10 mmol/L Glucose (basal chamber) or 10mM Mannitol apical chamber. The apical chamber also contains cholesterol acceptors (10 mM taurocholate, 2 mM phosphatidylcholine). ^3H-cholesterol labeled lipoproteins (4.10^6 CPM/ml) were added to the basal chamber. The integrity of the intestinal explants was determined by verifying the absence of leakage of non-permeable 70kDa FITC-dextran beads (Sigma-Aldrich, France) from the basal to the apical chamber. Medium in the apical chamber was collected and renewed every 30 min. Unless indicated, TICE represents the average of radioactivity over 3 periods of 30 min. Eventually intestinal explants were digested overnight at 37°C in an aqueous based solubilizer (Solvable - Perkin Elmer) in order to measure their radioactive content.

**Subjects:** Human intestinal biopsies were obtained from patients undergoing bariatric surgery (Roux-en-Y gastric by-pass), with their informed consent and according to the French laws (Nantes University Hospital ID RCB: 2006-A00196-45; Reference CPP: 2006/31; BRD: 06/8-I).

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Plasma total Cholesterol concentrations were determined using a commercially available kit (BioMerieux, France).

**Western blots**

Intestinal mucosae were scraped and homogenized in a RIPA buffer containing protease inhibitor cocktail (Roche Diagnostic). Total lysate proteins were resolved on NuPAGE 4–12% BisTris gels in MES SDS buffer (Invitrogen) under reducing conditions and transferred using Iblot system onto a nitrocellulose membrane (Invitrogen). Membranes were probed with antibodies raised against the mouse LDLR antibody (R&D Systems Europe, Lille, France) and cyclophilin A (Millipore, France). Immunoreactive bands were revealed using the ECL plus kit (Amersham Biosciences).

**Cytotoxicity assay**: Cytotoxicity was routinely measured using LDH cytotoxicity assay kit in apical medium (Abcam, Paris, France).

**^3H-Cholesterol measurement**

Plasma (20 µl), intestinal perfusates (500 µl) and Ussing media (500 µl) were mixed with 5 ml of liquid scintillation (ECOLITE, Perkin Elmer) and radiolabeled cholesterol levels were assessed by liquid scintillation counting.
125I-LDL clearance
C57BL6J or LDLR/- 12h-fasted anesthetized mice received an intravenous injection of 125I-labelled LDL (10μg; 1252 CPM/ng). Blood was harvested at 2, 15 and 120 minutes after injection. After two hours, mice were exsanguinated by puncturing the right atrium. Heart were perfused with 10 ml of saline to clear plasma 125I-labelled LDL associated with tissues. Thereafter, liver and small intestine were harvested. Small intestine was divided in 3 equal proximal, medial, distal segments. Lumen of each segment was gently flushed with saline solution. Plasma, liver and intestinal segment radioactivity were measured with a GammaCounter (1480 Wizard 300 Automatic Gamma Counter, Wallac, Waltham, Massachusetts, USA).

Fecal cholesterol excretion
Food consumption over 3 days was measured by weight difference method. Feces was collected every 24 h during the same period of time. Feces were desiccated overnight and ground. One hundred mg of feces were resuspended in 2 ml H2O for 10 h at 4°C on a rotating wheel. Liquid extraction with chloroform/methanol was performed overnight at 4°C on a rotating wheel. Samples were centrifuged and the inferior phase containing the lipids was evaporated with N2 and dissolved in 100 μL ethanol (repeated agitation with a vortex over 8 hours) and sonicated for 5 min. Cholesterol was measured in these extracts using a commercially available kit (BioMerieux, France).

Cholesterol absorption measurement-Plasma dual isotope ratio method
A feeding needle with round tip was inserted into the stomach of non-fasted mice, and each animal was given an intragastric bolus of 1 μCi [14C] cholesterol in olive oil. Under anaesthesia, 1 μCi [3H] cholesterol in Endolipid was injected via the penile vein. After dosing, mice were returned to cages, where they were free to feed for an additional 3 days. Animals were then anesthetized, and bled from the heart. Proportions of [14C] cholesterol and [3H] cholesterol doses remaining in plasma at 3 days were determined by liquid scintillation counting. The plasma ratio of the 2 radiolabels was used for calculating the percent cholesterol absorption as described elsewhere 3.

Statistics
All results are reported as means ± SEM. Statistical significance was analyzed using a non-parametric Mann-Whitney test. The values of p< 0.05 were considered as significant.

Reference List
(1) Fraker PJ, Speck JC, Jr. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem Biophys Res Commun 1978;80:849-857.

SUPPLEMENTARY FIGURE LEGEND

Figure I. Both LDL and HDL contribute to TICE
(A) C57Bl6J mouse proximal intestinal explants were mounted on Ussing Chambers. $^3$H-cholesteryl oleate labelled LDL or HDL ($^3$H CO-LDL; or $^3$H CO-LDL; n=14-13 explants from 7 mice per group) were added to Krebs/glucose buffer in the basolateral chamber. After 30 min, TICE was determined by measuring the appearance of radioactivity in the apical chamber filled with Krebs buffer enriched with cholesterol acceptors (taurocholate/phosphatidylcholine; TC/PC: 10/2 mM). (B-E) Both LDL and HDL provide cholesterol for TICE in vivo. NaCl, $^3$H-LDL or $^3$H-HDL were injected in the penile vein of C57Bl6 mice (6-8 mice per group) and transintestinal $^3$H-cholesterol excretion (TICE) was measured over 2 hours by intestinal canulation (cf. Figure 1C). (B) Cholesterol levels and (D) $^3$H-cholesterol were measured (n= 3-4 mice per group) during the course of the experiment. (C) Cholesterol distribution in various classes of lipoproteins was determined by Fast protein liquid chromatography analysis 15 min after injection of the lipoproteins (pool of 3 mice). (E) $^3$H cholesterol in fractions of perfusion solution collected every 15 minutes. NS: non significant. Results are means ± SEM.

Figure II. PCSK9 deficiency stimulates TICE.
(A-B) PCSK9$^{+/+}$ (n=10) and PCSK9$^{-/-}$ (n=13) mice received an intravenous injection of $^3$H-cholesterol-LDL at t = 0 min and TICE was measured over 2 hours. (A) Plasma cholesterol and (B) $^3$H-cholesterol levels of PCSK9$^{+/+}$ and PCSK9$^{-/-}$ littermates before and after the injection. (C-D) PCSK9$^{+/+}$ (n=6) and PCSK9$^{-/-}$ (n=6) mice received an intravenous injection of $^3$H-cholesteryl oleate-LDL at t = 0 min and TICE was measured over 2 hours. (C) Plasma $^3$H-Cholesteryl oleate levels and (D) TICE during the course of the experiment. (E) Fecal cholesterol output in PCSK9$^{-/-}$ mice. Food intake, dried feces weight and fecal cholesterol output and cholesterol absorption were determined in PCSK9$^{-/-}$ and PCSK9$^{+/+}$ littermates (n =6 per group) over 3 days. Data represent means ± SEM. * p<0.05.

Figure III. PCSK9 modulates TICE in an LDLR dependent manner.
PCSK9$^{-/-}$ (n=6) and LDLR$^{-/-}$ (n=7) mice received an intravenous injection of NaCl or 100µg of recombinant human PCSK9 protein at t=0. Sixty minutes later, mice were injected with $^3$H-LDL. Plasma $^3$H-cholesterol levels in mice injected or not with PCSK9. Values represent means ± SEM.
**Figure IV. TICE in C57Bl/6J mice upon lovastatin treatment.**

C57Bl6J mice fed for 10 days with or without 0.2% lovastatin received an intravenous injection of $^3$H-LDL at $t = 0$ min and TICE was measured over 2 hours. (A) Plasma Cholesterol (C) Cholesterol content in FPLC fractions of mouse plasma at the end of the treatment before measuring TICE (B) plasma $^3$H -cholesterol during the course of the experiment ($n= 10-12$ mice per group). Results represent means ± SEM.

**Figure V. TICE in C57Bl/6 and LDLR$^{-/-}$ mice.**

TICE was measured in vivo with $^3$H-LDL in C57Bl6J and LDLR$^{-/-}$ mice over 2 hours. (A) Plasma Cholesterol and (B) plasma $^3$H -cholesterol during the course of the experiment ($n= 10-12$ mice per group). Results represent means ± SEM.

**Figure VI. ABCB1a/b cholesterol floppase contributes to TICE.**

FVB and ABCB1a/b$^{-/-}$ received an intravenous injection of $^3$H-cholesterol diluted in endolipid at $t = 0$ min and TICE was measured over 2 hours. (A) Plasma cholesterol and (B) plasma $^3$H-cholesterol during the course of the experiment ($n= 6$ mice per group). Results represent means ± SEM.
Minutes after I.V. injection

Plasma $^3$H cholesterol (CPM/µl)

Plasma cholesterol (g/l)

C57Bl6J

LDLR$^{-/-}$

0 30 60 90 120

0 500 1000 1500 2000 2500 3000 3500 4000 4500 5000

C57Bl6J

LDLR$^{-/-}$

0 2 30 60 120

0 2 30 60 120
A

Minutes after I.V. injection

Plasma Cholesterol (g/l)

0 0.2 0.4 0.6 0.8 1 1.2 1.4

0 30 60 90 120

FVB

ABCB1ab−/−

B

Minutes after I.V. injection

Plasma \(^1\)H cholesterol (CPM/ml)

0 30 60 90 120

WT FVB

Pgp KO
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FVB and ABCB1a/b$^{-/-}$ received an intravenous injection of $^3$H-cholesterol diluted in endolipid at $t = 0$ min and TICE was measured over 2 hours. (A) Plasma cholesterol and (B) plasma $^3$H-cholesterol during the course of the experiment (n= 6 mice per group). Results represent means ± SEM.
Fig B, C, D:

- Mice injected with $^{3}$H-LDL
- Mice injected with $^{3}$H-HDL
- Mice injected with NaCl

Fig E:

- TICE (CPM/fraction) vs. Minutes after I.V. injection
A. Plasma cholesterol (g/l)

B. Plasma cholesterol (CPM/µL)

C. Plasma $^3$H CO (CPM/µL)

D. TICE (CPM/min/100g BW)

E. Food intake (g/d), Excreted dried feces (mg/d), Fecal cholesterol Excretion (µmol/d/100g BW), Cholesterol absorption (%)
Plasma $^3$H cholesterol (CPM/µl)

- PCSK9^−
- PCSK9^− + PCSK9
- LDLR^−
- LDLR^− + PCSK9

TICE
**A**

Plasma Cholesterol (g/l)

- **C57Bl6J**
- **C57Bl6J + Lovastatin**

Minutes after I.V. injection

**B**

Plasma $^3$H cholesterol (CPM/µl)

- **C57Bl6J**
- **C57Bl6J + Lovastatin**

Minutes after I.V. injection

**C**

Plasma $^3$H cholesterol (CPM/µl)

- **C57Bl6J**
- **C57Bl6J + Lovastatin**

Minutes after I.V. injection
Minutes after I.V. injection

Plasma $^3$H cholesterol (CPM/µl)

Plasma cholesterol (g/l)

C57Bl6J
LDLR$^-$

0 30 60 90 120

Minutes after I.V. injection

C57Bl6J
LDLR$^-$
A

Minutes after I.V. injection

Plasma Cholesterol (g/l)

Plasma H\(_2\) cholesterol (CPM/ml)

FVB
ABCB1ab/−

B

Minutes after I.V. injection

Plasma H\(_2\) cholesterol (CPM/ml)

WT FVB
Pgp KO