Increased Fecal Neutral Sterol Loss Upon Liver X Receptor Activation Is Independent of Biliary Sterol Secretion in Mice

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Background & Aims: **Reverse cholesterol transport (RCT) is defined as high-density lipoprotein (HDL)-mediated flux of excess cholesterol from peripheral cells to liver, followed by secretion into bile and disposal via the feces. Various steps of this pathway are controlled by the liver X receptor (LXR). We addressed the role of the intestine in LXR-dependent stimulation of fecal cholesterol excretion.** ***Methods:*** **To segregate biliary from intestine-derived cholesterol, wild-type and Mdr2 P-glycoprotein-deficient mice (*Mdr2*$^{-/-}$), which are unable to secrete cholesterol into bile, were treated with the LXR agonist GW3965.** ***Results:*** **Treatment with GW3965 increased biliary cholesterol secretion by 74% in wild-type mice but had no effect in *Mdr2*$^{-/-}$ mice. LXR activation increased fecal neutral sterol excretion 2.1-fold in wild-type mice. Surprisingly, an identical increase was observed in *Mdr2*$^{-/-}$ mice. Fractional cholesterol absorption was reduced on LXR activation in both strains but was more pronounced in *Mdr2*$^{-/-}$ mice, coinciding with reduced *Npc111* expression. Intestinal gene expression of ATP-binding cassette transporters (Abc) *Abca1*, *Abcg1*, *Abcg5*, and *Abcg8* was strongly induced upon LXR activation in both strains, whereas expression of *HMGCoA reductase*, controlling cholesterol synthesis, remained unaffected. Additionally, LXR activation stimulated the excretion of plasma-derived [^{3}H]cholesterol into the fecal neutral sterol fraction in *Mdr2*$^{-/-}$ mice.** ***Conclusions:*** **Increased fecal cholesterol loss upon LXR activation is independent of biliary cholesterol secretion in mice. An important part of excess cholesterol is excreted directly via the intestine, supporting the existence of an alternative, quantitatively important route for cholesterol disposal.**

Cholesterol accumulation in macrophages (foam cells) in the arterial vessel wall is considered a primary event in the development of atherosclerosis. Removal of excess cholesterol from these cells, as well as from other peripheral cells, is therefore of crucial importance. This pathway, referred to as *reverse cholesterol transport* (RCT), is usually defined as the high-density lipoprotein (HDL)-mediated flux of cholesterol from peripheral cells to the liver, followed by its secretion into bile and disposal via the feces. The ATP-binding cassette transporter (Abc) a1 (Abca1) facilitates the obligatory first step of RCT, ie, the efflux of cholesterol from peripheral cells toward HDL. HDL cholesterol is subsequently taken up by the liver, mainly via scavenger receptor-BI (Sr-Bi) and, finally, may be excreted into bile, either as free cholesterol or after conversion to bile salts. Hepatobiliary elimination of cholesterol was shown to be mediated, at least partially, by the half-transporters Abcg5 and Abcg8.[1] Part of biliary cholesterol, which mixes with dietary cholesterol in the lumen of the small intestine, is taken up by the Niemann-Pick C1 Like 1 (Npc111) protein, which has recently been shown to play a role in cholesterol absorption,[2] and transported back to the liver by the chylomicron-remnant pathway. Another part, however, is lost into feces. It has become clear that cholesterol absorption is not a passive process but depends on the combined actions of transporter proteins involved in uptake (Npc111) and efflux (Abcg5, Abcg8).

Fecal excretion of cholesterol in mice can be enhanced via activation of the nuclear liver X receptor (LXR), for which oxysterols have been identified as natural ligands. LXR regulates expression of a number of genes crucially involved in RCT, including the members of the ATP-binding cassette transporter family mentioned above. Activation of LXR in mice leads to elevated HDL levels, increased biliary cholesterol excretion, reduced intestinal

Abbreviations used in this paper: Abc, ATP-binding cassette transporter; Acat2, acyl-coenzyme A:cholesterol acyltransferase 2; ApoA-1, apolipoprotein A-1; BBM, brush border membrane; CETP, cholesteryl ester-transfer protein; FPLC, fast protein liquid chromatography; Hmgcr, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LXR, liver X receptor; Mdr2, multidrug resistance P-glycoprotein 2; Npc111, Niemann-Pick C1 Like 1; RCT, reverse cholesterol transport; Sr-b1, scavenger receptor BI.

0016-5085/05/$30.00
doi:10.1053/j.gast.2004.10.006

cholesterol absorption efficiency, and, finally, to increased neutral sterol loss via the feces.[3]

Recent data, however, indicate that the concept of RCT requires extensive rethinking.[1] Overexpression of cholesteryl ester-transfer protein (CETP) in mice, resulting in low HDL levels, showed that neither the HDL levels nor the level of CETP activity dictated the magnitude of centripetal cholesterol flux (ie, RCT) to the liver in mice.[4] Studies in apolipoprotein A-1 (*ApoA-1*)$^{-/-}$ mice, which have strongly decreased plasma HDL, revealed that neither HDL nor ApoA-1 levels are important determinants of centripetal cholesterol flux.[5] More recently, we reported that the absence of plasma HDL, and thus of HDL-mediated RCT, in *Abca1*-null mice does not at all affect hepatobiliary cholesterol transport.[3,6]

Recent data from our laboratory indicate that, other than the liver, the intestine may play a role as an excretory organ in RCT[3]: Upon pharmacologic LXR activation, fecal cholesterol excretion in various mouse models (C57BL/6J, DBA/1, Abca1$^{-/-}$ on a DBA/1 background) was increased to a much larger extent than could be explained by stimulation of biliary cholesterol secretion alone. To resolve the apparent discrepancies between the "classical" biliary route for removal of excess cholesterol and these findings, we now used an animal model in which biliary cholesterol secretion is strongly reduced, ie, Mdr2 P-glycoprotein (Pgp)-deficient (*Mdr2*$^{-/-}$) mice. Mdr2 (or Abcb4 according to new nomenclature) mediates the ATP-dependent translocation of phospholipids at the canalicular membrane of hepatocytes. Consequently, Mdr2 P-glycoprotein deficiency in mice leads to an inability to secrete phospholipids into the bile. Because of the tight coupling of phospholipid and cholesterol excretion, these mice also show a strongly impaired biliary cholesterol secretion.[7,8]

The first question we addressed was whether LXR activation, known to strongly increase biliary cholesterol output in wild-type mice,[3] would stimulate biliary cholesterol secretion in a phospholipid-independent manner. For this purpose, *Mdr2*$^{-/-}$ and wild-type mice were treated with the synthetic LXR agonist GW3965.[9] Upon GW3965 treatment, biliary cholesterol secretion remained nearly undetectable in *Mdr2*$^{-/-}$ mice, whereas it increased in wild-type mice. This allowed us to assess whether activation of LXR would stimulate fecal neutral sterol output independent of its effects on biliary cholesterol secretion. It was found that wild-type and *Mdr2*$^{-/-}$ mice treated with a synthetic LXR agonist showed a similarly increased fecal neutral sterol output without induction of genes involved in cholesterol synthesis, indicating that this increase must result from intestinal sources. This conclusion was supported by the finding that *Mdr2*$^{-/-}$ mice showed increased secretion of plasma-derived, radiolabeled cholesterol into the feces upon treatment with the LXR agonist. We therefore postulate that, other than the liver, the intestine plays an important and up to now underestimated role in cholesterol disposal.

Materials and Methods

Animals and Diet

Female *Mdr2*$^{-/-}$ mice (4–6 months) on an FVB background were obtained from the Central Animal Facility, Academic Medical Center, Amsterdam, The Netherlands. Age-matched FVB wild-type mice were purchased from Harlan (Horst, The Netherlands). Mice received standard mouse chow, containing 0.017 wt/wt percentage cholesterol (Arie Blok BV, Woerden, The Netherlands), or chow supplemented with the synthetic LXR agonist GW3965, 35 mg/kg/day (based on 26-g body weight and consumption of 3 g chow/day) for 10 days. GW3965 was kindly provided by GlaxoSmithKline, Stevenage, United Kingdom. All experiments were performed with the approval of the Ethical Committee for Animal Experiments of the University of Groningen.

Experimental Methods

Animals were housed in groups, and feces were collected on day 8 until day 10 of treatment. After 10 days, animals were anesthetized by intraperitoneal injection of Hypnorm (fentanyl/fluanisone, 1 mL/kg) and diazepam (10 mg/kg). After puncturing the gallbladder and disposal of its content, hepatic bile was collected for 30 minutes from the common bile duct via the gallbladder. During bile collection, body temperature was stabilized using a humidified incubator. Bile flow was determined gravimetrically assuming a density of 1 g/mL for bile. After bile collection, animals were killed by cardiac puncture. Blood was collected in EDTA-containing tubes. Livers were excised and weighed. The small intestine was rinsed with cold phosphate-buffered saline containing 100 μmol/L phenylmethylsulforylfluoride (PMSF) and divided into 3 equal parts. Parts of both the liver and the intestine were snap-frozen in liquid nitrogen and stored at −80°C for mRNA isolation and biochemical analysis. Samples for microscopic evaluation were frozen in isopentane and stored at −80°C or fixed in paraformaldehyde for H&E, oil red O, and Ki-67 staining.

Fractional cholesterol absorption was measured in a separate experiment using the fecal dual-isotope method. Animals were housed individually 1 week prior to the experiment. Wild-type and *Mdr2*$^{-/-}$ mice were fed normal chow or chow supplemented with GW3965 (35 mg/kg per day). After 6 days, mice received by gavage 150 μL medium-chain triglyceride oil containing 1 μCi [^{14}C]cholesterol (Amersham Bioscience, Buckinghamshire, England) and 2 μCi [^{3}H]sitostanol (American Radiolabeled Chemicals, St.