ABCG5 and ABCG8 require MDR2 for secretion of cholesterol into bile

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Abstract   The major pathway for the removal of cholesterol from the body is via secretion into the bile. Three members of the ATP binding cassette (ABC) family, ABCG5 (G5), ABCG8 (G8), and ABCB4 (MDR2), are required for the efficient biliary export of sterols. Here, we examined the interdependence of these three ABC transporters for biliary sterol secretion. Biliary lipid levels in mice expressing no MDR2 ($Mdr2^{-/-}$ mice) were compared with those of $Mdr2^{-/-}$ mice expressing 14 copies of a human G5 (hG5) and hG8 transgene ($Mdr2^{-/-};hG5G8Tg$ mice). $Mdr2^{-/-}$ mice had only trace amounts of biliary cholesterol and phospholipids. The $Mdr2^{-/-};hG5G8Tg$ mice had biliary cholesterol levels as low as those of $Mdr2^{-/-}$ mice. Thus, MDR2 expression is required for G5G8-mediated biliary sterol secretion. To determine whether the reduction in fractional absorption of dietary sterols associated with G5G8 overexpression is secondary to the associated increase in biliary cholesterol, we compared the fractional absorption of sterols in $Mdr2^{-/-};hG5G8Tg$ and $hG5G8Tg$ animals. Inactivation of MDR2 markedly attenuated the reduction in fractional sterol absorption associated with G5G8 overexpression.■ These results are consistent with the notion that increased biliary cholesterol secretion contributes to the reduction in fractional sterol absorption associated with G5G8 overexpression.—Langheim, S., L. Yu, K. von Bergmann, D. Lutjohann, F. Xu, H. H. Hobbs, and J. C. Cohen. **ABCG5 and ABCG8 require MDR2 for secretion of cholesterol into bile.** *J. Lipid Res.* 2005. **46:** 1732–1738.

Supplementary key words   ATP binding cassette transporters G5 and G8 • ATP binding cassette transporter B4 • sitosterol • campesterol • cholesterol absorption • bile acid • fecal sterol

The ATP binding cassette transporters ABCG5 (G5) and ABCG8 (G8) are expressed almost exclusively in the liver and intestine, where they limit the accumulation of sterols by promoting sterol excretion into bile and reducing dietary sterol uptake (1, 2). Mutations in G5 or G8 cause si-tosterolemia, a rare autosomal recessive disorder characterized by the accumulation of plant sterols in blood and tissues (1, 3, 4). Metabolic studies have demonstrated that sitosterolemic individuals have increased fractional absorption of sterols (5) and decreased biliary sterol secretion (6), consistent with a physiological role for G5 and G8 in both cell types. Metabolic studies in mice lacking G5 and G8 ($G5G8^{-/-}$) confirm the important role that G5 and G8 play in the enterohepatic trafficking of neutral sterols (2). $G5G8^{-/-}$ animals have markedly reduced biliary cholesterol secretion and increased fractional absorption of plant sterols, although not cholesterol (2). Consequently, these mice accumulate high levels of plant sterols, which results in dysregulation of cholesterol homeostasis in the liver and adrenal gland (7). Thus, G5 and G8 are essential for normal cholesterol homeostasis.

Expression of G5 and G8 is restricted almost exclusively to enterocytes and hepatocytes in humans and mice (1), but the relative expression levels in these organs differ between the two species. In chow-fed mice, both G5 and G8 are more highly expressed in the intestine than in the liver, whereas the opposite pattern is observed in humans (1). Mice expressing a human genomic fragment containing both G5 and G8 (8) have a pattern of expression that is similar to that in humans (i.e., higher in the liver than in the intestine), which is consistent with the difference in relative expression levels of G5 and G8 in the two tissues being mediated by *cis*-acting sequences at the *G5G8* locus. The significance of these interspecies differences in relative expression levels of G5 and G8 is not known, and the relative importance of hepatic and intestinal expression of G5 and G8 in sterol homeostasis has not been determined.

Abbreviations: G5, ATP binding cassette transporter G5; G8, ATP binding cassette transporter G8; MDR2, ATP binding cassette transporter B4.
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Overexpression of G5 and G8 in the livers of $G5G8^{-/-}$ mice by adenoviral transgenesis is sufficient to restore biliary cholesterol levels (9), but the effects of G5G8 expression in the intestine have not been determined directly. Overexpression of G5 and G8 in transgenic mice is associated with a reduction in fractional sterol absorption (8), but it is not known whether this decrease is a direct result of increased G5G8 activity in enterocytes or a secondary effect of the increase in the amount of cholesterol secreted into the bile, which competes with dietary cholesterol for access to intestinal sterol transporters. Several lines of evidence suggest that cholesterol absorption from the intestine is saturable and that increased biliary cholesterol secretion is associated with decreased fractional absorption of dietary cholesterol (10–12). However, studies of cholesterol absorption in animals with intestine- or liver-specific ablation of G5G8 activity have not been reported. Therefore, it is not known whether the decrease in fractional sterol absorption observed in $G5G8$ transgenic mice is secondary to increased biliary cholesterol secretion in these mice.

The intestinal and hepatic functions of G5 and G8 may be uncoupled in mice lacking the biliary phospholipid transporter $Abcb4$ ($Mdr2^{-/-}$ mice). Biliary cholesterol secretion is abolished in these animals, presumably secondary to the failure of biliary phospholipid transport (13), but cholesterol absorption is normal (14). In this study, we used $Mdr2^{-/-}$ mice to assess the role of intestinal G5 and G8 in limiting sterol absorption. To increase intestinal G5G8 expression, we crossed mice carrying 14 copies of a human G5G8 transgene (8) with $Mdr2^{-/-}$ animals. Expression of the $G5G8$ transgene did not increase biliary cholesterol levels in $Mdr2^{-/-}$ mice, but the effect of the transgene on fractional sterol absorption was attenuated, although not abolished. These data indicate that G5 and G8 reduce the absorption of dietary sterols both directly, by promoting sterol excretion in the enterocyte, and indirectly, by increasing the secretion of biliary sterols that compete with exogenous sterols for uptake in the intestine.

MATERIALS AND METHODS

Animals and diets

Hemizygous transgenic mice containing 14 copies of a genomic fragment encoding the human G5 and G8 genes were generated as described previously (8) and crossed with $Mdr2^{-/-}$ and $Mdr2^{+/+}$ mice in the FVB/NJ strain (stock numbers 002539 and 001800; Jackson Laboratory, Bar Harbor, ME) to generate four different groups of mice: wild type, $hG5G8Tg$, $Mdr2^{-/-}$, and $Mdr2^{-/-};hG5G8Tg$. The mice were individually housed in plastic cages in a temperature-controlled facility with a 12 h light cycle (light from 6 AM to 6 PM). Mice were fed a cereal-based rodent chow diet containing 0.02% cholesterol and 4% fat (Diet 7001; Harland Teklad, Madison WI). A total of 13 mmol/kg phosphatidylcholine (Sigma, St. Louis, MO) was added to the diet as a powder to replenish the phospholipids in the intestinal lumen of the $Mdr2^{-/-}$ mice.

Plasma sterol analysis

Venous blood was obtained from the vena cava, and plasma was isolated by centrifugation. Plasma sterol levels were assayed by gas chromatography and mass spectrometry as described previously (15, 16).

Biliary lipid analysis

Bile was collected from the gallbladders of anesthetized mice using a 30 gauge needle, and the sterols were extracted as described (8). The concentration of biliary cholesterol was measured using GC-MS (15), and the biliary phospholipids were measured using a colorimetric assay (17). Briefly, the methanol-diluted bile samples (25 μl) were air-dried before the addition of 50 μl of perchloric acid (70%). The samples were incubated at 190–210°C for 90 min in a sand bath. The mixture was allowed to cool, and then 400 μl of distilled water was added to each tube. Subsequently, 2 ml of malachite green-ammonium molybdate (A7302 M6880; Sigma) (ratio 3:1) and 100 μl of Tween 20 (final solution 1.5%) were added to the mixture. After 30 min, the optical densities of the samples were read at 660 nm and the concentration of phospholipids was calculated as described (17). Biliary bile acid levels were measured using an enzymatic assay (17).

Measurement of fractional absorption of dietary sterols

To measure the fractional absorption of sterols, mice were fed a chow diet with added phosphatidylcholine (13 mmol/kg; Sigma). After 1 week, 50 μl of a deuterated sterol/stanol-oil mixture was gavaged into the stomach of each mouse. The stable isotope solution was prepared by adding 4 mg each of $[26,26,26,27,27,27-^2H_6]$cholesterol, $[26,26,26,27,27,27-^2H_6]$cholestanol, and $[5,6,22,23-^2H_4]$sitostanol and 10 mg of $[2,2,4,4,6-^2H_5]$campesterol/sitosterol (40:60) (Medical Isotopes, Inc., Pelham, NH) to 2 ml of plant oil (Livio, Union Deutsche Lebensmittelwerke, Hamburg, Germany). Feces were collected for 3 days, pooled, and processed as described (5, 15, 18, 19).

RNA isolation and measurements of RNA levels using real-time PCR

The liver and intestine were excised, and the intestine was cut into three sections of equal length before submerging the tissues in liquid nitrogen. Total RNA was extracted from the livers and jejuna of five male mice of each genotype using RNA Stat-60 (Tel-Test, Inc., Friendswood, TX). Quantitative real-time PCR was performed to assay the relative amounts of selected mRNAs, as described (20).

Hepatic membrane preparation and immunoblotting

A total of 100–200 mg of mouse liver was homogenized using a Polytron in 1.2 ml of buffer A (250 mM sucrose, 2 mM MgCl$_2$, and 20 mM Tris-HCl, pH 7.5) containing proteinase inhibitors (Complete Protease Inhibitor cocktail; Roche Diagnostics). The crude preparation was centrifuged at 2,000 g for 10 min at 4°C. The supernatant was collected and recentrifuged at 120,000 g for 45 min at 4°C. The membrane pellet was resuspended in a solution containing 80 mM NaCl, 2 mM CaCl$_2$, 1% Triton X-100, 50 mM Tris-HCl, pH 8, and proteinase inhibitors as described above. The protein concentrations of the tissue membranes were determined using the bicinchoninic acid kit (Pierce, Rockford, IL).

Aliquots containing equal amounts of membrane proteins from each animal in a group were pooled. A total of 50 μg of the pooled membrane proteins was size-fractionated on 8% SDS-polyacrylamide gels and transferred to a nitrocellulose filter (Hybond-C Extra nitrocellulose filters; Amersham Biosciences, Piscataway, NJ). The filter was blocked overnight in the incubation buffer [PBS supplemented with 0.05% Tween 20, 5% dry milk powder, and 5% newborn calf serum (all from Sigma)]. The fil-
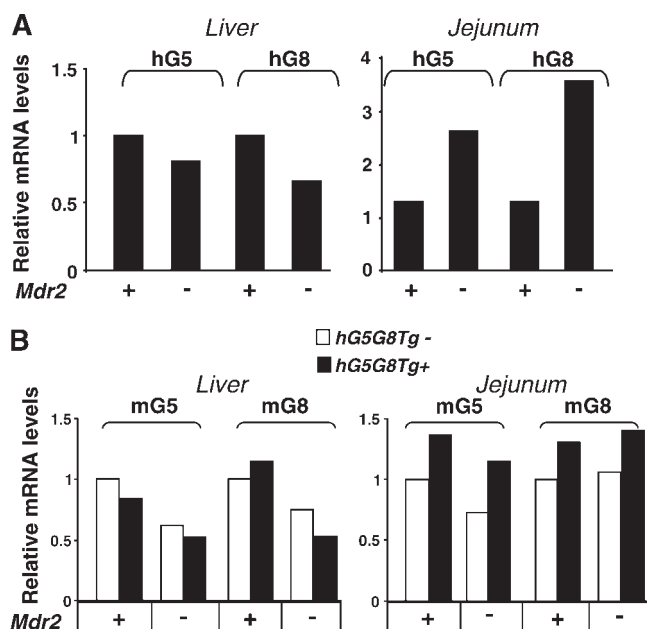
Fig. 1. Levels of hepatic and intestinal human (A) and mouse (B) mRNA transcripts in wild-type, *hG5G8Tg*, *Mdr2⁻/⁻*, and *Mdr2⁻/⁻; hG5G8Tg* male mice (n = 5). Total RNA was extracted from livers of five mice using RNA Stat-60 (Tel-Test), and equal amounts from mice in each group were pooled. Quantitative real-time PCR was performed as described using oligonucleotides specific for both the human (h) and mouse (m) forms of ATP binding cassette transporter G5 (G5) and G8. Cyclophilin was used as an internal control for these studies. The level of mRNA in the wild-type mice was set arbitrarily as 1, and the levels in the other mice were adjusted accordingly.

ter was then washed three times for 10 min each in PBS containing 0.05% Tween 20 (PBS-T, catalog number P-3563; Sigma) and incubated with a rabbit polyclonal antibody against the N-terminal region of mouse G5 at 1:1,000 dilution in the incubation buffer (2) for 1 h. After three washes with PBS-T, the filter was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories) at 1:5,000 dilution for 30 min in the incubation buffer, followed by three washes in PBS-T. The filter was then incubated with the SuperSignal substrates (Pierce) for 2 min and exposed to Kodak X-Omat Blue films for 5–30 s at room temperature. The same filter was stripped and the immunoblotting procedure was performed using a monoclonal antibody that recognizes both mouse and human G8 at 1:500 dilution (9) and horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson Immunoresearch Laboratories) at 1:5,000

dilution, or a rabbit polyclonal anti-BIP antibody (Stressgen Biotechnologies Corp., Victoria, Canada) at 1:2,000 dilution and horseradish peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories) at 1:5,000 dilution.

Statistical methods

For selected variables, including fractional cholesterol absorption, mean values of each group were compared using unpaired two-tailed *t*-tests.

RESULTS

Development of *Mdr2⁻/⁻* mice expressing high levels of human G5 and G8

Inactivation of *Mdr2* in mice is associated with the near absence of both phospholipids and cholesterol in the bile (13). To determine whether high-level G5 and G8 expression, which is associated with a dramatic increase in biliary cholesterol levels in wild-type mice (8), can overcome the defect in biliary cholesterol secretion, we developed *Mdr2⁻/⁻* mice expressing a human G5 and G8 transgene (*Mdr2⁻/⁻;hG5G8Tg*).

First, we examined the expression levels of the G5G8 transgene in the livers and intestines of wild-type and *Mdr2⁻/⁻* mice (**Fig. 1**). The mRNA levels for hG5 and hG8 transcripts were reduced by ∼20% in the livers of *Mdr2⁻/⁻* mice compared with wild-type animals (Fig. 1A). This may be attributable to the adverse effects of MDR2 deficiency on hepatic integrity, as indicated by the increase in serum transaminases (**Table 1**). In contrast to the liver, the levels of hG5 and hG8 mRNA were ∼2-fold higher in the jejunum of *Mdr2⁻/⁻* mice (Fig. 1A). We also examined the effect of the G5G8 transgene on the endogenous mG5 and mG8 mRNA levels. The levels of mG5 and mG8 were reduced by ∼40% in the livers of *Mdr2⁻/⁻* mice, similar to the findings in a prior report (21). Expression of the G5G8 transgene did not have a consistent effect on hepatic levels of mG5 and mG8 mRNA, whereas the levels of these transcripts were slightly but consistently higher in the jejunum of the transgenic animals (Fig. 1B).
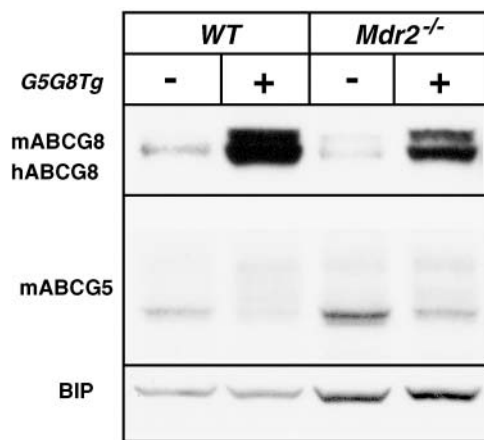
Immunoblot analysis of the liver samples was performed using an antibody that reacts with both mouse and human G8. The protein levels of G8 were lower in the *Mdr2⁻/⁻* mice (**Fig. 2**), as were the levels of G8 mRNA (Fig. 1A). In contrast to these results, the levels of immunodetectable

TABLE 1. Body weights and blood chemistries in wild-type, *hG5G8Tg*, *Mdr2⁻/⁻*, and *Mdr2⁻/⁻;hG5G8Tg* male mice (n = 4 per group)

Parameter	Wild Type	*hG5G8Tg*	*Mdr2⁻/⁻*	*Mdr2⁻/⁻;hG5G8Tg*
Body weight (g)	25.6 ± 1.4	27.3 ± 1.5	29.3 ± 0.8	29.3 ± 0.7
Liver cholesterol (mg/g)	2.3 ± 0.07	2.2 ± 0.03	2.2 ± 0.1	2.6 ± 0.1
Liver triglycerides (mg/g)	11.8 ± 2.7	10.9 ± 1.7	10.3 ± 1.9	6.2 ± 0.1
Bilirubin (mg/dl)	0.2	0.2	0.2	0.2–0.5
Serum alanine aminotransferase (U/dl)	27–28	24–34	57–137	251–318
Serum aspartate aminotransferase (U/dl)	35–40	31–33	69–129	186–335

Plasma was isolated from the blood, and liver function tests were performed at the Aston Center Pathology Laboratory (Dallas, TX). The hepatic lipids were measured using the cholesterol kit (Roche Molecular Biochemical, Mannheim, Germany) and Infinity Triglycerides Reagent (Sigma) as described previously (26). Values shown are means ± SEM.

Fig. 2. Immunoblot analysis of mouse and human G5 and G8 in the livers of the same mice used in the experiment described in Fig. 1. A total of 50 μg of membrane proteins pooled from each group of mice was fractionated on an 8% SDS-PAGE gel, and immunoblot analysis was performed using rabbit polyclonal antibody raised against mouse G5 (2) and a monoclonal antibody that recognizes both the mouse and human forms of G8. As a control, the amount of BIP was assayed using a rabbit anti-BIP polyclonal antibody (Stressgen Biotechnologies). WT, wild type.
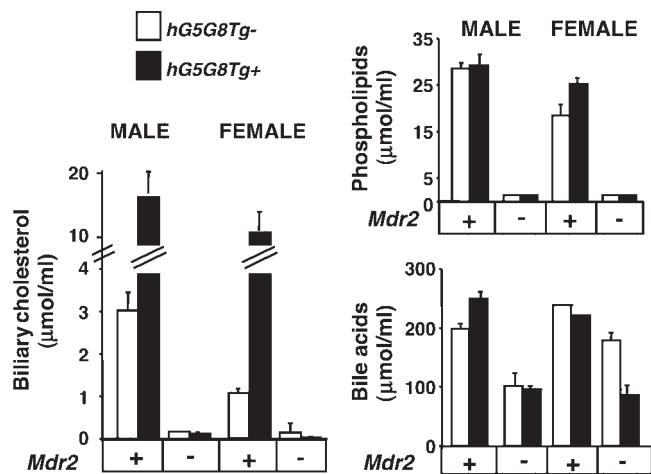

Fig. 3. Levels of biliary sterols in wild-type (n = 5), $hG5G8Tg$ (n = 5), $Mdr2^{-/-}$ (n = 3), and $Mdr2^{-/-};hG5G8Tg$ (n = 3) male and female mice aged 2–4 months. Mice were maintained on a chow diet supplemented with phosphatidylcholine (13 mmol/kg) for 3 weeks before being killed after a 4 h fast. The bile was collected, and the biliary cholesterol, phospholipids, and bile acids were measured as described in Materials and Methods. Error bars represent means ± SEM.

mouse G5 were slightly higher in the $Mdr2^{-/-}$ mice (Fig. 2), despite these mice having lower hepatic levels of G5 mRNA (Fig. 1). Thus, the absence of MDR2 expression had different effects on the mRNA levels of *G5* and *G8*, suggesting that there may be posttranscriptional regulation of G5.

Overexpression of hG5 and hG8 does not increase biliary cholesterol levels in $Mdr2^{-/-}$ mice

To determine the effect of increased G5 and G8 expression in the $Mdr2^{-/-}$ mice, we compared the levels of biliary cholesterol in wild-type, $hG5G8Tg$, $Mdr2^{-/-}$, and $Mdr2^{-/-};hG5G8Tg$ mice (**Fig. 3**). In both the wild-type and $hG5G8Tg$ mice, males had higher biliary cholesterol levels than their female counterparts. Biliary cholesterol levels were increased by 6.3-fold (19 vs. 3 μmol/ml) and 10-fold (10.8 vs. 1.08 μmol/ml) in male and female mice, respectively, expressing the G5G8 transgene, consistent with our previous results (8).

Inactivation of $Mdr2^{-/-}$ mice was associated with very low levels of biliary cholesterol, as described previously (13). Expression of the G5G8 transgene did not increase biliary cholesterol in $Mdr2^{-/-}$ mice. As expected, none of the $Mdr2^{-/-}$ mice had any detectable phospholipids in the bile. Inactivation of *Mdr2* was associated with a reduction in the biliary concentration of bile acids. Previously, Oude Elferink and colleagues found a slight increase in the rate of bile acid secretion in $Mdr2^{-/-}$ mice (from 309+/−88 to 394+/−88 nmol/min/100 g) compared with wild-type animals (22). Expression of the G5G8 transgene was not consistently associated with changes in the levels of bile acids in either wild-type or $Mdr2^{-/-}$ mice (Fig. 3).

Oude Elferink and colleagues also demonstrated that infusion of the hydrophobic bile acid cholate into the $Mdr2^{-/-}$ mice was associated with an increase in the secre-

tion of biliary cholesterol (22). To determine whether cholate would restore biliary cholesterol secretion in $Mdr2^{-/-}$ mice overexpressing G5 and G8, we supplemented the chow diet with 0.1% sodium cholate. After 3 weeks on this diet, the animals were killed and the bile was collected. Biliary cholesterol levels remained very low after cholate treatment, even in $Mdr2^{-/-}$ animals expressing the G5G8 transgene (**Fig. 4**).

The effect of hG5G8 overexpression on sterol absorption is attenuated in $Mdr2^{-/-}$ mice

Next, we measured the fractional absorption of dietary cholesterol, sitosterol, and campesterol in the four groups of mice by administering deuterated sterols and a nonabsorbed sterol (sitostanol) intragastrically to the animals
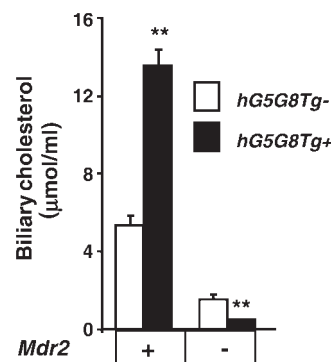
Fig. 4. Effect of cholate feeding on biliary cholesterol levels in wild-type, $hG5G8Tg$, $Mdr2^{-/-}$, and $Mdr2^{-/-};hG5G8Tg$ female mice (n = 4 in each group). All mice were fed chow diets supplemented with 0.1% cholate for 21 days. The mice were killed after a 4 h fast, and the bile was collected using a 30 gauge needle. The cholesterol was extracted and measured using GC-MS. ** $P < 0.01$. Error bars represent means ± SEM.

(n = 5 in each group). We then collected feces for 3 days and calculated the fractional absorption of cholesterol, sitosterol, and campesterol by measuring the ratio of deuterated sterols to the nonabsorbable marker sitostanol. Expression of the G5G8 transgene was associated with a 40% reduction in the fractional absorption of cholesterol in wild-type mice (**Fig. 5**), as reported previously (8). The fractional absorption of cholesterol was similar in $Mdr2^{-/-}$ and control mice, consistent with the findings of Kruit et al. (14). The reduction in the fractional absorption of cholesterol associated with the expression of hG5 and hG8 was attenuated in the $Mdr2^{-/-}$ mice compared with the wild-type animals, and the reduction in the fractional absorption of plant sterols associated with G5G8 expression was largely abolished in the $Mdr2^{-/-}$ mice. Taken together, these findings indicate that the effect of G5 and G8 overexpression on plant sterol absorption is largely mediated at the level of the liver, whereas the reduction in cholesterol absorption in these animals required increased activity of G5G8 in both liver and intestine.

Plasma sterol levels in $Mdr2^{-/-}$ mice

Mice lacking MDR2 had slightly lower plasma cholesterol levels and a 2- to 2.5-fold increase in plasma sitosterol and campesterol levels compared with wild-type animals. The plasma campesterol levels were higher than those of sitosterol (**Fig. 6**), consistent with the more efficient absorption of campesterol (2). In wild-type animals, expression of the G5G8 transgene was associated with a striking reduction in plasma plant sterol levels (8). In contrast, expression of the $hG5G8$ transgene did not decrease plasma plant sterol levels in the $Mdr2^{-/-}$ animals. Because the fractional absorption of campesterol and sitosterol was similar in wild-type and $Mdr2^{-/-}$ mice, these data are consistent with the increased plasma levels of plant sterols in
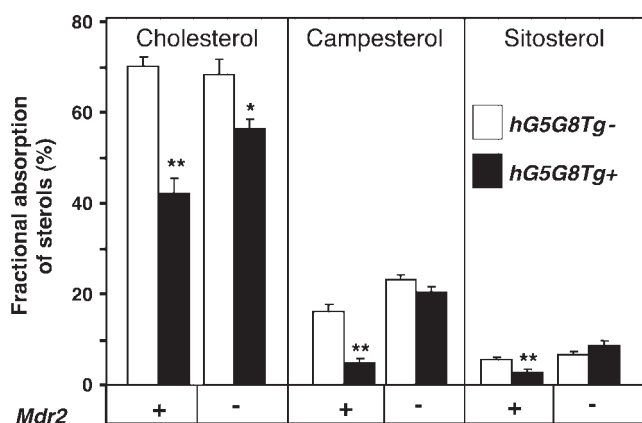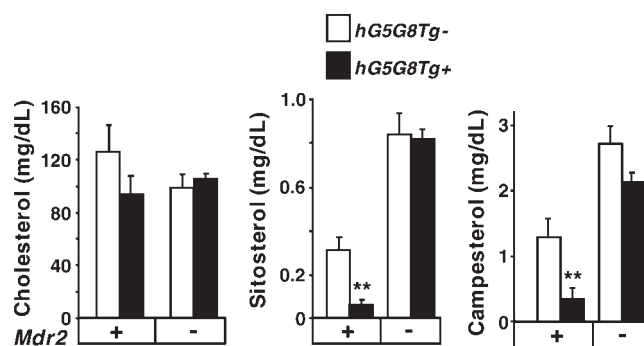

Fig. 6. Levels of plasma sterols in wild-type, $hG5G8Tg$, $Mdr2^{-/-}$, and $Mdr2^{-/-};hG5G8Tg$ male mice (n = 5). Venous blood was collected from the mice described in the legend for Fig. 5 after a 4 h fast. The plasma was isolated by centrifugation, and the sterols were measured as described in Materials and Methods. ** $P < 0.01$. Error bars represent means ± SEM.

these animals being attributable to the severely compromised ability of these mice to secrete sterols into bile.

Overexpression of hG5G8 in $Mdr2^{-/-}$ mice is not associated with increased fecal neutral sterol excretion

Feces were collected from individually housed mice for 3 days, and the levels of cholesterol and acidic sterols were measured as described previously (8). Consistent with our previous findings, the $hG5G8Tg$ mice had a 2- to 3-fold increase in fecal sterol excretion compared with the wild-type mice. Despite the marked reduction in the amount of cholesterol secreted into the bile in the $Mdr2^{-/-}$ mice, the amount of neutral sterols excreted was similar to that of wild-type animals in the absence of hG5G8 (**Fig. 7**). Moreover, expression of the $hG5G8$ transgene failed to produce any significant increase in fecal neutral sterol excretion in the $Mdr2^{-/-}$ mice (Fig. 7). Fecal bile acid excretion was slightly but not significantly increased in the h$G5G8Tg$ mice. Acidic sterol excretion was reduced by ~30% in the

Fig. 5. Fractional absorption of sterols in wild-type, $hG5G8Tg$, $Mdr2^{-/-}$, and $Mdr2^{-/-};hG5G8Tg$ male mice (n = 7). Mice of the indicated genotype were individually housed and fed a chow diet supplemented with phosphatidylcholine (13 mmol/kg). The mice were given by gavage 50 μl of a deuterated sterols-oil mixture as described in Materials and Methods. The feces were collected for 3 days after the gavage and processed as described in Materials and Methods. * $P < 0.05$; ** $P < 0.01$. Error bars represent means ± SEM.
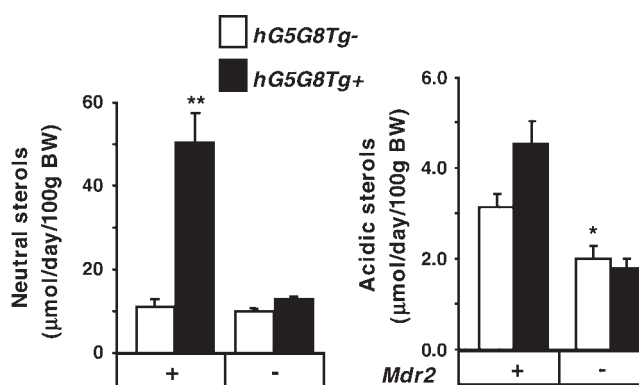

Fig. 7. Fecal excretion of the neutral and acidic sterols in wild-type, $hG5G8Tg$, $Mdr2^{-/-}$, and $Mdr2^{-/-};hG5G8Tg$ male mice (n = 5). The fecal samples were from the same mice described in the legend for Fig. 5. Fecal neutral and acidic sterols were extracted and quantified by GC, and the fecal excretion rates of the sterols were then determined and expressed as micromoles per day per 100 g of body weight (BW). * $P < 0.05$; ** $P < 0.01$. Error bars represent means ± SEM.

$Mdr2^{-/-}$ animals and did not change with expression of the $hG5G8$ transgene.

DISCUSSION

In mice with normal MDR2 activity, overexpression of G5 and G8 is associated with a marked reduction in the fractional absorption of neutral sterols, a 5-fold increase in biliary cholesterol levels, and a dramatic reduction in plasma levels of the major plant sterols campesterol and sitosterol (8). The major finding of this study is that biliary cholesterol secretion, which is almost completely abolished in $Mdr2^{-/-}$ mice, was not increased by overexpression of hG5 and hG8 and that overexpression of hG5 and hG8 in the $Mdr2^{-/-}$ mice did not decrease the fractional absorption or plasma levels of plant sterols. In mice lacking $Mdr2$, expression of the $hG5G8$ transgene leads to only a modest reduction in fractional cholesterol absorption. Taken together, these findings indicate that G5 and G8 require $Mdr2$ to influence the absorption, secretion, and plasma levels of neutral sterols.

G5 and G8 prevent the accumulation of noncholesterol sterols by limiting their absorption from the intestine and promoting their excretion in the bile (8). Increased expression of hG5 and hG8 is associated with a reduction in the fractional absorption of campesterol and sitosterol (8), but it is not known whether this decrease is a direct consequence of G5G8 action in the enterocyte, where the heterodimer promotes the efflux of newly absorbed sterols back into the intestinal lumen, or a secondary effect of increased biliary cholesterol secretion (12). Inhibition of biliary cholesterol secretion by genetic inactivation of $Mdr2$ completely prevented the decrease in plant sterol absorption in G5G8 transgenic mice, whereas the decrease in cholesterol absorption was only partly attenuated. This finding suggests that the effect of hG5G8 overexpression on plant sterol absorption is attributable primarily to increased biliary cholesterol secretion. Our results are most consistent with the increased levels of biliary cholesterol that enter the gut lumen in the $hG5G8Tg$ mice competing with the dietary plant sterols for uptake into enterocytes, resulting in a decrease in the fractional uptake of plant sterols.

It remains possible that the intestinal function of the $G5G8$ transgene is ameliorated in the $Mdr2^{-/-}$ mice. Whereas real-time PCR assays indicated that intestinal mRNA levels of hG5 and hG8 were higher in $Mdr2^{-/-}$ animals (Fig. 1), we were unable to obtain reliable immunoblots to confirm increased levels of G5 and G8 protein in the intestines of these mice (data not shown). Furthermore, $Mdr2$ deficiency may impair intestinal G5 and G8 function even in the presence of increased levels of hG5 and hG8, as observed in the liver. Therefore, further studies in mice with liver-specific overexpression of G5 and G8 will be required to confirm the conclusion that the reduced fractional sterol absorption observed in $hG5G8Tg$ mice is secondary to increased biliary cholesterol secretion.

Previous studies demonstrated that biliary cholesterol secretion is markedly impaired in $Mdr2^{-/-}$ mice (13). The results of the present study indicate that biliary cholesterol levels remain extremely low in these animals even when hG5 and hG8 are expressed at high levels. In wild-type animals, cholate administration either orally or by intravenous infusion increases biliary cholesterol secretion in wild-type or $Mdr2^{-/-}$ mice (23, 24). However, we found no increase in the levels of biliary cholesterol in $Mdr2^{-/-}$ mice expressing the human G5G8 transgene, confirming the requirement of a functional MDR2 for G5G8-mediated secretion of cholesterol into bile.

A paradoxical finding in $Mdr2^{-/-}$ mice was that fecal neutral sterol excretion is normal despite a marked reduction in the amount of cholesterol secreted into the bile of these animals. These results are similar to those recently reported by Kruit et al. (14), who found that activation of the liver X receptor was associated with an increase in fecal neutral sterol excretion in $Mdr2^{-/-}$ mice, despite the absence of any change in biliary cholesterol secretion rates. Kruit and colleagues (14) suggested that direct excretion of cholesterol from the intestine may constitute an important pathway for ridding the body of cholesterol. In contrast to the finding of no significant reduction in fecal sterol excretion in $Mdr2^{-/-}$ mice compared with wild-type mice, fecal neutral sterol excretion was markedly reduced in mice lacking G5 and G8 (2), which also have very low levels of biliary cholesterol. Moreover, treatment of the $G5G8^{-/-}$ mice with a potent liver X receptor agonist was not associated with an increase in fecal sterol excretion (25). Thus, the increased fecal neutral sterol excretion in the liver X receptor agonist-treated $Mdr2^{-/-}$ mice may be mediated by the action of G5 and G8 in the intestine. Further studies will be required to examine this interesting possibility.⬛⬛

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