

Ezetimibe normalizes metabolic defects in mice lacking ABCG5 and ABCG8

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Abstract The ATP binding cassette transporters ABCG5 (G5) and ABCG8 (G8) limit the accumulation of neutral sterols by restricting sterol uptake from the intestine and promoting sterol excretion into bile. Humans and mice lacking G5 and G8 (*G5G8*^{-/-}) accumulate plant sterols in the blood and tissues. However, despite impaired biliary cholesterol secretion, plasma and liver cholesterol levels are lower in *G5G8*^{-/-} mice than in wild-type littermates. To determine whether the observed changes in hepatic sterol metabolism were a direct result of decreased biliary sterol secretion or a metabolic consequence of the accumulation of dietary non-cholesterol sterols, we treated *G5G8*^{-/-} mice with ezetimibe, a drug that reduces the absorption of both plant- and animal-derived sterols. Ezetimibe feeding for 1 month sharply decreased sterol absorption and plasma levels of sitosterol and campesterol but increased cholesterol in both the plasma (from 60.4 to 75.2 mg/dl) and the liver (from 1.1 to 1.87 mg/g) of the ezetimibe-treated *G5G8*^{-/-} mice. Paradoxically, the increase in hepatic cholesterol was associated with an increase in mRNA levels of HMG-CoA reductase and synthase. **¶** Together, these results indicate that pharmacological blockade of sterol absorption can ameliorate the deleterious metabolic effects of plant sterols even in the absence of G5 and G8.—Yu, L., K. von Bergmann, D. Lütjohann, H. H. Hobbs, and J. C. Cohen. **Ezetimibe normalizes metabolic defects in mice lacking ABCG5 and ABCG8.** *J. Lipid Res.* 2005. 46: 1739–1744.

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Mutations in the ATP binding cassette transporters ABCG5 (G5) and ABCG8 (G8) cause sitosterolemia (1, 2), an autosomal recessive genetic disorder characterized by increased plasma and tissue levels of plant sterols. Patients with sitosterolemia have an increased fractional absorption of dietary sterols and an impaired ability to secrete

cholesterol and plant-derived sterols into bile (3–6). Plasma cholesterol levels are highly variable in sitosterolemic individuals, ranging from below normal to severely elevated (7, 8). Many sitosterolemic individuals deposit cholesterol and plant sterols in the skin, tendons, and coronary arteries and suffer premature coronary heart disease (9–13). The dysregulation of sterol metabolism in sitosterolemia is associated with a distinctive pattern of response to different classes of lipid-lowering agents. Sitosterolemic individuals respond poorly to HMG-CoA reductase inhibitors (statins) but exhibit exuberant responses to dietary cholesterol restriction and to bile acid sequestrants (8, 14–16), which interrupt the enterohepatic recirculation of bile acids.

The hallmark of sitosterolemia is increased plasma levels of sitosterol, the most abundant dietary plant sterol. Other sterols, including plant-derived campesterol and stigmasterol and shellfish-derived brassicasterol, also accumulate in this disorder (3, 17). In normal individuals, these sterols are poorly absorbed and preferentially secreted into bile (18–22) and thus constitute <1% of circulating and tissue sterols. In contrast, noncholesterol sterols constitute 15% of tissue sterols in sitosterolemic subjects (9).

It is not known whether the abnormalities in cholesterol metabolism in sitosterolemia are a direct consequence of abnormal cholesterol trafficking or a secondary effect of plant sterol accumulation in tissues. Recently, we showed that the accumulation of plant sterols in mice lacking G5 and G8 (*G5G8*^{-/-}) was associated with abnormal cholesterol homeostasis in the adrenal gland, an organ that does not express G5 or G8 (23). Cholesterol concentrations were reduced by ~90% in the adrenals of *G5G8*^{-/-} mice compared with wild-type littermates, yet no counterregu-

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latory increases in the expression of the LDL receptor and the enzymes of cholesterol biosynthesis were observed. Treatment of the $G5G8^{-/-}$ mice with ezetimibe, a drug that inhibits sterol absorption, resulted in a decrease in the levels of plant sterols and an increase in the level of cholesterol in the adrenal glands, suggesting that the accumulation of noncholesterol sterols may contribute to dysregulation of cholesterol metabolism in the $G5G8^{-/-}$ mice.

To determine whether the accumulation of noncholesterol sterols contributes to impaired cholesterol homeostasis in the liver and circulation of $G5G8^{-/-}$ mice, we examined the effects of ezetimibe treatment on hepatic and circulating cholesterol levels in these animals.

MATERIALS AND METHODS

Materials

Sterols were from Sigma-Aldrich (St. Louis, MO) or Steraloids, Inc. (Newport, RI). Deuterated [26,26,26,27,27,27- 2H_6]cholesterol and [2,2,4,4,6- 2H_5]campesterol-sitosterol (40:60) were purchased from Medical Isotope, Inc. Deuterated cholestanol, campestanol, and sitostanol were synthesized by hydrogenation of the deuterated sterols using microwaves (24).

Animals and diets

Mice homozygous for disruption of *Abcg5* and *Abcg8* ($G5G8^{-/-}$) were generated as described (25). The mice used in these studies were offspring of $G5G8^{+/-}$ mice of mixed genetic background (129S6/SvEv \times C57BL/6J) and were housed in plastic cages in a temperature-controlled room (22°C) with a daylight cycle from 6 AM to 6 PM. The mice were fed ad libitum a cereal-based rodent chow diet (Diet 7001; Harlan Teklad, Madison, WI) containing 0.02% cholesterol and 4% fat. All animal procedures were performed with the approval of the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center.

Ezetimibe treatment

Diets containing 0.005% (w/w) ezetimibe (kindly provided by Drs. Stephen Turley and John Dietschy at the University of Texas Southwestern Medical Center) were prepared by mixing powdered chow diet (Diet 7001; Harlan Teklad) with ezetimibe and stored at 4°C. Female mice were housed individually for 1 week before initiation of the ezetimibe diet and then fed for 1 month with either the ezetimibe diet or the chow diet dispensed from a feeder jar.

Plasma, bile, and tissue collection

Blood samples were collected from the retro-orbital plexus before the initiation of ezetimibe treatment and at day 15. At day 30, mice were anesthetized and killed by exsanguination from the vena cava. The plasma was isolated by centrifugation (4,000 rpm for 10 min). Bile was collected from the gallbladders of anesthetized mice using a 30 gauge needle. Livers and kidneys were excised, rinsed in saline, dried on Whatman papers, and frozen in liquid nitrogen for lipid analysis. Total RNAs were extracted from livers using the RNA Stat-60 kit (Tel-Test, Inc.), and real-time PCR was performed as described (25, 26).

Sterol chemistries

Sterol levels in plasma, bile, liver, and kidney were measured by GC-MS as described previously (21, 27, 28).

Determination of dietary sterol absorption and fecal cholesterol excretion

A mixture containing 4 mg of [26,26,26,27,27,27- 2H_6]cholesterol, 4 mg of [26,26,26,27,27,27- 2H_6]cholestanol, 10 mg of [2,2,4,4,6- 2H_5]campesterol-sitosterol (40:60), and 4 mg of [2,2,4,4,6- 2H_5]sitostanol was prepared (25) and dissolved in 2 ml of plant oil (Livio; Union Deutsche Lebensmittelwerke, Hamburg, Germany). A single dose of the deuterated sterol/stanol oil mixture (50 μ l) was gavaged into the stomach of each mouse. Feces were collected for 3 days after the gavage, pooled, dried in a Speedvac Concentrator (Savant Instruments, Inc., Farmingdale, NY), and homogenized using a mortar. Two milliliters of chloroform-methanol (2:1, v/v), 50 μ g of 5 α -cholestane, and 1 μ g of epicoprostanol were added to a 50 mg aliquot of stool from each mouse. After vortexing and centrifugation at 3,000 g, 1 ml of the supernatant was collected and the solvents were evaporated. The residual lipids were hydrolyzed for 1 h at 62°C after the addition of 1 ml of 90% ethanol and 1 N NaOH. One milliliter of double-distilled water was added, and the sterols and stanols were extracted twice with 4 ml of cyclohexane. The combined organic phases were evaporated, and the residual sterols and stanols were dissolved in 100 μ l of *n*-decane, silylated by the addition of 40 μ l of pyridine-hexamethyldisilazan-trimethylchlorosilane (9:3:1, v/v/v), and incubated at 65°C for 1 h.

Sterols and stanols were separated by GC on a 30 m DB-XLB capillary column (J&W Scientific, Folsom, CA) using a 250 μ m film of cross-linked methyl silicone. Selected ion monitoring was performed at *m/z* 376 for [2H_6]coprostanol, *m/z* 464 for [2H_6]cholesterol, *m/z* 477 for [2H_5]campesterol, *m/z* 491 for [2H_5]sitosterol, *m/z* 466 for [2H_6]cholestanol, and *m/z* 493 for [2H_5]sitostanol. The fractional absorption of cholesterol (including coprostanol and coprostanone), cholestanol, campesterol, and sitosterol was calculated as described previously (6, 25, 29). Fecal cholesterol excretion rate was calculated from total fecal content of cholesterol (including coprostanol and coprostanone) in each sample and expressed as micromoles per day per 100 g of body weight.

Statistical analysis

All data are reported as means \pm SEM. The differences between the mean values of control and treated groups were tested for statistical significance by two-tailed Student's *t*-tests.

RESULTS

Ezetimibe inhibits dietary sterol absorption in $G5G8^{-/-}$ mice

To determine whether the absence of G5 and G8 affected the magnitude of reduction in sterol absorption associated with ezetimibe treatment, we measured the fractional absorption of cholesterol, cholestanol, campesterol, and sitosterol in $G5G8^{-/-}$ mice after 1 month of treatment with ezetimibe. As expected, ezetimibe dramatically decreased the fractional absorption of these sterols in both wild-type and $G5G8^{-/-}$ mice (Fig. 1). Thus, G5 and G8 are not required for ezetimibe-mediated inhibition of dietary sterol absorption.

Ezetimibe increases fecal cholesterol excretion independent of G5 and G8

To determine whether the decreased dietary cholesterol absorption in mice fed ezetimibe results in increased

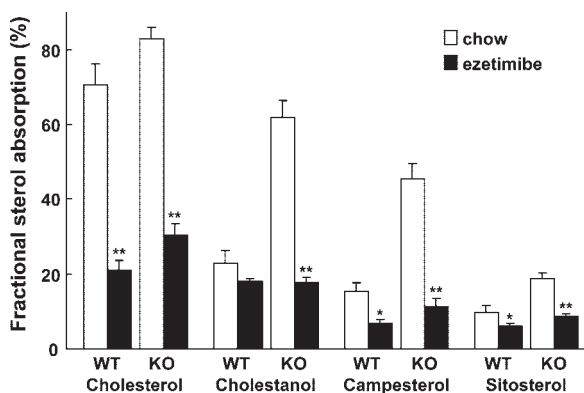


Fig. 1. Fractional absorption of dietary sterols in chow-fed and ezetimibe-fed mice. Three to 4 month old female wild-type and ABCG5- and ABCG8-deficient ($G5G8^{-/-}$) mice ($n = 6$ per group) were housed individually and fed chow diet or chow diet plus 0.005% ezetimibe for 27 days before administration by gavage of 50 μ l of oil containing deuterated cholesterol, cholestanol, campesterol, sitosterol, and sitostanol. Feces were then collected for 3 days with continuous ezetimibe feeding. The deuterated sterol amounts in fecal samples were determined by GC-MS as described in Materials and Methods. Deuterated sitostanol was used as a nonabsorbable fecal marker by which the fractional absorption of the other deuterated sterols was calculated. WT, wild-type mice; KO, $G5G8^{-/-}$ mice. Values shown are means \pm SEM. * $P < 0.05$, ** $P < 0.001$, chow versus ezetimibe. The fractional absorption of cholesterol was repeated using a different set of animals, and similar results were obtained.

fecal cholesterol excretion, feces were collected for the last 3 days of ezetimibe treatment and the cholesterol content of the pooled samples was measured. Cholesterol excretion rates were higher in both wild-type and $G5G8^{-/-}$ mice after ezetimibe feeding (Fig. 2).

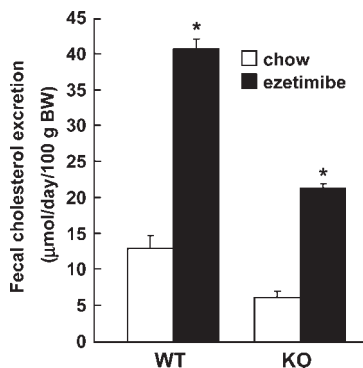


Fig. 2. Fecal cholesterol excretion rates in mice on chow and ezetimibe diets. The same fecal samples described for Fig. 1 were used for the determination of fecal cholesterol content. After saponification, fecal total sterols were extracted and subjected to GC-MS analysis as described in Materials and Methods. Fecal cholesterol including coprostanol and coprostanone were calculated using 5 α -cholestanol as an internal quantitative recovery standard and expressed as micromoles per day per 100 g of body weight (BW). WT, wild-type mice; KO, $G5G8^{-/-}$ mice. Values shown are means \pm SEM. * $P < 0.0001$, chow versus ezetimibe. Similar results were observed in a separate experiment using different animals.

Ezetimibe reduces plasma plant sterol levels and increases plasma cholesterol levels in $G5G8^{-/-}$ mice

Ezetimibe treatment markedly reduced plasma concentrations of campesterol and sitosterol ($\sim 60\%$ at day 15 and $\sim 75\%$ at day 30) in the ezetimibe-treated $G5G8^{-/-}$ mice (Fig. 3A). Plasma levels of plant stanols (campestanol and sitostanol) were also greatly increased in $G5G8^{-/-}$ mice and were reduced by ezetimibe (data not shown). A similar percentage reduction in plasma plant sterol levels was observed in wild-type mice, although the absolute change was much smaller.

Plasma cholesterol levels were significantly lower in the chow-fed $G5G8^{-/-}$ mice than in the chow-fed wild-type mice (Fig. 3B), consistent with our previous data (21, 25).

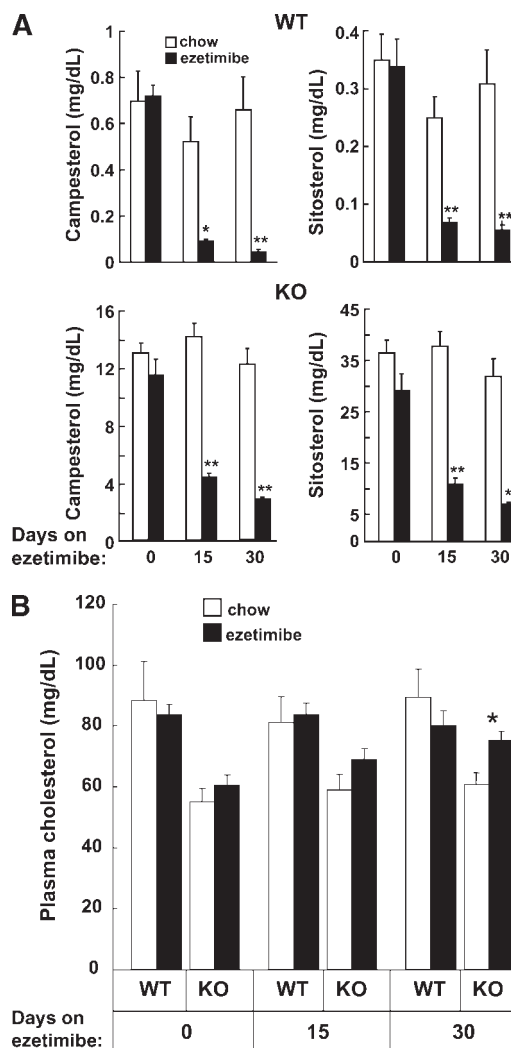


Fig. 3. Plasma levels of plant sterols (A) and cholesterol (B) in chow-fed and ezetimibe-fed mice. Three to 4 month old female wild-type and $G5G8^{-/-}$ mice were housed individually and fed chow diet or chow diet plus 0.005% ezetimibe for 1 month. Plasma samples were obtained from each group ($n = 6$) at the indicated time points and analyzed for sterol levels as described in Materials and Methods. WT, wild-type mice; KO, $G5G8^{-/-}$ mice. Values shown are means \pm SEM. * $P < 0.005$, ** $P < 0.001$, chow versus ezetimibe. Identical results were achieved in a separate experiment with a different set of mice.

Ezetimibe treatment resulted in a progressive increase in plasma cholesterol levels in the $G5G8^{-/-}$ mice (from 60.4 mg/dl at day 0 to 69.1 mg/dl at day 15 to 75.2 mg/dl at day 30), but no significant changes were observed in the wild-type mice (Fig. 3B).

Ezetimibe reduces plant sterol levels and increases hepatic and biliary levels of cholesterol in $G5G8^{-/-}$ mice

Ezetimibe treatment significantly reduced the levels of sitosterol and campesterol in the livers and kidneys of wild-type and $G5G8^{-/-}$ mice (Fig. 4A, B). The levels of cholesterol in the livers and kidneys of wild-type mice were not affected by ezetimibe treatment, whereas those of $G5G8^{-/-}$ mice were increased significantly in both organs (Fig. 4A, B).

To determine the effect of ezetimibe on biliary cholesterol levels, we measured the level of cholesterol in bile obtained from the gallbladders of wild-type and $G5G8^{-/-}$ mice (Fig. 5). The biliary cholesterol levels increased by 136% (11 vs. 26 mg/dl) with ezetimibe treatment in the

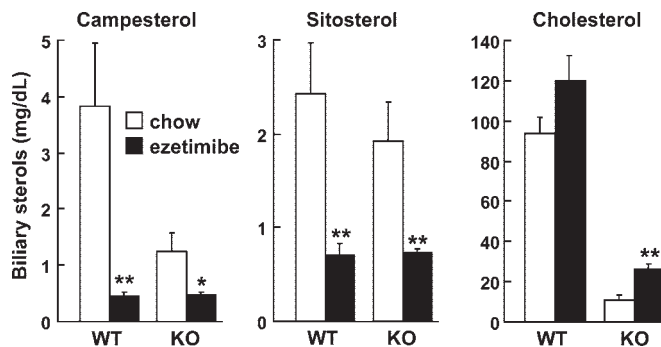


Fig. 5. Gallbladder cholesterol concentrations in mice on chow or ezetimibe diet. Gallbladder bile was collected with 30 gauge needles from the mice described for Fig. 2 and analyzed for sterol concentrations using GC-MS as described in Materials and Methods. WT, wild-type mice; KO, $G5G8^{-/-}$ mice. Values shown are means \pm SEM. * $P < 0.05$, ** $P < 0.01$, chow versus ezetimibe. Similar results were found with the mice described for Fig. 3.

$G5G8^{-/-}$ mice. A mild increase in biliary cholesterol levels was also seen in the ezetimibe-treated wild-type mice (Fig. 5). The biliary concentrations of both sitosterol and campesterol decreased in both wild-type and $G5G8^{-/-}$ mice after ezetimibe treatment (Fig. 5), presumably reflecting the depletion of these sterols in the liver.

Ezetimibe treatment results in increased levels of mRNAs encoding enzymes in the cholesterol biosynthetic pathway

Hepatic expression of enzymes in the cholesterol biosynthetic pathway is suppressed in $G5G8^{-/-}$ mice (21). To determine whether ezetimibe treatment ameliorated the suppression of these enzymes, we examined the levels of the mRNAs encoding HMG-CoA reductase, HMG-CoA synthase, and sterol-regulatory element binding protein 2 (SREBP-2) (21). Ezetimibe treatment of $G5G8^{-/-}$ mice increased the mRNA levels of each of these transcripts to levels observed in wild-type animals (Fig. 6A). A modest increase in the levels of these same mRNAs was also seen in the wild-type mice after ezetimibe treatment, similar to a previous report (30). Hepatic levels of desmosterol, a precursor sterol in the cholesterol biosynthetic pathway, were increased by ezetimibe treatment (Fig. 6B), consistent with increased cholesterol synthesis in this organ.

DISCUSSION

The major finding of this study is that the suppression of hepatic cholesterol synthesis in $G5G8^{-/-}$ mice is largely ameliorated by blocking the intestinal absorption of sterols with ezetimibe. Treatment with a specific inhibitor of sterol absorption for 1 month resulted in a remarkable decline in plasma and tissue levels of plant sterols and a substantial correction in plasma and tissue levels of cholesterol in the $G5G8^{-/-}$ animals. The suppression of hepatic mRNA levels of cholesterol synthetic genes was also normalized by ezetimibe treatment. These findings indicate

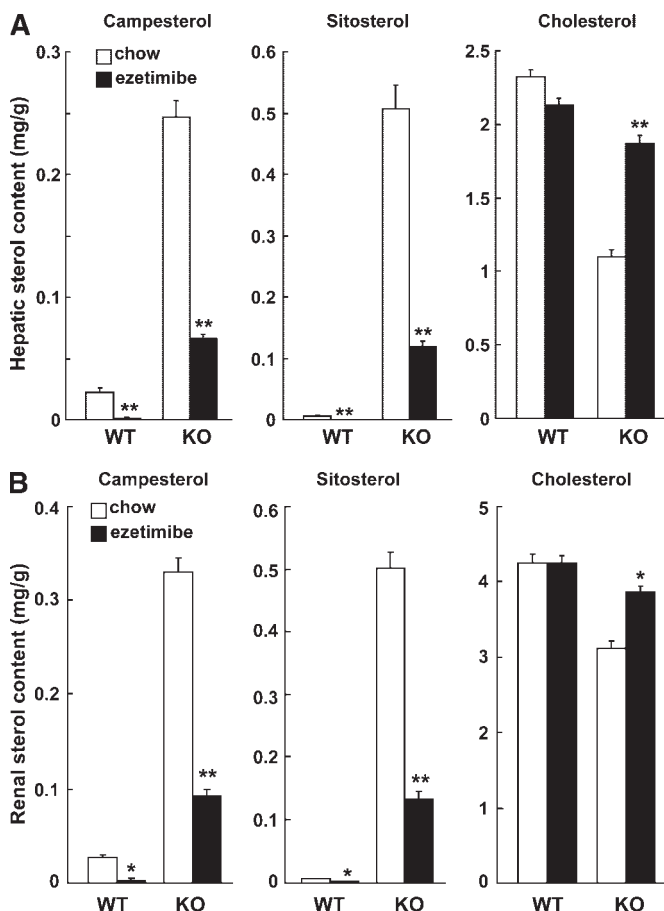


Fig. 4. Sterol contents in livers (A) and kidneys (B) of chow-fed and ezetimibe-fed mice. The same mice described for Fig. 2 were killed, and their livers and kidneys were harvested for the determination of sterol contents as described in Materials and Methods. WT, wild-type mice; KO, $G5G8^{-/-}$ mice. Values shown are means \pm SEM. * $P < 0.001$, chow versus ezetimibe. The experiment was repeated in a second set of mice, and similar results were obtained.

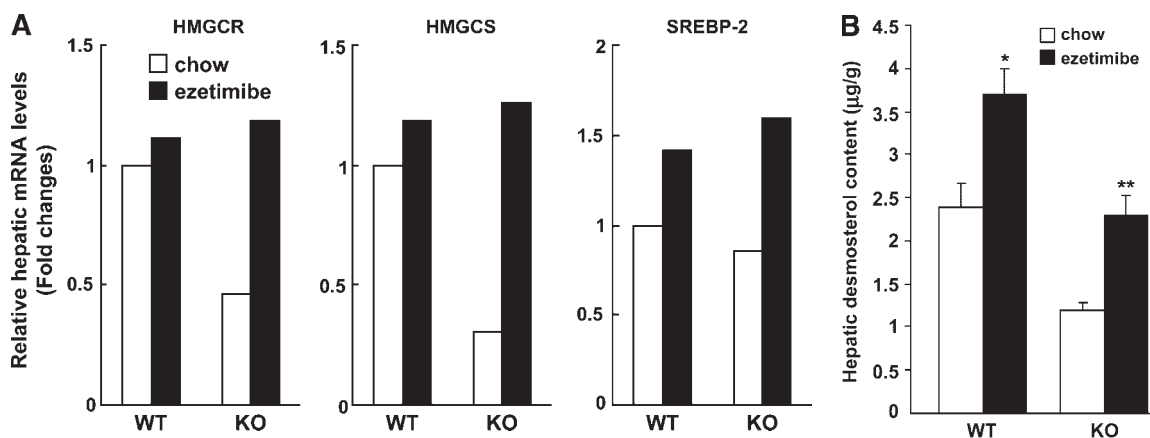


Fig. 6. Hepatic mRNA levels (A) and desmosterol content (B) in mice fed chow or ezetimibe. Five to 6 month old female wild-type and $G5G8^{-/-}$ mice were housed individually and fed chow diet or chow diet plus 0.005% ezetimibe for 1 month. The mice were then killed, and the livers were harvested and snap-frozen in liquid nitrogen. Total RNAs were extracted from liver samples, and an equal amount of total RNA from each animal in each group ($n = 6$) was pooled and used for quantitative real-time PCR as described in Materials and Methods. The experiment was repeated twice with different sets of animals, and similar results were observed. Hepatic desmosterol content was determined as described in Materials and Methods. WT, wild-type mice; KO, $G5G8^{-/-}$ mice; HMGCR, HMG-CoA reductase; HMGCS, HMG-CoA synthase; SREBP-2, sterol-regulatory element binding protein. Values shown are means \pm SEM. * $P < 0.01$, ** $P < 0.001$, chow versus ezetimibe.

that the accumulation of noncholesterol sterols disrupts cholesterol homeostasis in the liver, as well as in other tissues of $G5G8^{-/-}$ mice, and that high tissue levels of these sterols can be effectively reduced by blocking sterol absorption from the intestine, despite the markedly reduced rate of biliary secretion of sterols in these animals.

The plasma and liver levels of cholesterol are reduced by $\sim 50\%$ in the $G5G8^{-/-}$ mice (25). The low tissue levels of cholesterol in these animals are associated with reduced cholesterol synthesis: hepatic mRNA levels for multiple enzymes in the cholesterol biosynthetic pathway are reduced by $\sim 50\%$ compared with those in wild-type mice (21). Sitosterolemia is also associated with a marked reduction in cholesterol synthesis in humans (6, 31), resulting in a blunted response to statin drugs in these patients (15, 16). Recent studies of the adrenal glands of $G5G8^{-/-}$ animals indicate that certain plant-derived sterols, including stigmasterol, suppress cholesterol synthesis by inhibiting the SREBP pathway (23). The present results extend these findings to the liver and demonstrate that the accumulation of noncholesterol sterols, rather than failure to secrete cholesterol, is responsible for the suppression of cholesterol synthesis and low cholesterol levels in this organ.

Treatment with ezetimibe reduces plasma levels of plant sterols in sitosterolemia (32). This study demonstrates that ezetimibe treatment also reduces noncholesterol sterol levels in tissues, even in the absence of G5 and G8 expression. These data provide further evidence that cholesterol and plant sterols enter the enterocyte via a common pathway and that blockade of this pathway not only prevents the further accumulation of noncholesterol sterols in tissues but promotes the depletion of the noncholesterol sterols already deposited in the tissues of $G5G8^{-/-}$ mice. Excretion of these sterols is achieved at least in part by secretion into the bile: although the efficiency of biliary

sterol secretion is greatly decreased, significant amounts of both sitosterol and campesterol are present in the gallbladder bile of $G5G8^{-/-}$ animals.

Ezetimibe treatment for 1 month significantly increased biliary cholesterol concentrations in $G5G8^{-/-}$ mice, but the levels of biliary cholesterol remained far lower than in the wild-type littermates (Fig. 5). The finding that biliary cholesterol secretion remained low in ezetimibe-treated $G5G8^{-/-}$ mice even after plasma and liver cholesterol levels returned to normal suggests that the reduced biliary cholesterol secretion in these animals is a direct effect of $G5G8$ deficiency and is not secondary to the reduced availability of cholesterol in the liver.

In contrast to humans, in which sitosterolemia is associated with severe atherosclerosis in some individuals, no evidence of an increased susceptibility to atherosclerosis was found in the $G5G8^{-/-}$ mice despite the dramatic increase in plant sterol levels in these animals (33). Consequently, it is difficult to evaluate the effects of ezetimibe on the clinical consequences of sitosterolemia in this mouse model. Nevertheless, the finding that ezetimibe reduces tissue levels of plant sterols and normalizes most of the metabolic defects associated with $G5G8$ deficiency suggests that this drug represents the optimal therapeutic intervention for the treatment of this potentially devastating inherited disease. **FIG 6**

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