Hepatic ABCG5/G8 overexpression reduces apoB-lipoproteins and atherosclerosis when cholesterol absorption is inhibited

Federica Basso,* Lita A. Freeman,[1,*] Carol Ko,* Charles Joyce,* Marcelo J. Amar,* Robert D. Shamburek,* Terese Tansey,* Fairwell Thomas,* Justina Wu,* Beverly Paigen,[†] Alan T. Remaley,* Silvia Santamarina-Fojo,* and H. Bryan Brewer, Jr.[§]

Molecular Disease Section,* National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD; Jackson Laboratory,[†] Bar Harbor, ME; and Cardiovascular Research Institute,[§] Washington Hospital Center, Washington, DC

Abstract **We previously reported that liver-specific overexpression of ABCG5/G8 in mice is not atheroprotective, suggesting that increased biliary cholesterol secretion must be coupled with decreased intestinal cholesterol absorption to increase net sterol loss from the body and reduce atherosclerosis. To evaluate this hypothesis, we fed low density lipoprotein receptor-knockout (LDLr-KO) control and ABCG5/G8-transgenic (ABCG5/G8-Tg)×LDLr-KO mice, which overexpress ABCG5/G8 only in liver, a Western diet containing ezetimibe to reduce intestinal cholesterol absorption. On this dietary regimen, liver-specific ABCG5/G8 overexpression increased hepatobiliary cholesterol concentration and secretion rates (1.5-fold and 1.9-fold, respectively), resulting in 1.6-fold increased fecal cholesterol excretion, decreased hepatic cholesterol, and increased (4.4-fold) de novo hepatic cholesterol synthesis versus LDLr-KO mice. Plasma lipids decreased (total cholesterol, 32%; cholesteryl ester, 32%; free cholesterol, 30%), mostly as a result of reduced non-high density lipoprotein-cholesterol and apolipoprotein B (apoB; 36% and 25%, respectively). ApoB-containing lipoproteins were smaller and lipid-depleted in ABCG5/G8-Tg×LDLr-KO mice. Kinetic studies revealed similar ^{125}I-apoB intermediate density lipoprotein/LDL fractional catabolic rates, but apoB production rates were decreased 37% in ABCG5/G8-Tg×LDLr-KO mice. Proximal aortic atherosclerosis decreased by 52% (male) and 59% (female) in ABCG5/G8-Tg×LDLr-KO versus LDLr-KO mice fed the Western/ezetimibe diet. Thus, increased biliary secretion, resulting from hepatic ABCG5/G8 overexpression, reduces atherogenic risk in LDLr-KO mice fed a Western diet containing ezetimibe. These findings identify distinct roles for liver and intestinal ABCG5/G8 in modulating sterol metabolism and atherosclerosis.**—Basso, F., L. A. Freeman, C. Ko, C. Joyce, M. J. Amar, R. D. Shamburek, T. Tansey, F. Thomas, J. Wu, B. Paigen, A. T. Remaley, S. Santamarina-Fojo, and H. B. Brewer, Jr. **Hepatic ABCG5/G8 overexpression reduces apoB-lipoproteins and atherosclerosis when cholesterol absorption is inhibited.** ***J. Lipid Res.*** **2007.** 48: **114–126.**

Manuscript received 7 August 2006 and in revised form 4 October 2006.

Published, JLR Papers in Press, October 23, 2006.
DOI 10.1194/jlr.M600353-JLR200

Supplementary key words ABC transporters • ABCG subfamily • ezetimibe • non-HDL-cholesterol • biliary sterol secretion • hepatic cholesterol homeostasis • ApoB Kinetics

ABCG5 and ABCG8 (ABCG5/G8) are ABCG half-transporters with a key role in excreting cholesterol and other sterols from the body. ABCG5/G8 were recently identified as the genes defective in sitosterolemia (1–3), a rare genetic disorder characterized by increased plasma and tissue levels of cholesterol and other neutral sterols. The accumulation of sterols in these patients is attributable to both increased fractional intestinal absorption and decreased biliary secretion of neutral sterol and is associated with the development of xanthomas and premature coronary artery disease (4–12). In mice, ABCG5/G8 deficiency increases plasma sitosterol and decreases bile cholesterol (13), whereas ABCG5/G8 overexpression increases biliary cholesterol excretion and decreases dietary cholesterol absorption (14). ABCG5/G8 are expressed mainly in liver (15–18), where they function as heterodimers at the apical membranes of hepatocytes (19, 20), and in small intestine (3, 15–18), where they have been localized to intestinal microvilli in the gut lumen (20). ABCG5/G8 are also expressed to a lesser degree in the colon and gallbladder (21). Sitosterolemia in ABCG5-deficient mice is aggravated on activation of the liver X receptor (22). Thus, by secreting excess sterols from hepatocytes via the biliary system into the gut lumen, and from enterocytes into the

Abbreviations: ABCG5/G8-Tg, ABCG5/G8-transgenic; apoB, apolipoprotein B; apoB-Lp, apolipoprotein B-containing lipoprotein; apoE-KO, apolipoprotein E-knockout; CE, cholesteryl ester; FC, free cholesterol; FCR, fractional catabolic rate; FPLC, fast-protein liquid chromatography; HDL-C, high density lipoprotein-cholesterol; IDL, intermediate density lipoprotein; LDLr-KO, low density lipoprotein receptor-knockout; LRP, LDL receptor-related protein; NPC1L1, Niemann-Pick C1-Like 1; TC, total cholesterol; TG, triglyceride.

[1] To whom correspondence should be addressed.
e-mail: litaf@mail.nih.gov

gut lumen, hepatic and intestinal ABCG5/G8 appear to act in concert to excrete excess sterols from the body.

Until recently, it was difficult to uncouple the relative influence of hepatic versus intestinal ABCG5/G8 on sterol excretion. The generation of a transgenic mouse model overexpressing human ABCG5/G8 only in liver and not in the intestine provided a useful system in which to investigate the relative importance of ABCG5/G8 in these two tissues. Liver-specific overexpression of ABCG5/G8 increased biliary secretion of cholesterol and plant sterols and reduced plasma plant sterol levels. However, hepatic ABCG5/G8 overexpression did not alter the fractional intestinal cholesterol absorption (23). Because most of the biliary cholesterol secreted into the intestine by hepatic ABCG5/G8 was reabsorbed and transferred back to the liver, the increased output of liver cholesterol via the bile did not induce a net loss of hepatic cholesterol in the liver of transgenic mice. Hepatic overexpression of ABCG5/G8, therefore, did not alter the plasma levels of cholesterol or apolipoprotein B-containing lipoproteins (apoB-Lps). Consequently, overexpression of ABCG5/G8 only in liver did not alter the development of aortic lesions in either low density lipoprotein receptor-knockout (LDLr-KO) or apolipoprotein E-knockout (apoE-KO) mice (23). In contrast, overexpression of human ABCG5 and ABCG8 in both liver and intestine was found to protect against diet-induced hypercholesterolemia and the development of aortic lesions in LDLr-KO mice (24). Although this difference has been suggested to be a function of the levels of ABCG5/G8 expression in the two independent mouse models, it is more likely that not only increased biliary cholesterol secretion but also decreased intestinal absorption are required for hepatic ABCG5/G8 to decrease liver cholesterol concentrations and to trigger the compensatory mechanisms that reduce plasma levels of proatherogenic apoB-Lps and atherosclerosis.

In this study, we provide direct evidence to support this hypothesis. We used ezetimibe, which blocks intestinal cholesterol absorption through the Niemann-Pick C1-Like 1 (NPC1L1) protein (25, 26), to decrease the reabsorption of cholesterol secreted from liver into bile of ABCG5/G8-transgenic (ABCG5/G8-Tg)×LDLr-KO versus LDLr-KO mice fed a Western diet. In the presence of ezetimibe, hepatic ABCG5/G8 expression increased cholesterol excretion from the body of ABCG5/G8-Tg×LDLr-KO mice compared with LDLr-KO mice, decreased plasma apoB-Lp levels by reducing apoB-Lp synthesis, and thus reduced atherosclerosis. These data, in conjunction with our previous results (23), demonstrate that a reduction in the amount of cholesterol reabsorbed by the intestine is required for moderate increases in hepatic ABCG5/G8 expression to decrease liver cholesterol concentration and to reduce plasma levels of the proatherogenic apoB-Lps and atherosclerosis.

MATERIALS AND METHODS

Animals and diets

LDLr-KO mice overexpressing the human ABCG5 and ABCG8 genes only in liver were generated as described previously (23). We refer to these mice, previously termed Liver-G5/G8-Tg mice, as ABCG5/G8-Tg×LDLr-KO mice. Control LDLr-KO and transgenic ABCG5/G8-Tg×LDLr-KO mice (2–5 months of age) were fed a Western diet (TD 88137; Harlan Teklad, Madison, WI) (0.2% cholesterol and 21.2% fat, derived from anhydrous milkfat) supplemented with 15 mg/kg/day ezetimibe. Animals were maintained on the Western plus ezetimibe diet for 24 weeks before euthanasia. Blood sampling for lipid analyses was performed every 2 weeks. Food intake was measured in a representative group of four mice for each study group. All experiments were performed according to a research protocol approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute, National Institutes of Health.

RNA isolation and Northern blot analysis

Total RNA was isolated (TRIzol; Invitrogen, Carlsbad, CA) from age- and sex-matched mice according to the manufacturer's instructions. RNA (20–30 μg) was subjected to Northern analysis as described (23).

Analysis of plasma lipids

Plasma was obtained after a 4 h fast, and lipid levels were determined as described previously (27, 28). High density lipoprotein-cholesterol (HDL-C) was determined as the cholesterol remaining in the plasma after precipitation of the apoB-Lps with dextran sulfate (Bayer Corp., Tarrytown, NJ).

Western blot analysis

Plasma levels of apoA-I and apoB were quantified by densitometric scanning after electrophoresis on NuPAGE 4–12% Bis-Tris acrylamide gels (Invitrogen), transfer onto Immobilon-P PVDF membranes (Millipore, Bedford, MA), and incubation with rabbit anti-mouse apoB and apoA-I polyclonal antibodies (Biodesign International, Saco, ME) followed by the secondary antibody (donkey anti-rabbit antibody) conjugated to horseradish peroxidase (Amersham, Piscataway, NJ), as described previously (23).

Fast-protein liquid chromatography and native agarose gel electrophoresis

Plasma lipoproteins were analyzed by native agarose gel electrophoresis and fast-protein liquid chromatography (FPLC; 50 μl of plasma pooled from four mice), as described previously (29).

Bile cannulation and bile assays

Mice were weighed, fasted for 4–6 h, and anesthetized with intraperitoneal avertin (1.25%; 0.015–0.017 ml/g body weight) at the middle of the dark cycle. The cystic duct, exposed by an upper midline abdominal incision, was clamped, and the common bile duct was cannulated with a PE-10 polyethylene catheter, as described previously (23). Bile was collected for 1 h by gravity. Animals were maintained at 37°C with a heat lamp. Total bile was extracted with chloroform-methanol (2:1), and biliary cholesterol was analyzed by GC as described (23).

Cholesterol absorption

Cholesterol absorption was assessed by the plasma dual-isotope ratio method as described (23).

Hepatic sterol and fecal neutral sterol content

Approximately 100 mg (wet weight) of liver and 50 mg (dry weight) of ground stool were homogenized in 1 ml of water.

Lipids were extracted by adding 21 ml of chloroform-methanol (2:1) and 4.5 ml of water (30), and cholesterol was measured by GC (Shimadzu, Columbia, MD). Fecal excretion of cholesterol and its breakdown products was calculated by adding coprostanol, cholestanol, and cholesterol measured in feces by GC (Shimadzu). The GC measurements were confirmed by a modified enzymatic method (31).

Cholesterol synthesis

Cholesterol synthetic rates were determined by intraperitoneal injection of mice with 10 mCi of [^{3}H]water during the middle of the dark cycle, as described previously (32, 33).

ApoB metabolic studies

Purified mouse intermediate density lipoprotein (IDL)/LDL were collected after ultracentrifugation at a density of 1.019–1.063 g/ml. Apolipoproteins were labeled with ^{125}I using the iodine monochloride method (34). The integrity of the radiolabeled lipoproteins was assessed by native agarose gel electrophoresis and FPLC. Autologous lipoproteins were injected (1.5×10^6 dpm of labeled particle per mouse), and retained plasma counts were expressed as a percentage of radioactivity remaining in plasma at 3 min after saphenous vein injection.

Analysis of aortic lesions

The heart and the attached section of ascending aorta were dissected en bloc and prepared as described previously (35). Three millimeter sections of the aortic root and ascending aorta were stained with Oil Red O for neutral lipids and with hematoxylin for nucleic tissue. Five sections per animal were evaluated to determine the mean cross-sectional area of lesions for each animal.

Statistical analysis

All data are expressed as means ± SEM. Statistically significant differences between control and transgenic mice were assessed by Student's *t*-test and defined as a two-tailed $P < 0.05$. For all data presented in **Table 1**, the variances were determined to be equal by the Bartlett test. Nonparametric data were analyzed by the Mann-Whitney test (Instat Software; Graphpad, Inc., San Diego, CA).

RESULTS

We previously used a 140 kb bacterial artificial chromosome (BAC) to generate transgenic mice that selectively overexpressed human ABCG5 and ABCG8 only in liver (23). Although biliary cholesterol secretion was enhanced in these mice, much of the cholesterol secreted into bile was reabsorbed, cholesterol balance across the liver and elimination of cholesterol from the body were not altered, and hepatic ABCG5/G8 overexpression did not protect against the development of aortic lesions in C57Bl/6, apoE-KO, or LDLr-KO mice. Here, we directly tested the hypothesis that decreasing intestinal cholesterol absorption by ezetimibe feeding will prevent reabsorption of the excess biliary cholesterol secreted in mice overexpressing ABCG5/G8 in liver, altering the cholesterol balance across the liver, causing a net loss of cholesterol from the body, and allowing hepatically expressed ABCG5/G8 to protect against atherosclerosis.

Ezetimibe blocks fractional intestinal cholesterol absorption and downregulates hepatic ABCG5/G8 levels

To ensure that ezetimibe was in fact blocking absorption, we first measured fractional intestinal cholesterol absorption using the plasma dual-isotope method. Table 1 shows that ezetimibe reduced intestinal cholesterol absorption to ~5% in both LDLr-KO and ABCG5/G8-Tg×LDLr-KO mice (all mouse groups, $P < 0.01$). **Figure 1** illustrates the effects of ezetimibe feeding (15 mg/kg/day for 24 weeks) on ABCG5/G8 expression in liver of LDLr-KO and ABCG5/G8-Tg×LDLr-KO mice. Northern analysis of liver RNA using probes specific for human ABCG5/G8 or mouse ABCG5/G8 shows that the addition of ezetimibe to the Western diet reduced hepatic mRNA levels of both human ABCG5/G8 (Fig. 1, upper panels) and endogenous mouse ABCG5/G8 (Fig. 1, lower panels). The downregulation of ABCG5/G8 expression by ezetimibe in mice fed a Western diet is consistent with the findings reported by Repa and colleagues (36, 37), in which mice fed

TABLE 1. Hepatobiliary cholesterol concentrations and secretion rates, fractional intestinal cholesterol absorption, and fecal cholesterol excretion in liver of ABCG5/G8-Tg×LDLr-KO and LDLr-KO mice on a Western diet plus ezetimibe

Mouse	Number	Bile Volume	Bile Cholesterol Content	Bile Cholesterol Secretion Rate	Bile Cholesterol Secretion Rate	Dietary Cholesterol	Intestinal Cholesterol (Bile + Diet)	Intestinal Cholesterol Absorption	Absorbed Cholesterol	Fecal Cholesterol Excretion
		μl/h/100 g	*μM*	*nm/h/30 g*	*mg/day/30 g*			*%*	*mg/day/30 g*	
LDLr-KO	7	280 ± 24	319 ± 44	27.48 ± 4.73	0.26 ± 0.05	3.71 ± 0.29	3.97 ± 0.18	4.38 ± 0.06	0.19 ± 0.004	3.18 ± 0.17
ABCG5/G8-Tg×LDLr-KO	9	349 ± 20	486 ± 36[a]	52.28 ± 6.43[a]	0.54 ± 0.08[a]	4.74 ± 0.42	5.28 ± 0.47[b]	4.63 ± 0.09	0.22 ± 0.004[c]	5.06 ± 0.33[c]
P		0.11	0.03	0.03	0.04	0.11	0.05	0.71	0.001	0.007

ABCG5/G8-Tg, ABCG5/G8-transgenic; LDLr-KO, low density lipoprotein receptor-knockout. Biliary cholesterol concentration and secretion rates were measured by GC analysis. The amount of dietary cholesterol ingested daily was determined in individual mice during 3 consecutive days. The intestinal cholesterol content was calculated by adding the daily amount of cholesterol secreted into the bile and ingested in the diet. Fractional intestinal cholesterol absorption, determined using a dual-isotope method, was multiplied by the total intestinal cholesterol to obtain the amount (mg) of absorbed cholesterol. Fecal cholesterol and its breakdown products, coprostanol and cholestanol, were measured in the feces by GC. Data are expressed as means ± SEM. *P* values were determined using an unpaired *t*-test.

[a] $P < 0.05$.
[b] $P = 0.05$.
[c] $P < 0.01$.

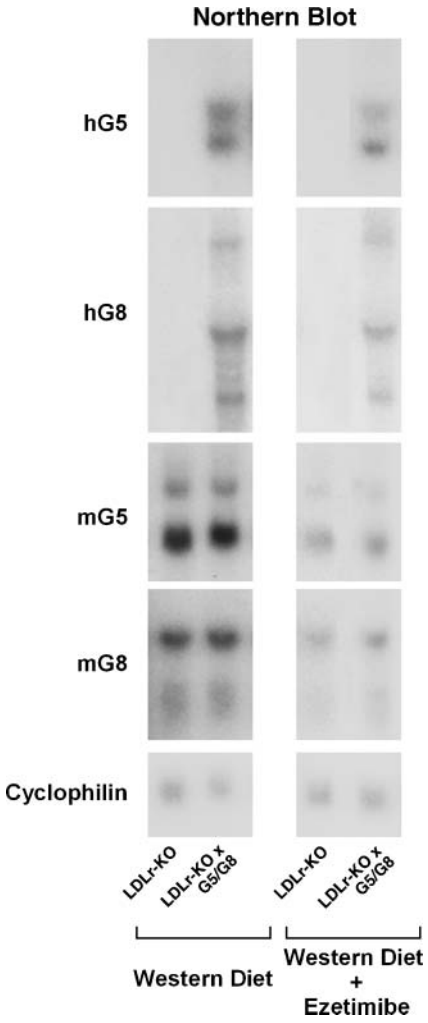

Fig. 1. Overexpression of human ABCG5 and ABCG8 in liver of low density lipoprotein receptor-knockout (LDLr-KO) mice on a Western diet with or without ezetimibe. Northern analysis of hepatic ABCG5 and ABCG8 expression in LDLr-KO or ABCG5/G8-transgenic (ABCG5/G8-Tg)×LDLr-KO mice fed a Western diet or a Western diet plus ezetimibe for 24 weeks is shown. RNA was extracted from livers of individual male mice (n = 4 for each genotype), separated on the same 1% agarose glyoxal gel, transferred to the same membrane, and sequentially hybridized to four different radiolabeled cDNA probes that recognize only human ABCG5 (hG5), human ABCG8 (hG8), mouse ABCG5 (mG5), or mouse ABCG8 (mG8), as reported (23). A radiolabeled cyclophilin cDNA probe was then hybridized to the same blot for a loading control. The panels shown here are representative of four independent mice per study group.

a high-cholesterol diet supplemented with the ezetimibe analog SCH 58053 (36) or a basal diet containing 0.2% (w/w) cholesterol plus 10% olive oil supplemented with ezetimibe (37) had decreased ABCG5/G8 mRNA levels compared with mice fed similar diets lacking SCH 58053 or ezetimibe. In our study, human ABCG5/G8 was still overexpressed in ABCG5/G8-Tg×LDLr-KO versus LDLr-KO mice on the Western diet plus ezetimibe (Fig. 1). Figure 1 also demonstrates that the increased ABCG5/G8 mRNA levels in ABCG5/G8-Tg×LDLr-KO versus LDLr-KO mice fed the Western diet plus ezetimibe were completely determined by the expression of the human transgenes, because expression of the endogenous mouse ABCG5/G8 transporters was similar between the transgenic and control mouse groups (Fig. 1), as reported previously for ABCG5/G8-Tg mice in the C57Bl/6 background (23).

Hepatic ABCG5/G8 expression increases biliary cholesterol excretion and fecal cholesterol excretion in LDLr-KO mice fed a Western diet supplemented with ezetimibe

We next measured the biliary cholesterol concentration and secretion rates after cannulation of the common bile duct (Table 1). Consistent with the downregulation of hepatic ABCG5/G8 mRNA observed after the addition of ezetimibe to the Western diet (Fig. 1), the biliary cholesterol secretion rates in ezetimibe-treated LDLr-KO and ABCG5/G8-Tg×LDLr-KO mice (0.26 ± 0.05 and 0.54 ± 0.08 mg/day/30 g, respectively) (Table 1) were reduced compared with those in previously studied non-ezetimibe-treated LDLr-KO and ABCG5/G8-Tg×LDLr-KO mice (23). Despite this decrease in secretion rates in both mouse strains fed ezetimibe, the biliary cholesterol concentration (484 ± 36 vs. 319 ± 44 μM; $P = 0.03$) and secretion rates (52.28 ± 6.43 vs. 27.48 ± 4.73 nm/h/30 g, or 0.54 ± 0.08 vs. 0.26 ± 0.05 mg/day/30 g; $P = 0.03$) still remained higher in the ABCG5/G8-Tg×LDLr-KO mice compared with LDLr-KO controls (Table 1). Moreover, fecal cholesterol excretion was increased by 1.6-fold (5.06 ± 0.33 vs. 3.18 ± 0.17 mg/day/30 g; $P < 0.01$) in ABCG5/G8-Tg×LDLr-KO compared with LDLr-KO mice on a Western diet plus ezetimibe (Table 1). No differences in the biliary phospholipid and bile acid secretion rates between ABCG5/G8-Tg×LDLr-KO and LDLr-KO mice were noted (data not shown).

Thus, on the Western diet, ezetimibe reduces the fractional intestinal cholesterol absorption and decreases the biliary cholesterol secretion of both LDLr-KO and transgenic mice. However, biliary cholesterol secretion is still enhanced in transgenic mice compared with control mice by 2-fold. Consequently, fecal cholesterol excretion is increased in ABCG5/G8-Tg×LDLr-KO mice compared with controls.

Hepatic ABCG5/G8 expression alters cholesterol balance across the liver in LDLr-KO mice fed ezetimibe

Measurements of daily dietary cholesterol intake, biliary cholesterol secretion, intestinal cholesterol absorption, and fecal cholesterol excretion were then used to determine the hepatic cholesterol balance of ABCG5/G8-Tg×LDLr-KO versus LDLr-KO mice fed a Western diet supplemented with ezetimibe (Table 1). The milligrams of dietary cholesterol ingested daily were similar for ABCG5/G8-Tg×LDLr-KO and LDLr-KO mice (4.74 ± 0.42 vs. 3.71 ± 0.29 mg/day/30 g; $P = 0.1$). However, the daily biliary secretion of cholesterol in ABCG5/G8-Tg×LDLr-KO mice (0.54 ± 0.08 mg/day/30 g) was two times greater (by 0.28 mg/day) than that of LDLr-KO mice (0.26 ± 0.05 mg/day/30 g; $P = 0.03$). Consequently, the amount of

intestinal cholesterol available for absorption from dietary plus biliary sources (Table 1) was higher in ABCG5/G8-Tg×LDLr-KO mice (5.28 ± 0.47 mg/day/30 g) versus that in control LDLr-KO mice (3.97 ± 0.18 mg/day/30 g). However, because ezetimibe blocked fractional intestinal cholesterol absorption to 5%, the actual milligram amount of cholesterol absorbed each day in both groups of mice was very low (0.22 ± 0.004 mg/day/30 g in ABCG5/G8-Tg×LDLr-KO vs. 0.19 ± 0.004 mg/day/30 g in control LDLr-KO mice). Thus, the output of hepatic cholesterol through the bile in control LDLr-KO mice was 0.26 mg of cholesterol per day, but ~0.19 mg of cholesterol were reabsorbed from the intestine and returned to the liver. Consequently, there was a net output of hepatic cholesterol into the intestine of ~0.07 mg/day in LDLr-KO controls. In ABCG5/G8-Tg×LDLr-KO mice, the secretion of hepatic cholesterol through the bile was 0.54 mg/day, and only 0.22 mg of cholesterol were reabsorbed from the intestine and returned to the liver each day. These studies demonstrate that the net output of hepatic cholesterol in the intestine for the ABCG5/G8-Tg×LDLr-KO mice was 0.3 mg/day, an amount significantly greater than that for control LDLr-KO mice. Consistently, fecal cholesterol excretion was increased in ABCG5/G8-Tg×LDLr-KO compared with LDLr-KO mice (Table 1).

Thus, blocking intestinal reabsorption of secreted biliary cholesterol by ezetimibe enhances fecal cholesterol excretion, resulting in a loss of liver cholesterol via the enterohepatic circulation in ABCG5/G8-Tg×LDLr-KO mice with only hepatic ABCG5/G8 expression.

Loss of cholesterol in ABCG5/G8-Tg×LDLr-KO mice through bile results in decreased hepatic cholesterol, with a compensatory upregulation of hepatic cholesterol synthesis and downregulation of hepatic ABCA1 and ABCG1 mRNA levels

The net loss of cholesterol in the feces of ABCG5/G8-Tg×LDLr-KO mice resulted in a decreased hepatic concentration of total cholesterol (TC) (2.47 ± 0.03 vs. 3.47 ± 0.25 μg/mg liver; $P < 0.01$) (**Fig. 2A**) and cholesteryl ester (CE) (0.61 ± 0.09 vs. 1.46 ± 0.20 μg/mg liver; $P < 0.01$) (Fig. 2B) in ABCG5/G8-Tg×LDLr-KO versus LDLr-KO mice fed a Western diet supplemented with ezetimibe. Northern blot hybridization of liver RNA demonstrated compensatory upregulation of liver HMG-CoA reductase (Fig. 2C), leading to enhanced de novo hepatic cholesterol synthesis, as measured by the incorporation of tritiated water (increased by 4.4-fold; 3,190 ± 576 vs. 730 ± 41 nmol/h/g; $P = 0.01$) in ABCG5/G8-Tg×LDLr-KO compared with control LDLr-KO mice (Fig. 2D). Endogenous expression of ABCA1 and ABCG1 was downregulated in ABCG5/G8-Tg×LDLr-KO mice compared with control mice, whereas there was no difference in LDL receptor-related protein hepatic expression (Fig. 2C).

Lipid profile is atheroprotective in ABCG5/G8-Tg×LDLr-KO mice fed ezetimibe

Previous studies have shown that the addition of ezetimibe or the ezetimibe analog SCH 58053 to a lipid-rich diet markedly decreases plasma TC levels in LDLr-KO mice (36, 37). Consistently, total plasma cholesterol levels in female and male LDLr-KO mice fed a Western diet for 10 weeks (1,766 ± 68 and 2,247 ± 304 mg/dl, respectively) decreased markedly in female and male LDLr-KO mice fed a Western diet plus ezetimibe for 10 weeks (524 ± 23 and 1,381 ± 101 mg/dl, respectively) and decreased even further in female and male mice fed a Western diet plus ezetimibe for 24 weeks (675 ± 58 and 919 ± 32 mg/dl, respectively).

We next evaluated the effects of ABCG5/G8 overexpression on the plasma lipids and lipoproteins in mice fed a Western diet supplemented with ezetimibe (**Fig. 3**, females; **Fig. 4**, males). The plasma lipids were reduced significantly in female ABCG5/G8-Tg×LDLr-KO compared with female LDLr-KO control mice [TC, 461 ± 60 vs. 675 ± 58 mg/dl; free cholesterol (FC), 118 ± 15 vs. 170 ± 16 mg/dl; CE, 343 ± 45 vs. 505 ± 43 mg/dl; non-HDL-C, 379 ± 59 vs. 595 ± 62 mg/dl; $P \leq 0.02$ for all] at 24 weeks of Western diet plus ezetimibe therapy (Fig. 3A). No significant differences in plasma triglyceride (TG), phospholipid, HDL-C, or apoA-I were noted. Figure 3B, C illustrate the changes in plasma TC and non-HDL-C over time: significant reductions in plasma cholesterol and non-HDL-C were evident starting at 9 weeks and sustained through 24 weeks of the Western diet plus ezetimibe therapy. Consistent with the decrease in plasma concentrations of apoB-Lps, immunoblot analysis of plasma apoB levels revealed a 25% reduction of circulating apoB-48 in ABCG5/G8-Tg×LDLr-KO mice compared with control LDLr-KO mice (359 ± 15 vs. 269 ± 10 mg/dl; $P = 0.02$) (Fig. 3A, inset). FPLC analysis also revealed decreased plasma levels of apoB-Lp cholesterol in female ABCG5/G8-Tg×LDLr-KO mice (Fig. 3D). The absolute lipid values for males in ABCG5/G8-Tg×LDLr-KO and LDLr-KO mice were higher than those of their female counterparts (Fig. 4A); however, the percentage reductions in TC (24%), CE (24%), non-HDL-C (29%), and apoB (33%) ($P < 0.05$ for all) were similar to those observed in females. In addition, decreases in TG and phospholipid levels reached statistical significance in males. FPLC analysis also revealed decreased plasma levels of apoB-Lp cholesterol in male ABCG5/G8-Tg×LDLr-KO mice (Fig. 4B). Thus, the reductions in TC of transgenic mice were attributable to decreased plasma levels of the proatherogenic apoB-Lps in mice fed a Western diet plus ezetimibe.

In **Fig. 5A, B**, the lipoprotein particle size and composition in both groups of mice were analyzed. The major lipoprotein particles in the IDL/LDL fraction isolated from LDLr-KO mice were >400, 363, 309, and 242 Å (Fig. 5A). In ABCG5/G8-Tg×LDLr-KO mice, the 363 Å particle was reduced significantly. No major differences were evident in the particle size of the VLDL fraction in LDLr-KO and ABCG5/G8-Tg×LDLr-KO mice. The lipid and apoB contents of both VLDL and IDL/LDL fractions were reduced in ABCG5/G8-Tg×LDLr-KO versus LDLr-KO mice (Fig. 5B). However, only VLDL, a minor component of total plasma non-HDL-C in ABCG5/G8-Tg×LDLr-KO mice (Figs. 3D, 4B), was lipid-depleted compared with the VLDL fractions in LDLr-KO controls.

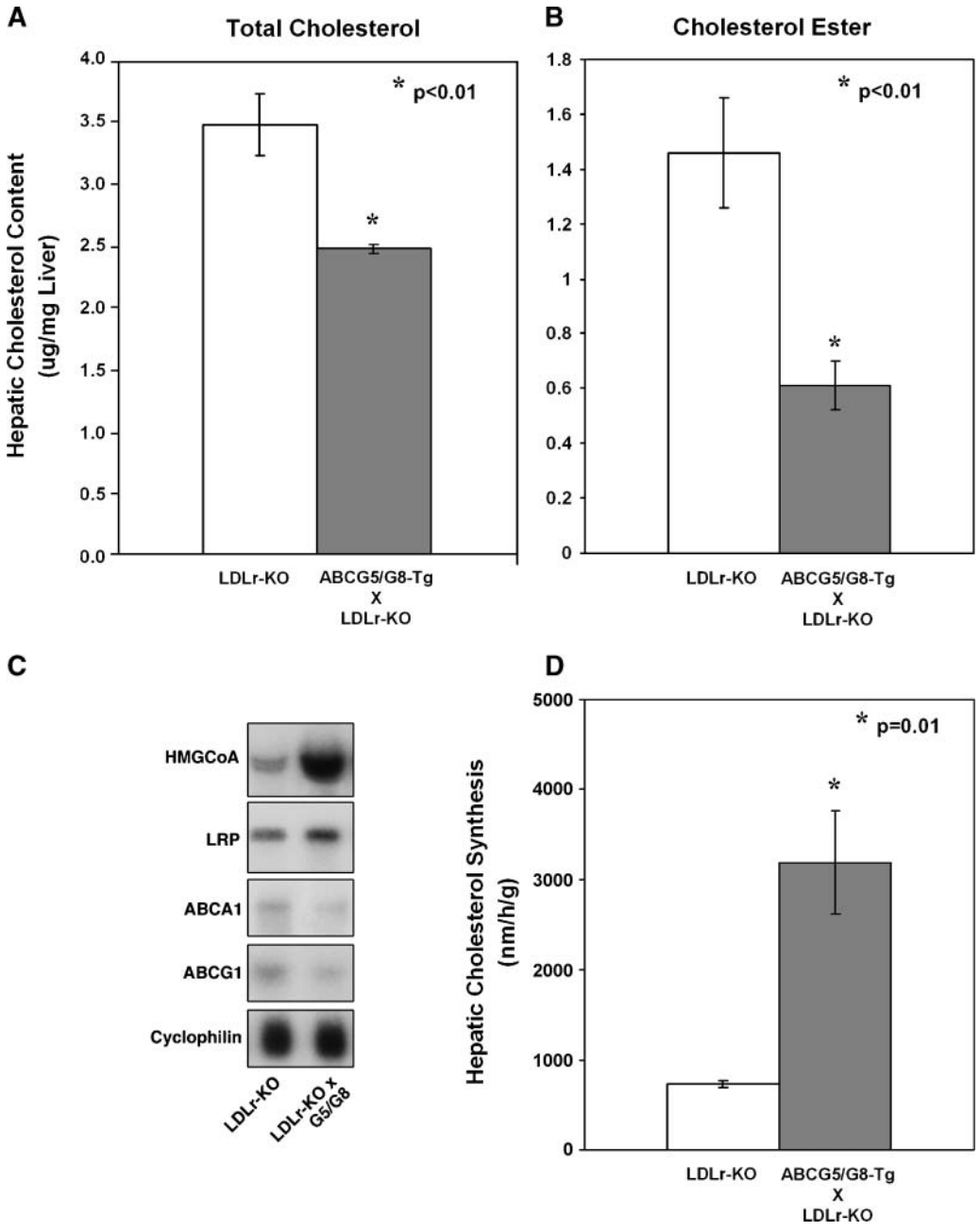

Fig. 2. Decreased hepatic cholesterol content alters the expression of ABCA1, ABCG1, and HMG-CoA reductase in ABCG5/G8-Tg×LDLr-KO mice. Eight month old LDLr-KO (n = 6) and ABCG5/G8-Tg×LDLr-KO (n = 12) male mice were euthanized after 24 weeks on the Western diet plus ezetimibe. Livers were isolated, and liver homogenates and total RNA were prepared from each mouse. A: Hepatic cholesterol content of each mouse study group, determined by GC and independently confirmed by enzymatic assays as described in Materials and Methods. B: Hepatic cholesteryl ester (CE) content, determined using an enzymatic sterol assay. C: Northern blot hybridization study of hepatic HMG-CoA reductase, LDL receptor-related protein (LRP), ABCA1, ABCG1, or cyclophilin mRNA isolated from the same study mice. D: Measured hepatic cholesterol synthesis in a separate group of 8 month old male LDLr-KO (n = 4) and ABCG5/G8-Tg×LDLr-KO (n = 4) mice fed the Western diet plus ezetimibe for 24 weeks after intraperitoneal injection of $^{3}H_2O$ as a cholesterol precursor. Lipid extracts of tissues were saponified, free cholesterol (FC) was precipitated by digitonin, and [^{3}H]cholesterol was counted on a β-counter. Counts were back-calculated to nmol cholesterol/h/g wet liver tissue as described in Materials and Methods. Data are expressed as means ± SEM.

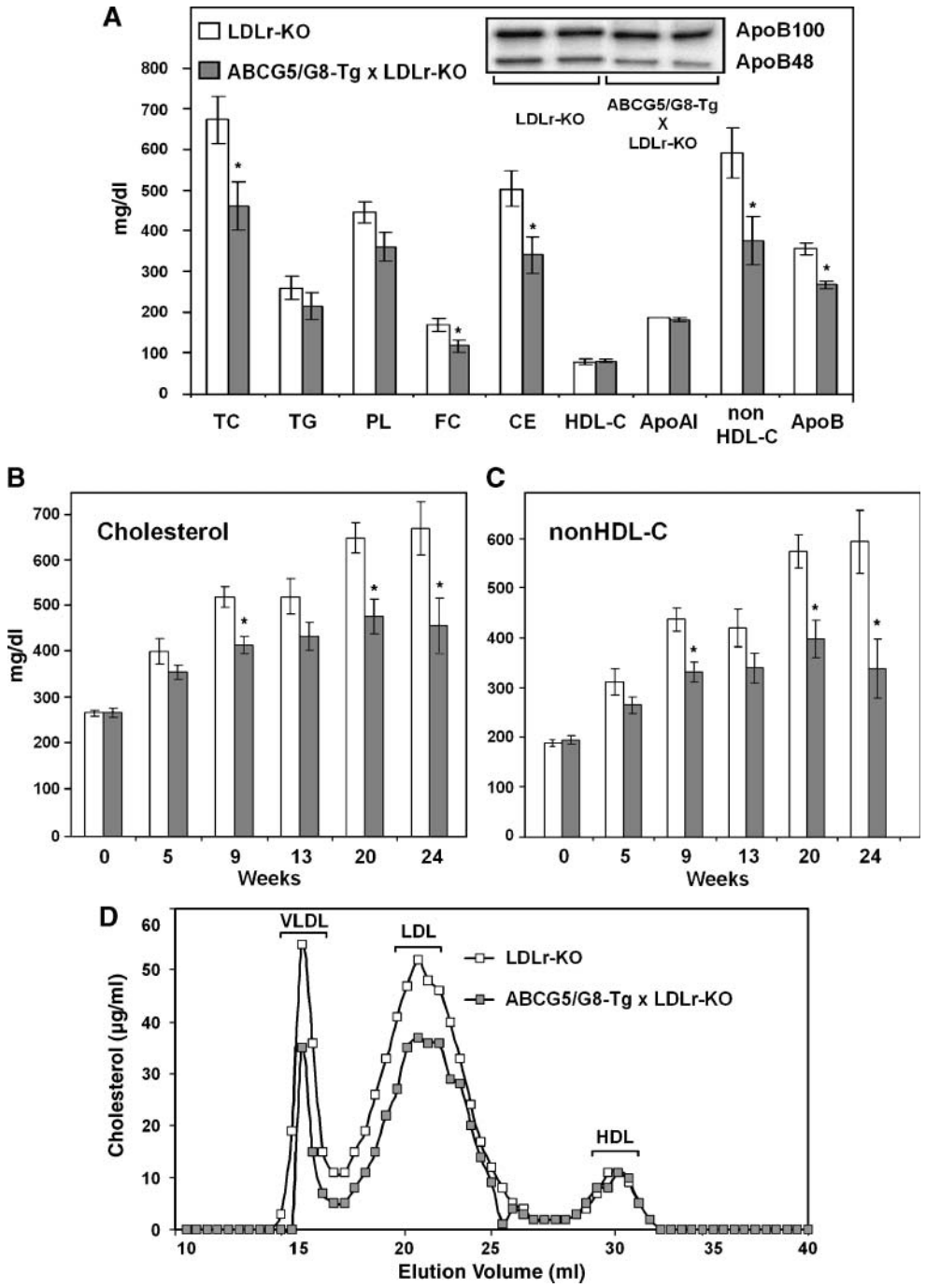

Fig. 3. Lipid, lipoprotein, and apolipoprotein analysis of female ABCG5/G8-Tg×LDLr-KO mice on a Western diet plus ezetimibe. A: Plasma lipids of fasted female LDLr-KO (n = 7) and ABCG5/G8-Tg×LDLr-KO (n = 7) mice after 24 weeks of a Western diet plus ezetimibe were quantified. Apolipoprotein B-100 (apoB-100) and apoB-48 from 2 µl (1:40 dilution) of plasma from two LDLr-KO and two ABCG5/G8-Tg×LDLr-KO mice were detected by immunoblot analysis using an antibody that recognizes both apoB-100 and apoB-48 (inset). B, C: Time course of accumulation of total cholesterol (TC) and non-high density lipoprotein-cholesterol (non-HDL-C) in the same female study mice measured after 0, 5, 9, 13, 20, or 24 weeks on a Western diet plus ezetimibe. D: Distribution of TC in plasma lipoproteins after fast-protein liquid chromatography (FPLC) separation of 50 µl of pooled plasma from female fasted mice (n = 5 for each genotype). FPLC fractions corresponding to VLDL, intermediate density lipoprotein (IDL)/LDL, and HDL are shown. PL, phospholipid; TG, triglyceride. * $P \leq 0.02$. Data are expressed as means ± SEM.

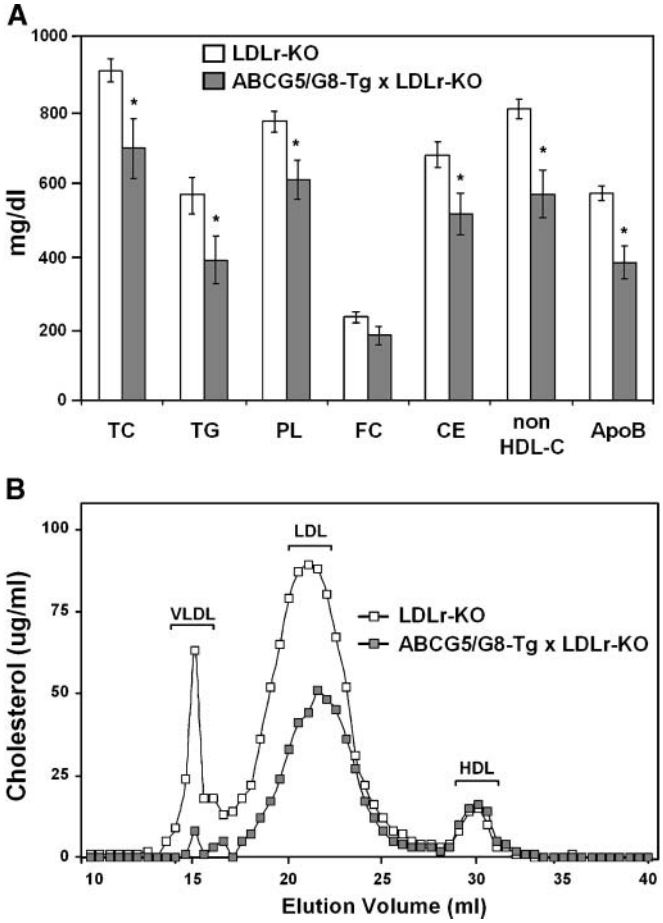

Fig. 4. Lipid and lipoprotein analysis of male ABCG5/G8-Tg×LDLr-KO mice on a Western diet plus ezetimibe. A: Plasma lipids of fasted male LDLr-KO (n = 5) and ABCG5/G8-Tg×LDLr-KO (n = 6) mice after 20 weeks of a Western diet plus ezetimibe were quantified. B: Distribution of TC in plasma lipoproteins after FPLC separation of 50 μl of pooled plasma from male fasted mice (n = 5 for each genotype). FPLC fractions corresponding to VLDL, IDL/LDL, and HDL are shown. PL, phospholipid. * $P \leq 0.02$. Data are expressed as means ± SEM.

In the absence of the LDLr, the plasma concentration of IDL/LDL-cholesterol would be regulated by its production rate and/or its fractional catabolic rate (FCR) through non-LDLr pathways. To determine the mechanisms underlying the decreased apoB-Lp cholesterol concentrations in ABCG5/G8-Tg×LDLr-KO mice after ezetimibe feeding, apoB-Lp production and catabolism were determined (**Fig. 6A**). Injection of autologous iodinated IDL/LDL indicated that the catabolism of apoB in these particles was not changed in the two groups of mice (FCR, 1.37 ± 0.07 vs. 1.83 ± 0.28 pools/day). The production rate, however, was reduced in ABCG5/G8-Tg×LDLr-KO versus LDLr-KO mice (15.39 ± 0.54 vs. 24.58 ± 0.06 mg/kg/day; $P = 0.001$). These combined data indicate that the reason for the reduced apoB and apoB-Lp levels in ABCG5/G8-Tg×LDLr-KO mice is the decreased production of apoB-Lps (Fig. 6A, inset), which are lipid-depleted (Fig. 5B).

Thus, the reduction of intestinal cholesterol absorption induced by ezetimibe in ABCG5/G8-Tg×LDLr-KO mice decreases plasma concentrations of apoB-Lps primarily by decreasing their production rates. Furthermore, the VLDL particles that accumulate in ABCG5/G8-Tg×LDLr-KO mice fed ezetimibe are lipid-depleted, a compensatory mechanism that may be consistent with the decreased hepatic cholesterol content in these animals.

Decreased atherosclerosis in ABCG5/G8-Tg×LDLr-KO mice fed ezetimibe

We previously reported that on a Western diet, overexpression of ABCG5/G8 only in liver did not alter plasma apoB-Lp levels or reduce aortic atherosclerosis despite enhanced biliary cholesterol secretion. Analysis of lesions in the proximal aorta of ABCG5/G8-Tg×LDLr-KO mice fed the Western diet plus ezetimibe revealed that compared with control LDLr-KO male and female mice, mean aortic lesion area was decreased significantly by 52% and 59%, respectively [58,150 ± 7,890 vs. 121,100 ± 24,370 μm^2 for males ($P = 0.01$) and 61,331 ± 8,473 vs. 149,186 ± 23,102 μm^2 for females ($P = 0.002$)] (Fig. 6B). These data definitively establish that, in addition to enhanced biliary secretion, reduced intestinal cholesterol absorption is required for ABCG5/G8 to have an antiatherogenic effect.

DISCUSSION

We recently reported that liver-specific overexpression of ABCG5 and ABCG8 in transgenic mice increased the biliary secretion of cholesterol and plant sterols but did not change the fractional intestinal cholesterol absorption, plasma total cholesterol, apoB-Lp levels or aortic atherosclerosis in either apoE-KO or LDLr-KO mice (23). Although more hepatic cholesterol was removed from the liver and secreted into the bile in these liver-specific ABCG5/G8 transgenic mice (23), most of this excess cholesterol was reabsorbed from the intestine and transferred back to the liver, resulting in the recycling of cholesterol through the enterohepatic circulation. As a result, there was no difference in fecal cholesterol excretion between the two groups of mice, no difference in hepatic cholesterol content, no difference in apoB-Lp levels, and no difference in atherosclerosis. These data suggested that not only increased biliary cholesterol secretion but also decreased intestinal cholesterol absorption is required for hepatic ABCG5/G8 to decrease liver cholesterol concentrations and to trigger the compensatory mechanisms that reduce plasma levels of the proatherogenic apoB-Lps and atherosclerosis.

Here, we demonstrate that blocking intestinal cholesterol absorption with ezetimibe does in fact induce increased cholesterol excretion from the body, alter liver cholesterol transport, decrease apoB-Lp levels in plasma, and, most importantly, decrease atherosclerosis in ABCG5/G8-Tg×LDLr-KO mice that overexpress ABCG5/G8 only in liver compared with control LDLr-KO mice. Thus, block-

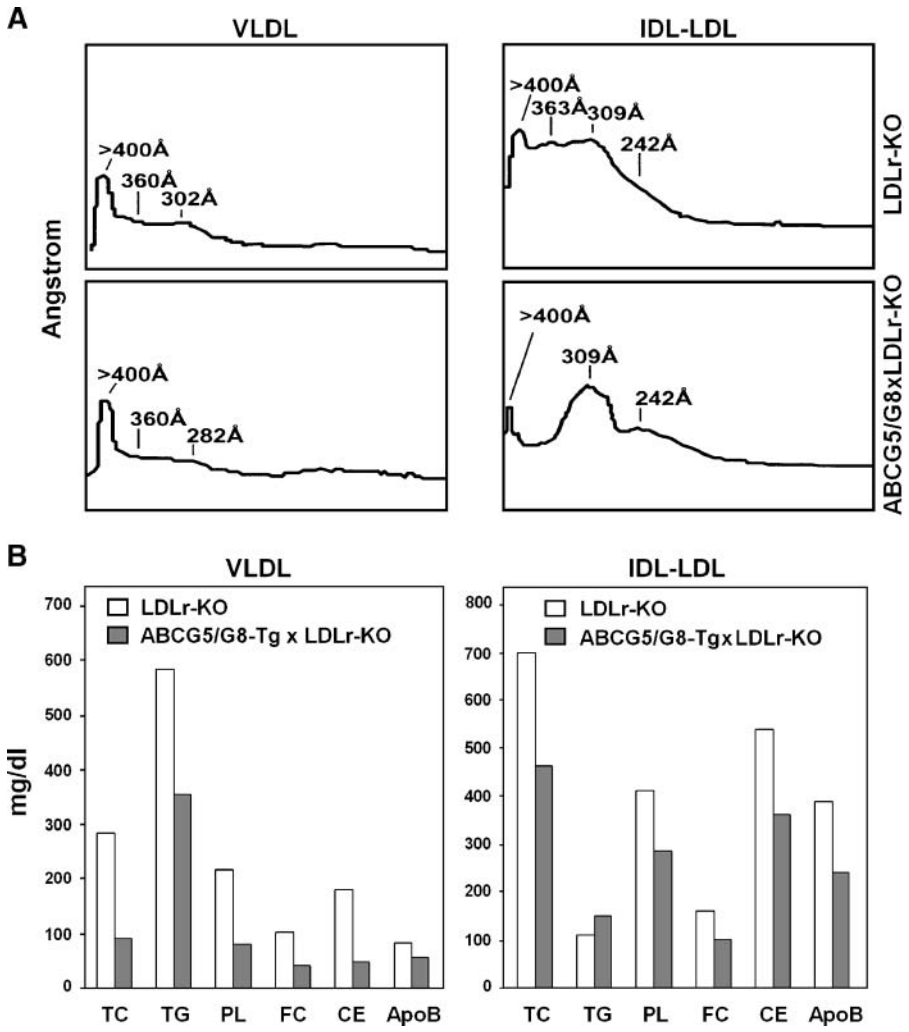

Fig. 5. Analysis of VLDL and IDL/LDL particle size and composition of ABCG5/G8-Tg×LDLr-KO and LDLr-KO mice after 24 weeks on a Western diet plus ezetimibe. A: Particle size of VLDL ($d < 1.006$; left panels) and IDL/LDL ($d = 1.006$–1.063; right panels) fractions isolated from plasma pooled from LDLr-KO ($n = 6$; upper panels) and ABCG5/G8-Tg×LDLr-KO ($n = 6$; lower panels) female mice analyzed by native Tris-Glycine 4–12% polyacrylamide gradient gel electrophoresis. The diameters (in Å) are indicated. B: TC, TG, phospholipid (PL), FC, CE, and apoB contents of VLDL and IDL/LDL fractions isolated from the same LDLr-KO and ABCG5/G8-Tg×LDLr-KO mice.

ing the reuptake of excess biliary cholesterol secreted by ABCG5/G8-Tg×LDLr-KO mice into the intestine can markedly alter cholesterol balance across the liver, creating an atheroprotective lipid profile that results in the decreased formation of aortic lesions. We show that ezetimibe blocks the reabsorption of biliary cholesterol secreted in these mice, resulting in more fecal cholesterol excretion and a negative hepatic cholesterol balance. We have also demonstrated that the negative hepatic cholesterol balance was partially but not completely compensated for by a marked increase in de novo hepatic cholesterol synthesis induced by the upregulation of HMG-CoA reductase. Biliary sterol secretion and hepatic expression of ABCA1 and ABCG1 were reduced as well. Despite these compensatory mechanisms aimed at conserving hepatic cholesterol, the hepatic cholesterol content of ABCG5/G8-Tg×LDLr-KO mice fed ezetimibe was reduced compared with that in LDLr-KO control mice. As anticipated, there were no apparent changes in hepatic LRP expression, and because our mice are LDLr-deficient, the decrease in apoB-Lp levels is not attributable to the upregulation of this receptor.

Our data support the prevailing concept of a dual role of hepatic and intestinal ABCG5/G8 acting in concert to excrete cholesterol from the body, with hepatic ABCG5/G8 excreting cholesterol from hepatocytes into bile and intestinal ABCG5/G8 excreting cholesterol from enterocytes into the gut lumen (13, 14). We note that the role of intestinal ABCG5/G8 in transporting cholesterol from enterocytes back into the gut lumen remains hypothetical, because, as yet, no direct evidence supports this concept. Alternative explanations for the lower fractional absorption of cholesterol observed in mice overexpressing ABCG5/G8 both in the liver and the intestine (13, 24) include dilution of the isotope tracer used to measure

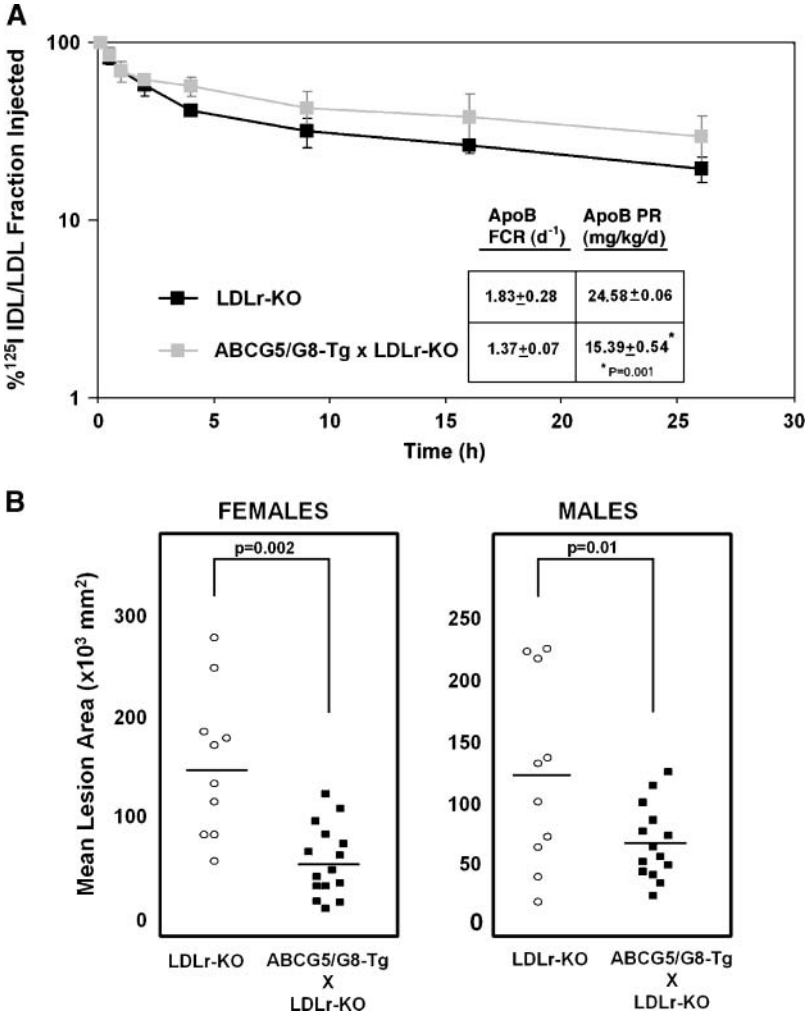

Fig. 6. Kinetic analyses of ^{125}I-apoB IDL/LDL and measurement of proximal aortic lesions in ABCG5/G8-Tg×LDLr-KO and LDLr-KO mice fed a Western diet plus ezetimibe for 24 weeks. A: Autologous mouse IDL/LDL was isolated and radiolabeled as described in Materials and Methods, and aliquots were injected into fasted female mice (n = 3 for each group). The clearance of ^{125}I-apoB IDL/LDL was expressed as a percentage of radioactivity remaining in plasma at 3 min after saphenous vein injection. ApoB fractional catabolic rates (FCRs; inset) were determined from the area under the plasma radioactivity curves using a multiexponential curve-fitting technique in the SAAM program, and apoB production rates (PRs; inset) in the steady state were calculated using the following equation: PR = apoB (mg/dl) × plasma volume × FCR (per day)/weight (kg), where the plasma volume = weight (kg) × 0.0315 × 10. Data are expressed as means ± SEM. B: Proximal aortic lesion area in LDLr-KO male (n = 10) and female (n = 10) mice and in ABCG5/G8-Tg×LDLr-KO male (n = 14) and female (n = 15) mice maintained for 24 weeks on the Western diet plus ezetimibe.

cholesterol absorption, caused by more cholesterol secreted into the bile of ABCG5/G8 transgenic mice, and a compensatory reduction of NPC1L1 expression in the gut, caused by increased intestinal cholesterol, as reported by Davis et al. (26).

Recently, Tremblay et al. (38) reported that ezetimibe reduced the LDL pool size in non-familial hypercholesterolemic patients by enhancing the catabolism of apoB-100 VLDL, IDL, and LDL and hypothesized that these changes were attributable to the upregulation of the LDLr. Similarly, Repa et al. (37) proposed that part of the mechanism by which ezetimibe decreases plasma TC and non-HDL-C levels in LDLr$^{+/+}$ control mice was enhanced hepatic LDLr expression; however, because plasma TC and non-HDL-C levels were also reduced in LDLr$^{-/-}$ mice, it was hypothesized that decreased LDL-cholesterol production rates contributed to the decrease in plasma non-HDL-C. These studies did not investigate the effect of hepatic ABCG5/G8 on the metabolism of apoB-Lps in mice fed ezetimibe.

In our study, direct measurement of lipid and apoB levels in apoB-Lp particles revealed that the lipid content of VLDL was decreased in ABCG5/G8-Tg×LDLr-KO mice versus LDLr-KO mice fed ezetimibe. Moreover, the IDL/LDL particles from ABCG5/G8-Tg×LDLr-KO mice were smaller compared with the apoB-Lp particles present in LDLr-KO mice. Thus, depletion of cholesterol in apoB-Lps contributes to the decrease in plasma apoB-Lp cholesterol in ABCG5/G8-Tg×LDLr-KO compared with LDLr-KO mice. In addition, the number of apoB-Lp particles decreased, as ABCG5/G8-Tg×LDLr-KO mice had a significant decrease in plasma apoB levels compared with LDLr-KO mice (Fig. 3). Kinetic analysis of ^{125}I-apoB IDL/LDL catabolism revealed similar FCR and a 37% reduction in production rate of apoB in IDL/LDL in ABCG5/G8-Tg×LDLr-KO mice. From these combined data, we conclude that the major mechanism involved in the reduction of plasma apoB-Lps and apoB-Lp cholesterol levels in ABCG5/G8-Tg×LDLr-KO mice compared with control LDLr-KO mice fed ezetimibe is the decreased production of IDL/LDL particles. Moreover, the apoB-Lps present in ABCG5/G8-Tg×LDLr-KO mice are lipid-depleted. These findings indicate that decreased production of apoB-Lps as well as the reduced lipid content of these particles in response to lower hepatic cholesterol content is partly responsible for the decreased apoB-Lp cholesterol levels in plasma of ABCG5/G8-Tg×LDLr-KO mice fed ezetimibe.

Several published reports support the concept that depletion of a hepatic pool of cholesterol can decrease VLDL secretion (39–43). In some cases, reduction of hepatic cholesterol levels decreases only the number and not the cholesterol or TG content of secreted VLDL particles. This has been shown in miniature pigs (44) and apoE*3-Leiden mice (45) after HMG-CoA reductase inhibition. In hamster hepatocytes in vitro, hepatic CE and hepatic apoB secretion were closely correlated after the addition of statin or ACAT inhibitor plus or minus oleate (46). A novel oxidosqualene:lanosterol cyclase (OSC) inhibitor that inhibits cholesterol synthesis but enhances oxysterol synthesis in miniature pigs reduced hepatic CE but not FC and inhibited VLDL-apoB production without altering its composition (47). These combined studies demonstrate that hepatic cholesterol or CE levels can alter apoB-Lp cholesterol secretion without changing the lipid content of the apoB-Lps.

In contrast, decreased hepatic cholesterol content can also lead to the direct secretion of lipid-depleted apoB-containing particles (small VLDL, IDL, or LDL) without altering the actual number of lipoprotein particles secreted by the liver (39, 48–50). Decreasing liver cholesterol content in mice treated with the hypolipidemic agent HOE 402 (51) or with dietary plant sterol esters (52) also decreases the amount of cholesterol incorporated into nascent VLDLs without changing the VLDL production rate, indicating that liver cholesterol concentrations can influence the incorporation of cholesterol into nascent VLDL. In ACAT-deficient mice, apoB-Lps are secreted but their lipid core contains primarily TGs rather than cholesterol (53). Conversely, increased hepatic cholesterol concentrations can also increase the cholesterol content of newly secreted apoB-Lps (54–57). Increased hepatic CE has been reported to increase the particle number as well as the CE content of secreted VLDL in rats (58). Finally, some (59–61), although not all (62, 63), studies in HepG2 cells and McA-RH7777 cells have found a relationship between hepatic cholesterol or CE levels and hepatic VLDL production. Thus, a number of reports have, in fact, provided evidence for the secretion of lipid-depleted particles in IDL/LDL and VLDL as well as a reduction in hepatic apoB-Lp secretion in response to decreased hepatic cholesterol. These studies, in conjunction with the findings reported here, indicate that decreasing hepatic cholesterol or CE content can lead to reduced VLDL-cholesterol secretion, and that not only the number but also the lipid content of apoB-Lp particles can be altered.

Are these effects of hepatic cholesterol on VLDL-cholesterol secretion regulatory or are they simply attributable to substrate availability? In a review of cholesterol and hepatic lipoprotein assembly and secretion, Kang and Davis (64) noted that "While there are no data indicating that FC acts as a specific regulator of the assembly and secretion of apoB-containing lipoproteins, there are several reports suggesting that its availability can become rate-limiting. It remains unproven whether or not the inhibition of cholesterol synthesis is actually responsible for preventing VLDL assembly/secretion or if it is a secondary effect due to a more general impairment of the secretory pathway."

Our finding that ezetimibe decreases apoB-Lps but not HDL in LDLr-KO mice agrees with a recent study published by Repa et al. (37). Ezetimibe fed to LDLr-KO mice led to a 91% reduction in fractional intestinal cholesterol absorption, a 4.7-fold increase in fecal neutral sterol excretion, and an 18% decrease in plasma TC concentrations. Geiss, Otto, and Parhofer (65) also recently demonstrated that in humans, ezetimibe reduced cholesterol in almost all LDL subfractions, although hepatic cholesterol content and secretion were not determined in their study. Davis et al. (66) recently reported that the decreased plasma apoB-Lp cholesterol levels associated with ezetimibe feeding in apoE-KO mice reduced aortic atherosclerosis. Interestingly, in this mouse model with intact LDLr function, HDL-C levels were increased. Here, we extend these combined findings using in vivo kinetic studies to demonstrate that in the absence of the LDLr, the major mechanism leading to ezetimibe-induced apoB-Lp cholesterol lowering is the decreased production of apoB-Lps that are lipid-depleted. We also demonstrate that the impact of increased hepatobiliary sterol secretion on fecal cholesterol excretion by mice overexpressing ABCG5/G8 only in liver is greatly amplified by ezetimibe, which blocks the intestinal reabsorption of the biliary and dietary cholesterol. Thus, ezetimibe alters the flow of sterols via the enterohepatic circulation as well as the balance of cholesterol across the liver. Most importantly, we demonstrate that the previously unaltered aortic atherosclerosis in LDLr-KO mice overexpressing ABCG5/G8 only in liver was markedly reduced by ezetimibe feeding.

Our results demonstrate that a reduction of intestinal cholesterol absorption, in conjunction with increased secretion of cholesterol from liver into bile, can have a major effect on the lipoprotein profile and atherosclerosis, supporting the following working model. Increased hepatic expression of ABCG5/G8 enhances the amount of cholesterol secreted from liver into bile. Without ezetimibe, this additional cholesterol is merely reabsorbed by the intestine, necessarily forming chylomicrons that enter the intestinal lymph, are transported back to the liver, and ultimately are either secreted back into the bile or secreted into the plasma via the plasma lipoproteins. However, with ezetimibe feeding, intestinal cholesterol reabsorption is decreased markedly and more cholesterol is excreted into feces, resulting in a negative hepatic cholesterol balance. As a result, the liver cholesterol concentration is decreased, prompting compensatory mechanisms to restore the hepatic cholesterol content, which, in turn, affect the plasma levels of proatherogenic apoB-Lps and decrease atherosclerosis.

In summary, a 2-fold increase in biliary cholesterol secretion, resulting from liver-only expression of ABCG5/G8, is not sufficient to protect against atherosclerosis (23). Decreasing intestinal absorption by ezetimibe feeding results in net hepatic cholesterol excretion from the body, decreasing plasma apoB-Lps and protecting against ath-

erosclerosis. These studies emphasize the distinct roles of ABCG5/G8 in biliary sterol secretion and intestinal cholesterol absorption and establish the importance of concerted alterations in both ABCG5/G8 functions to affect atherogenesis.

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