Delineation of molecular changes in intrahepatic cholesterol metabolism resulting from diminished cholesterol absorption

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Abstract **The absorption of cholesterol by the small intestine is a major route for the net entry of cholesterol into the body and can therefore affect the plasma low density lipoprotein-cholesterol (LDL-C) concentration. These studies used ezetimibe, a potent inhibitor of cholesterol absorption, to delineate the biochemical and molecular changes in intrahepatic metabolism and biliary lipid secretion when there is a major reduction in chylomicron cholesterol delivery to the liver. In female LDL receptor (LDLR)-deficient ($LDLR^{-/-}$) mice fed a basal diet containing ezetimibe (0–10 mg/day/kg body weight), cholesterol absorption was reduced up to 91%, fecal neutral sterol excretion was increased up to 4.7-fold, and plasma total cholesterol concentrations decreased by up to 18%. Blocking cholesterol absorption prevented the accumulation of very low density lipoproteins and LDL in the circulation of $LDLR^{-/-}$ mice fed a lipid-rich diet. In female $LDLR^{+/+}$ mice fed the lipid-rich diet with ezetimibe, the relative mRNA level for the LDLR in the liver was 2-fold greater than in matching mice given the lipid-rich diet alone. We conclude that in the mouse the reduction in plasma LDL-C levels induced by blocking cholesterol absorption reflects both a diminished rate of LDL-C production and a modest increase in hepatic LDLR expression.**—Repa, J. J., S. D. Turley, G. Quan, and J. M. Dietschy. **Delineation of molecular changes in intrahepatic cholesterol metabolism resulting from diminished cholesterol absorption.** ***J. Lipid Res.*** **2005.** 46: **779–789.**

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Epidemiological data, together with the results of numerous clinical trials involving lipid-lowering drugs, show unequivocally that an increased plasma low density lipoprotein-cholesterol (LDL-C) concentration is a major risk factor for atherosclerosis (1–3). The plasma LDL-C concentration is determined principally by the liver, because not only is it the site of formation of VLDL, the precursor of most of the LDL in the circulation, but it is also the organ in which the bulk of receptor-mediated clearance of LDL takes place (4). Although loss of LDL receptor (LDLR) function results in familial hypercholesterolemia (5), studies in patients with other primary forms of hypercholesterolemia have shown that the plasma LDL-C concentration is regulated by both the rate of LDL production and catabolism (6).

The liver also initially clears all of the cholesterol that is absorbed from the small intestine and carried into the circulation in chylomicrons (7, 8). In the average adult consuming a typical Western diet, ~1,200–1,700 mg of cholesterol enters the lumen of the small bowel daily. Approximately 300–500 mg of this cholesterol comes directly from the diet, and the remainder is derived largely from bile (9). Given that in the average individual approximately half of all the cholesterol entering the small intestine is absorbed (10–12), hundreds of milligrams of chylomicron cholesterol are delivered to the liver each day. Hence, with the central role that the liver plays in determining the rates of LDL-C production and clearance, shifts in the level of cholesterol absorption can potentially lead to a significant change in the steady-state plasma LDL-C concentration. The sustained delivery of excess cholesterol from the intestine to the liver results in a compensatory suppression of de novo synthesis. If this suppression of synthesis fails to compensate fully for the uptake of sterol, then two other adaptive mechanisms come into play. One of these is an increased rate of cholesterol esterification in the hepatocytes (13). The quantity of cholesteryl esters

Abbreviations: ABCG1, adenosine triphosphate binding cassette transporter G1; apoA-I, apolipoprotein A-I; CYP, cytochrome P450; LDL-C, cholesterol carried in low density lipoprotein; LDLR, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; LXR, liver X receptor; NPC1L1, Niemann-Pick C1-like 1; SR-BI, scavenger receptor class B type 1.

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formed is a function not only of the amount of excess cholesterol available for esterification but also of the species of fatty acid that predominates in the liver cell (14, 15). The highest rates of ester formation in the liver occur when there is an enrichment with oleic acid or linoleic acid (16, 17). The formation of these esters drives lipoprotein-cholesterol secretion from the liver almost as a linear function of the concentration of cholesteryl esters in the cells (17). In this manner, an increase in cholesterol absorption can lead to an accelerated rate of LDL-C production. In addition to these events, an increase in hepatic cholesterol content can also be accompanied by the downregulation of LDLR expression and activity, and hence a lower rate of clearance of LDL particles from the circulation (14, 18–20). A second adaptive mechanism that helps prevent the accumulation of excess unesterified cholesterol in the hepatocyte involves activation of the liver X receptor (LXR), which in turn increases the expression of genes that regulate biliary sterol secretion and bile acid synthesis (21).

Although the changes in hepatic cholesterol metabolism that occur in response to shifts in the enterohepatic flux of sterols have been well studied, to date such changes have not been systematically defined at a molecular level. Thus, the main objective of the present studies was to delineate the relative changes at the mRNA level for a constellation of proteins in the liver that play critical roles in the intrahepatic handling of cholesterol and in regulating LDL-C metabolism and biliary lipid secretion when the enterohepatic circulation of cholesterol is interrupted in a major way. These studies took advantage of the availability of ezetimibe, a novel and potent inhibitor of cholesterol absorption (12, 22, 23), and the LDLR-deficient ($LDLR^{-/-}$) mouse, a model in which changes in circulating LDL-C levels reflect only changes in the rate of LDL-C production (17). The data for $LDLR^{-/-}$ mice and matching $LDLR^{+/+}$ mice fed lipid-rich diets with or without ezetimibe show that markedly inhibiting cholesterol absorption decreases the plasma LDL-C concentration through a profound reduction in LDL-C production and a modest upregulation of hepatic LDLR expression.

MATERIALS AND METHODS

Animals and diets

$LDLR^{-/-}$ mice were generated from breeding stock initially provided by Dr. Joachim Herz at this institution. The mutation was maintained on a mixed-strain background (C57BL/6:129/SvJae). As described in detail elsewhere, in such animals that lack LDLR activity, the concentration of LDL-C in the plasma mirrors directly the rate of cholesterol secretion from the liver in lipoproteins (i.e., the VLDL-C production rate) (17). The animals were fed ad libitum a cereal-based rodent diet (Wayne Lab Blox, No. 8604; Harlan Teklad, Madison, WI) that contained 0.02% (w/w) cholesterol and 5% total lipid. This was defined as the basal diet. In all studies, the mice were fed the powdered form of this diet, which in some experiments was enriched with both cholesterol (0.2%, w/w) and olive oil (10%, w/w). In the initial dose-response experiments, the mice were fed the basal diet containing only ezetimibe at levels that provided approximate doses of 0, 2.5, 5.0, and 10.0 mg/day/kg body weight (based on the consumption of 160 g diet/day/kg). In the subsequent studies with the lipid-rich diet, ezetimibe was provided in the diet at a single dose of 5 mg/day/kg. Female $LDLR^{-/-}$ mice were used for all studies except one, which used female 129/SvJae $LDLR^{+/+}$ animals. These $LDLR^{+/+}$ mice were derived from our own colony. All mice were 3 to 4 months of age at the time of study and were fed their experimental diets for 16–21 days, as specified. They were housed as previously described and studied in the fed state at the end of the 12 h dark phase of the lighting cycle (24). All experiments were approved by the Institutional Animal Care and Use Committee.

Tissue and plasma cholesterol and triacylglycerol concentrations and lipoprotein composition

Plasma and tissue cholesterol concentrations were determined by enzymatic or gas chromatographic methods (24). Plasma triacylglycerol concentrations were measured using Infinity Triglycerides Liquid Stable reagent (ThermoTrace, Noble Park, Australia). The same reagent was used to quantitate hepatic total triacylglycerol concentrations. In this assay, an aliquot of liver (300–400 mg) was extracted in chloroform:methanol (2:1, v/v) in the presence of [^{14}C]triolein (American Radiolabeled Chemicals, Inc., St. Louis, MO). Duplicate 5 ml aliquots of this extract were dried under air, and the residue was redissolved in 1 ml of hexane: methyl-t-butyl ether (100:1.5, v/v). This solution was run over a Sep-Pak Vac RC silica cartridge (500 mg; Waters Corp., Milford, MA). After elution of the cholesteryl esters, the solvent was switched to hexane: methyl-t-butyl ether (96:4, v/v) for separation of the triacylglycerols (25). This fraction was dried under air and redissolved in chloroform: methanol (2:1, v/v). Aliquots of this solution were in turn dried and used for the measurement of the recovered internal standard and the mass of triacylglycerol. Fractionation of pooled plasma samples was performed using fast-protein liquid chromatography on a Superose 6 HR 10/30 column (Amersham Biosciences Corp., Piscataway, NJ). The cholesterol content of the resulting 45 fractions was measured enzymatically (24). Plasma apolipoprotein A-I (apoA-I) and apoE levels were assessed qualitatively by electrophoretically size-fractionating plasma proteins on SDS-polyacrylamide 3–15% gradient gels and immunoblotting using rabbit anti-rat apoA-I and apoE antisera (26). Plasma apoB levels were similarly determined (27). The relative abundance of the various apolipoprotein bands was estimated by densitometry (model 300A; Molecular Dynamics, Inc., Sunnyvale, CA).

Bile acid pool size and composition, fecal bile acid and neutral sterol excretion, biliary lipid composition, and intestinal cholesterol absorption

Details of the methods used to measure these parameters have been described previously (24). Intestinal cholesterol absorption was determined as a fractional value (percentage) using a fecal dual-isotope ratio method (24). The molar ratio of cholesterol in gallbladder bile was calculated with respect to bile acid, phospholipid, and cholesterol combined and expressed as a percentage value. The rate of fecal bile acid excretion was taken as a measure of the rate of bile acid synthesis.

Tissue cholesterol synthesis

The rates of cholesterol synthesis in the liver, entire small intestine, and residual carcass were measured in vivo using [^{3}H]water as described previously (24). The rates of sterol synthesis in the liver and small intestine were calculated as nanomoles of [^{3}H]water incorporated into digitonin-precipitable sterols per hour per gram wet weight of tissue. Whole animal synthesis rates were determined as micromoles of [^{3}H]water incorporated into

sterols per hour per 100 grams of body weight. Rates of fatty acid synthesis in the liver were expressed as micromoles of [^{3}H]water incorporated into fatty acids per hour per gram of tissue.

Relative mRNA expression analysis

mRNA levels were measured using a quantitative real-time PCR assay (28). Total RNA was treated with DNase I (RNase-free; Roche) and reverse-transcribed with random hexamers using SuperScript II RNase H-reverse transcriptase to generate cDNA. Primer Express Software (Perkin-Elmer Life Sciences) was used to design the primers, for which sequences are given in **Table 1**. Primers were validated by analysis of template titration and dissociation curves. PCR assays were performed on an Applied Biosystems Prism 7000 sequence detection system. The PCR mixture contained (in a final volume of 20 μl) 50 ng of reverse-transcribed RNA, 150 nM of each primer, and 10 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems). All analyses were determined by the comparative Ct (cycle number at threshold) method (User Bulletin No. 2, Perkin-Elmer Life Sciences) using cyclophilin as the internal control. Relative mRNA levels were determined by expressing the amount of mRNA found relative to that obtained for mice fed the basal diet alone, which in each case was arbitrarily set at 1.0.

Statistical analysis of data

All data are reported as means ± SEM for the specified number of individual animals and are deemed significantly different at $P < 0.05$. GraphPad Prism software (GraphPad, San Diego, CA) was used to perform all statistical analyses. In the ezetimibe dose-response studies (depicted in Figs. 1, 2), one-way ANOVA was performed followed by Dunnett's postcomparison test of all groups with the control treatment. For the studies evaluating the effects of ezetimibe with a low-fat or high-fat diet (shown in Figs. 3, 5–8), two-way ANOVA was used (with the factors diet and drug). If a significant interaction was observed, all groups were compared by one-way ANOVA followed by Newman-Keuls posthoc comparison. In all cases, if unequal variance was evident by Bartlett's test, log transformation of data was performed before statistical analysis.

RESULTS

In the first set of experiments, various parameters of cholesterol metabolism were measured in female LDLR$^{-/-}$ mice fed a plain chow diet containing ezetimibe at levels that provided approximate doses of 0, 2.5, 5.0, and 10.0 mg/day/kg. At a dose of only 2.5 mg/day/kg, fractional cholesterol absorption decreased by 73%, and it decreased by 92% at the highest dose of the drug (**Fig. 1A**). The marked inhibition of cholesterol absorption was reflected in a dose-related increase in fecal neutral sterol excretion, which in the case of the mice fed the highest dose of ezetimibe was 4.7-fold greater than in matching mice fed

TABLE 1. Primer sequences used for the measurement of mouse hepatic RNA levels by quantitative real-time PCR

Gene	GenBank Number	Primer Sequences	Ct[a]	Reference
Abcg1	NM_009593	F: 5′-gctgtgcgttttgtgctgtt R: 5′-tgcagctccaatcagtagtcctaa	25.1	–[b]
Abcg5	NM_031884	F: 5′-tggatccaacacctctatgctaaa R: 5′-ggcaggttttctcgatgaactg	22.0	52
Apob	XM_137955	F: 5′-cgtgggctccagcattcta R: 5′-tcaccagtcatttctgcctttg	16.6	53
Cyclophilin	M60456	F: 5′-tggagagcaccaagacagaca R: 5′-tgccggagtcgacaatgat	19.9	52
Cyp3a	NM_007820	F: 5′-aggagaacaagggcagcatt R: 5′-gcagttcctgggtccaattc	15.0	–[b]
Cyp7a1	NM_007824	F: 5′-agcaactaaacaacctgccagtacta R: 5′-gtccggatattcaaggatgca	21.9	–[b]
Cyp27a1	NM_024264	F: 5′-gcctcacctatgggatcttca R: 5′-tcaaagcctgacgcagatg	19.9	–[b]
Hmg CoA Red	NM_008255	F: 5′-cttgtggaatgccttgtgattg R: 5′-agccgaagcagcacatgat	22.6	53
Hmg CoA Syn	NM_145942	F: 5′-gccgtgaactgggtcgaa R: 5′-gcatatatagcaatgtctcctgcaa	20.0	53
Ldlr	NM_010700	F: 5′gaggaactggcggctgaa R: 5′-gtgctggatggggaggtct	29.4	–[b]
Lpl	NM_008509	F: 5′-ggactgagaatggcaagcaa R: 5′-ccactgtgccgtacagagaaa	24.6	–[b]
Lrp	NM_008512	F: 5′-tgggtctcccgaaatctgtt R: 5′-accaccgcattcttgaagga	20.6	–[b]
Scd-1	NM_009127	F: 5′-ccggagacccccttagatcga R: 5′-tagcctgtaaaagatttctgcaaacc	17.0	53
Sr-bI	NM_016741	F: 5′-tccccatgaactgttctgtgaa R: 5′-tgcccgatgcccttga	20.7	–[b]
Srebp-1c	NM_011480	F: 5′-ggagccatggattgcacatt R: 5′-ggcccgggaagtcactgt	20.5	53

Abcg1, adenosine triphosphate binding cassette transporter G1; Cyp, cytochrome P450; F, forward; Hmg CoA Red, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; Hmg CoA Syn, 3-hydroxy-3-methylglutaryl coenzyme A synthase; Ldlr, low density lipoprotein receptor; Lpl, lipoprotein lipase; Lrp, low density lipoprotein receptor-related protein; R, reverse; Scd-1, stearoyl-CoA desaturase-1; Sr-bI, scavenger receptor class B type 1; Srebp-1c, sterol regulatory element binding protein-1C.

[a] Average cycle number at threshold for the control group (chow diet, no ezetimibe).

[b] Not previously published.

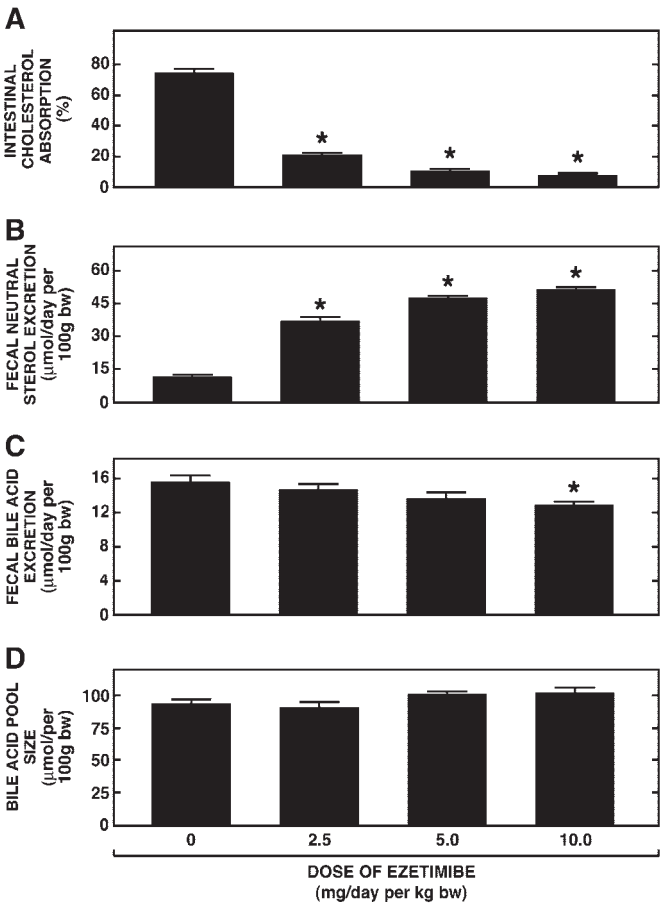

Fig. 1. Fractional cholesterol absorption, fecal neutral sterol excretion, and bile acid excretion and pool size in low density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice fed diets containing different levels of ezetimibe. Female $LDLR^{-/-}$ mice were housed individually and fed ad libitum for 17 days the basal diet containing ezetimibe at levels that provided doses of 0, 2.5, 5.0, and 10.0 mg/day/kg. A: Toward the end of the feeding period, the mice were dosed intragastrically with labeled sterols and their stools were collected over the next 3 days for the measurement of fractional cholesterol absorption. B and C: Another set of stools was collected from each mouse over the 3 days just before the cholesterol absorption measurements were done for the determination of rates of fecal neutral sterol (B) and bile acid (C) excretion. Fecal neutral sterols consisted of cholesterol and its derivatives coprostanol, epicoprostanol, and cholestanone. D: Separate matching groups of mice were used for the determination of bile acid pool size. Values represent means ± SEM of data from five to seven animals at each dose of ezetimibe. bw, body weight. * $P < 0.05$ compared with the value for the group not given ezetimibe.

chow alone (Fig. 1B). In contrast, the rate of bile acid synthesis, as measured by fecal bile acid excretion, did not change with ezetimibe treatment except at the highest dose, at which a marginal reduction was evident (Fig. 1C). Although there were no statistically significant changes in bile acid pool size (Fig. 1D), there was a trend toward cholic acid enrichment of the pool as the dose of ezetimibe was increased. The ratio of cholic acid to muricholic acid in the pool at the ezetimibe doses of 0, 2.5, 5.0, and 10.0 mg/day/kg equaled 2.59 ± 0.24, 3.18 ± 0.21, 3.23 ± 0.11, and 3.38 ± 0.37, respectively.

Separate, matching groups of mice were used for the measurement in vivo of the rate of sterol synthesis in the small intestine (**Fig. 2A**) and liver (Fig. 2B). At the highest dose of ezetimibe, the rate of synthesis in the small intestine and liver was increased 2.0- and 4.9-fold, respectively. Significant stimulation of synthesis was seen in both of these organs even at the dose of 2.5 mg/day/kg. A marginal increase in sterol synthesis in the residual carcass was also seen at this and higher doses of ezetimibe (data not shown). The data for synthesis in the intestine, liver, and carcass were used to calculate the rate of whole animal sterol synthesis. These rates, which were determined as micromoles of [^{3}H]water incorporated into sterols per hour per 100 grams of body weight, equaled 53.3 ± 2.8 in the mice fed the highest dose of the drug and 20.5 ± 1.6 in the mice fed chow only. The additional cholesterol was synthesized primarily in the liver (72%) and small intestine (11%). Despite the marked dose-related stimulation of hepatic sterol synthesis, the rate of fatty acid synthesis in the liver remained in the range of 30–39 μmol/h/g for all groups (data not shown). In the mice fed the highest dose of ezetimibe, the whole body cholesterol content was 210.7 ± 2.6 mg/100 g body weight versus 219.5 ± 2.5 mg/100 g body weight in the matching controls fed the basal diet alone. Significant reductions in the total cholesterol concentration in both the liver (Fig. 2C) and plasma (Fig. 2D) were seen at all doses of ezetimibe tested.

In the next set of studies, multiple parameters of hepatic lipid metabolism were determined in mice fed a lipid-rich diet alone or containing ezetimibe at a single dose (5 mg/day/kg). Matching groups of animals given the basal diet with or without ezetimibe at this dose were studied concurrently. The lipid-rich diet caused dramatic increases in the concentration of total cholesterol in both liver (**Fig. 3A**) and plasma (Fig. 3B). When ezetimibe was included in this diet, these increases were almost totally blocked. Hence, the hepatic and plasma cholesterol concentrations in these mice were similar to those found in the mice that were fed the basal diet with or without ezetimibe. The accumulation of triacylglycerol in the liver (Fig. 3C) and plasma (Fig. 3D) resulting from the lipid-rich diet was also significantly blunted by ezetimibe treatment.

Plasma from mice within each of these four groups was pooled and subjected to Western blot analysis (**Fig. 4A**) and fast-protein liquid chromatography (Fig. 4B). The lipid-rich diet caused a marked increase in the plasma content of both apoB (~4.5-fold) and apoE (~2.5-fold) but had no discernible effect on the level of apoA-I (Fig. 4A). These increases in the levels of apoB and apoE were prevented by ezetimibe. The plasma lipoprotein profile was also altered by ezetimibe, particularly in the mice fed the lipid-rich diet. As shown in Fig. 4B (upper panel), ezetimibe effected a broad reduction in the LDL fraction, as well as a slight decrease in the HDL fraction. These changes reflect the reduction in the plasma total cholesterol concentration seen at 5 mg ezetimibe/day/kg (Figs. 2D, 3B). In the mice fed the lipid-rich diet alone, there was a massive expansion of the pool of VLDL and LDL particles in the circulation (Fig. 4B, lower panel). This was

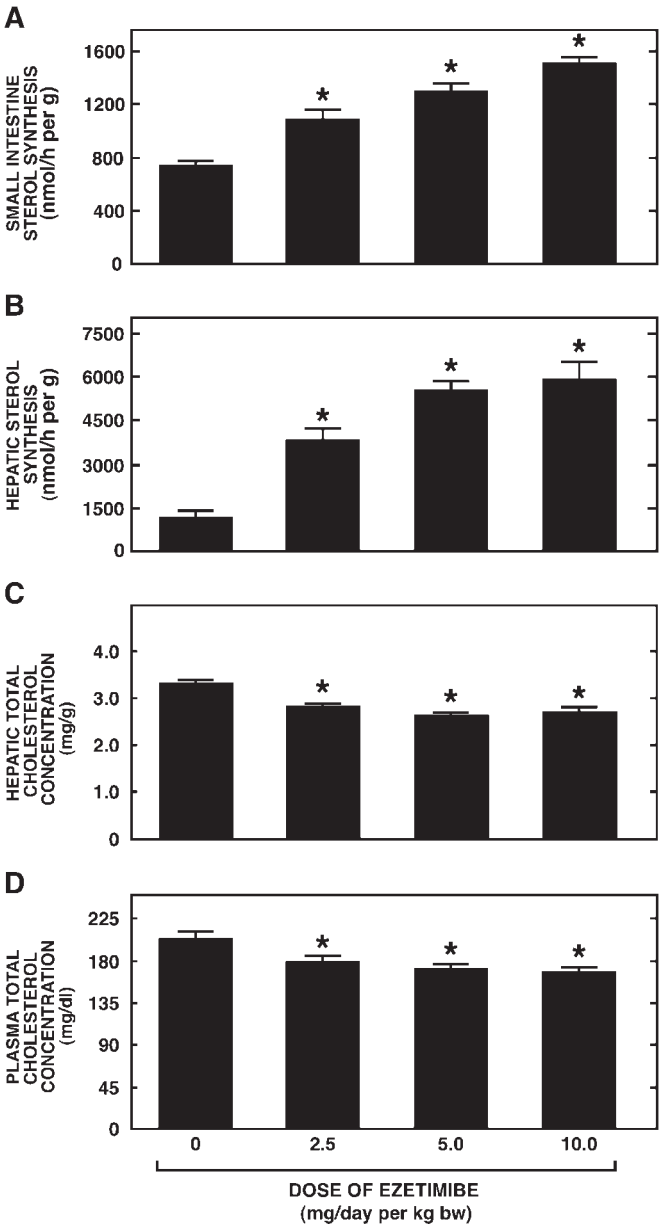

Fig. 2. Rate of sterol synthesis in small intestine and liver and total cholesterol concentration in liver and plasma of LDLR$^{-/-}$ mice fed diets containing different levels of ezetimibe. Female LDLR$^{-/-}$ mice were fed the same diets as those used in the study described in Fig. 1 for 16 days. The rate of sterol synthesis in the small intestine (A) and liver (B), and total cholesterol concentrations in liver (C) and plasma (D), were then measured. Rates of sterol synthesis are presented as nanomoles of [^{3}H]water incorporated into digitonin-precipitable sterols per hour per gram wet weight of tissue. Values represent means ± SEM of data from 6 or 7 animals (A–C) and from 13 animals (D) at each dose of ezetimibe. bw, body weight. * $P <$ 0.05 compared with the value for the group not given ezetimibe.

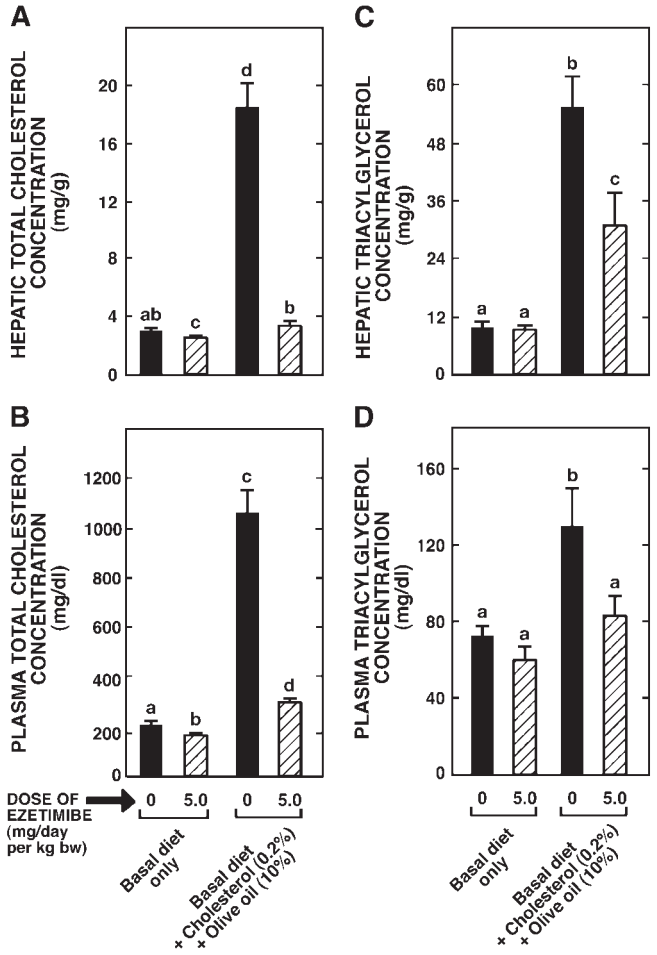

Fig. 3. Hepatic and plasma cholesterol and triacylglycerol concentrations in LDLR$^{-/-}$ mice fed a basal or lipid-rich diet with or without ezetimibe. Female LDLR$^{-/-}$ mice were fed for 21 days either the basal diet with or without ezetimibe (5 mg/day/kg) or the basal diet enriched with both cholesterol (0.2%, w/w) and olive oil (10%, w/w) with or without ezetimibe (5 mg/day/kg). Hepatic and plasma total cholesterol and triacylglycerol concentrations were then measured. Values represent means ± SEM of data from seven animals in each group. bw, body weight. Bars denoted by different letters are significantly different ($P < 0.05$).

essentially fully prevented by the addition of ezetimibe to the diet.

Gallbladder bile was harvested from the same mice used in the studies described in Figs. 3, 4. In the mice fed the basal diet with ezetimibe, there were no significant changes in the concentration of cholesterol (**Fig. 5A**), phospholipid (Fig. 5B), or bile acid (Fig. 5C) compared with those in mice given the basal diet alone. Hence, the molar ratio of cholesterol was unchanged (Fig. 5D). The lipid-rich diet fed alone clearly increased the absolute (Fig. 5A) and relative (Fig. 5D) cholesterol concentrations in the bile. These increases were prevented in mice given the lipid-rich diet with ezetimibe.

The relative mRNA expression of a constellation of genes involved in the regulation of hepatic cholesterol homeostasis was determined in the livers of the same mice used in the studies described in Figs. 3–5. The first group of genes investigated included four that predominantly regulate the rate of conversion of cholesterol to bile acids through several pathways. In the mice given the basal diet with ezetimibe, no significant changes were seen in either the rate of bile acid synthesis (measured as the rate of fecal bile acid excretion) (**Fig. 6A**) or the relative mRNA level for cytochrome P450 7A1 (CYP7A1; Fig. 6B), CYP27A1

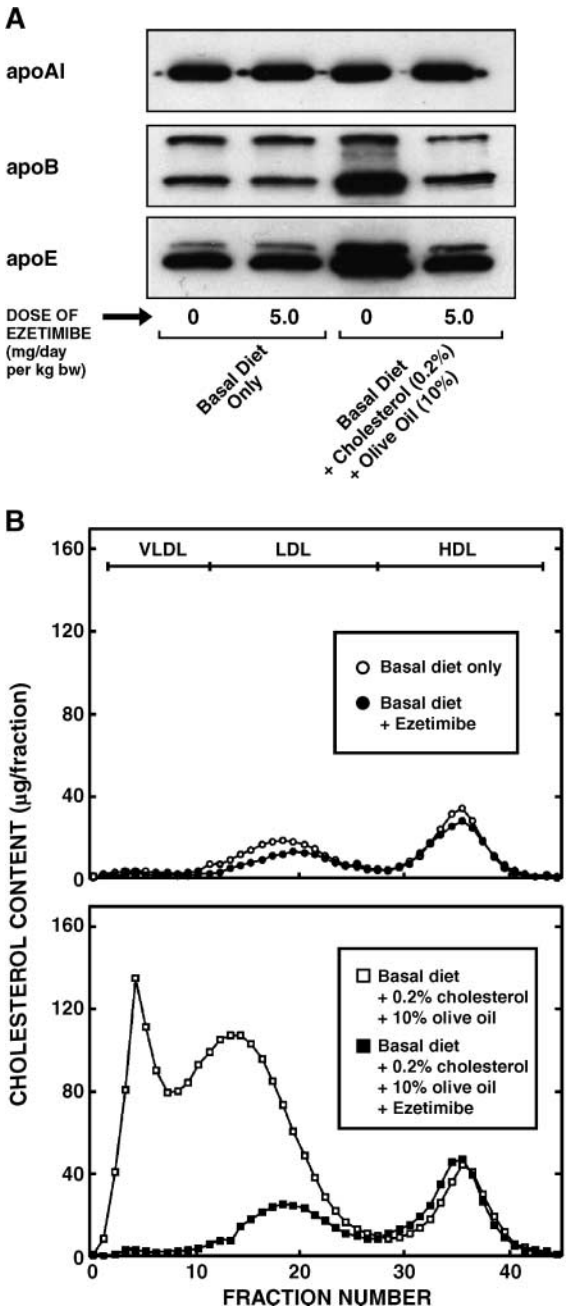

Fig. 4. Plasma lipoprotein profiles and apolipoprotein composition in LDLR$^{-/-}$ mice fed a basal or lipid-rich diet with or without ezetimibe. Plasma from the mice that were used in Fig. 3 was combined and subjected to Western blotting (A) or to fast-protein liquid chromatography (B). The results obtained represent the analysis of plasma combined from seven animals in each group. The Western blots were analyzed by densitometry. bw, body weight.

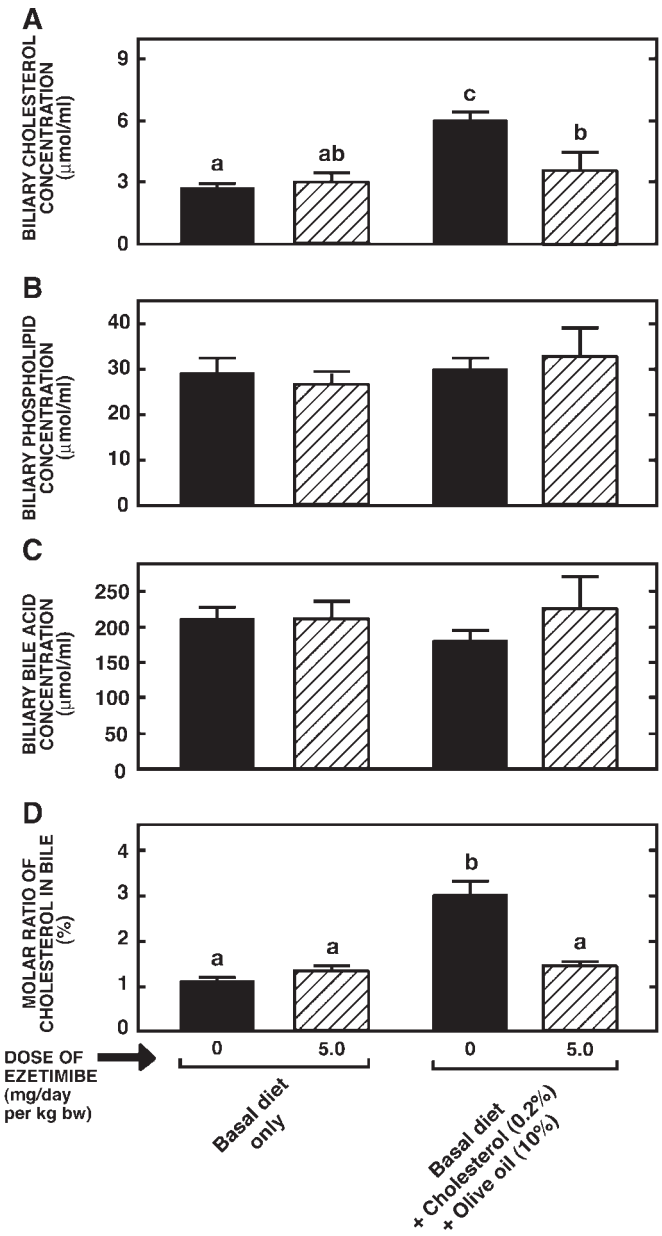

Fig. 5. Biliary lipid composition in LDLR$^{-/-}$ mice fed a basal or lipid-rich diet with or without ezetimibe. Gallbladder bile was harvested from mice that were fed the same diets as those used in the study described in Fig. 3. The absolute concentrations of cholesterol (A), phospholipids (B), and bile acid (C) were determined as described in Materials and Methods. These data were used to calculate the molar ratio of cholesterol relative to the combined content of bile acid, phospholipid, and cholesterol (D). Values represent means ± SEM of data from four to six animals in each group. bw, body weight. Bars denoted by different letters are significantly different ($P < 0.05$).

(Fig. 6C), CYP7B1 (Fig. 6D), and CYP3A11 (Fig. 6E). The feeding of the lipid-rich diet alone resulted in a significantly higher rate of bile acid excretion and mRNA level for CYP7A1. However, it did not affect the mRNA level for the other three genes involved in bile acid synthesis. These changes in bile acid synthesis and CYP7A1 message were blocked when ezetimibe was given with the lipid-rich diet.

The data in **Fig. 7** show the relative mRNA expression of 10 other genes in these same mice. Consistent with the hepatic sterol synthesis data in Fig. 2B, ezetimibe treatment led to a significant increase in mRNA level for both HMG-CoA reductase (Fig. 7A) and HMG-CoA synthase (Fig. 7B). This was seen in mice fed either the basal or the lipid-rich diet. In the latter case, the increase was particularly striking because the lipid-rich diet alone caused a decisive reduction in the expression of mRNA for both of

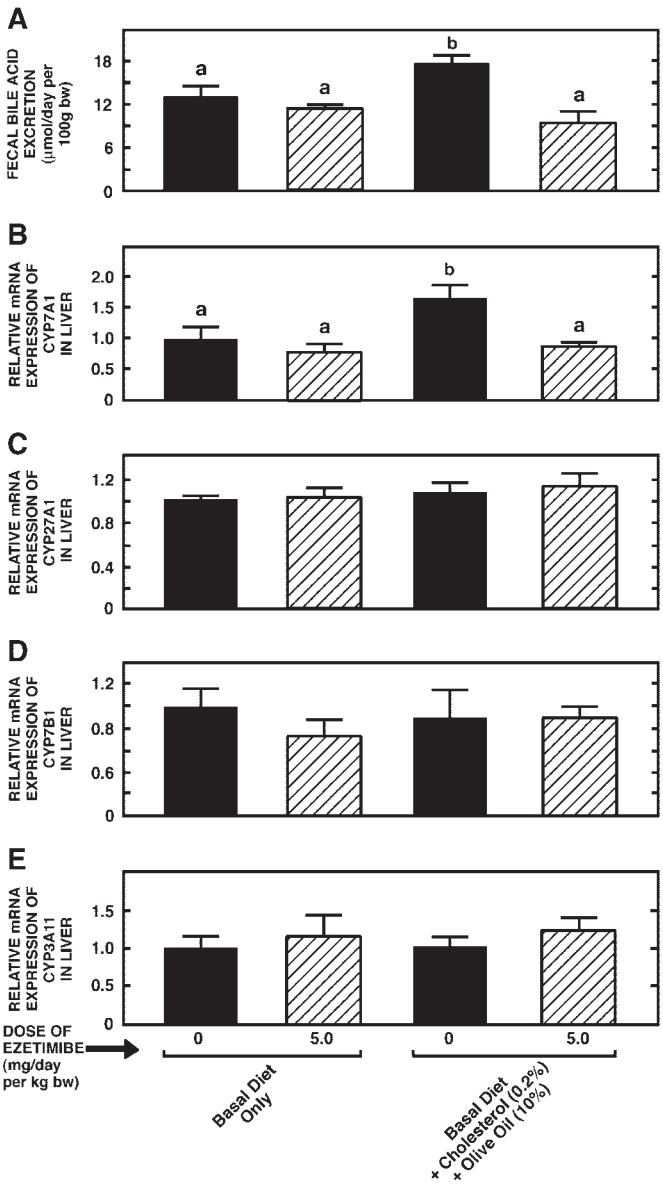

Fig. 6. Rate of fecal bile acid excretion and expression of mRNA for various proteins involved in bile acid synthesis in the livers of LDLR$^{-/-}$ mice fed a basal or lipid-rich diet with or without ezetimibe. Stools were collected over 3 days from the same animals that were used in the study described in Fig. 3. These were used to measure the rate of fecal bile acid excretion (A), which was taken to represent the rate of bile acid synthesis. At the end of the study, aliquots of liver from each mouse were taken for the measurement of the relative mRNA levels for cytochrome P450 7A1 (CYP7A1; B), CYP27A1 (C), CYP7B1 (D), and CYP3A11 (E) using real-time quantitative PCR. Values represent means ± SEM of data from seven animals in each group. Bars denoted by different letters are significantly different ($P < 0.05$).

these genes. In contrast, there was no consistent change in the mRNA expression for scavenger receptor class B type 1 (SR-BI; Fig. 7C), LDLR-related protein (LRP; Fig. 7D), and apoB (Fig. 7E) as a function of either the type of diet or the presence of ezetimibe.

The other five genes represented in Fig. 7 are all targets of the LXR (29, 30). In the mice given the basal diet with ezetimibe, there was no change in the mRNA level for adenosine triphosphate binding cassette transporter G1 (ABCG1; Fig. 7F), ABCG5 (Fig. 7G), sterol regulatory element binding protein-1C (Fig. 7H), stearoyl-CoA desaturase-1 (Fig. 7I), and LPL (Fig. 7J). However, the mRNA level for all five of these genes increased markedly in response to feeding the lipid-rich diet. The inclusion of ezetimibe in this diet kept the mRNA expression for all five genes at the same level as was seen in mice given the basal diet alone.

The final study measured the cholesterol concentration and the mRNA level for several proteins in the livers of LDLR$^{+/+}$ mice fed the basal or lipid-rich diet with or without ezetimibe at a dose of 5 mg/day/kg. As shown in **Fig. 8A**, ezetimibe was highly effective in preventing the marked increase in hepatic cholesterol levels caused by the lipid-rich diet. Irrespective of whether the drug was fed with the basal or lipid-rich diet, it produced a dramatic compensatory increase in hepatic cholesterol synthesis, as judged from the relative mRNA level for HMG-CoA synthase (Fig. 8B). Qualitatively, these responses were almost identical to those described earlier for the LDLR$^{-/-}$ mice (Figs. 3A, 7B). In the LDLR$^{+/+}$ mice, there were also changes in the mRNA level for the LDLR (Fig. 8C), but these were modest compared with those for HMG-CoA synthase. Thus, in the group fed just the lipid-rich diet, the relative mRNA for LDLR was 27% lower than that in matching mice given the basal diet alone. The addition of ezetimibe to the lipid-rich diet resulted in relative mRNA levels for LDLR that were approximately double those in the group fed the lipid-rich diet alone. In contrast to the mRNA levels for LDLR, those for LRP (Fig. 8D) in these same four groups of mice did not vary consistently as a function of either the type of diet or the presence of ezetimibe.

DISCUSSION

The use of the LDLR$^{-/-}$ mouse as a model for pharmacologic, pathologic, and dietary studies is well described (17, 31–34). Given their lack of LDLR activity, these mice can be used to directly determine the extent to which changes in the plasma LDL-C concentration reflect changes in the rate of LDL-C production when the enterohepatic flux of sterols is experimentally manipulated. Female LDLR$^{-/-}$ mice fed a basal low-cholesterol rodent diet manifest a total plasma cholesterol concentration of ~200 mg/dl, of which ~50% is contained in LDL (33). Treating them with ezetimibe dramatically reduced fractional cholesterol absorption (Fig. 1A) and significantly decreased the plasma total cholesterol concentration (Fig. 2D). Analysis of the plasma lipoprotein composition by HPLC revealed most of the reduction to be in the LDL fraction. Thus, in these mice, as in patients with homozygous familial hypercholesterolemia (35), the LDL-C-lowering action of ezetimibe clearly involved a decrease in LDL production. This finding was explored further by giving ezetimibe to LDLR$^{-/-}$ mice fed a diet enriched with cholesterol and olive oil, a formulation known to drive hepatic lipoprotein

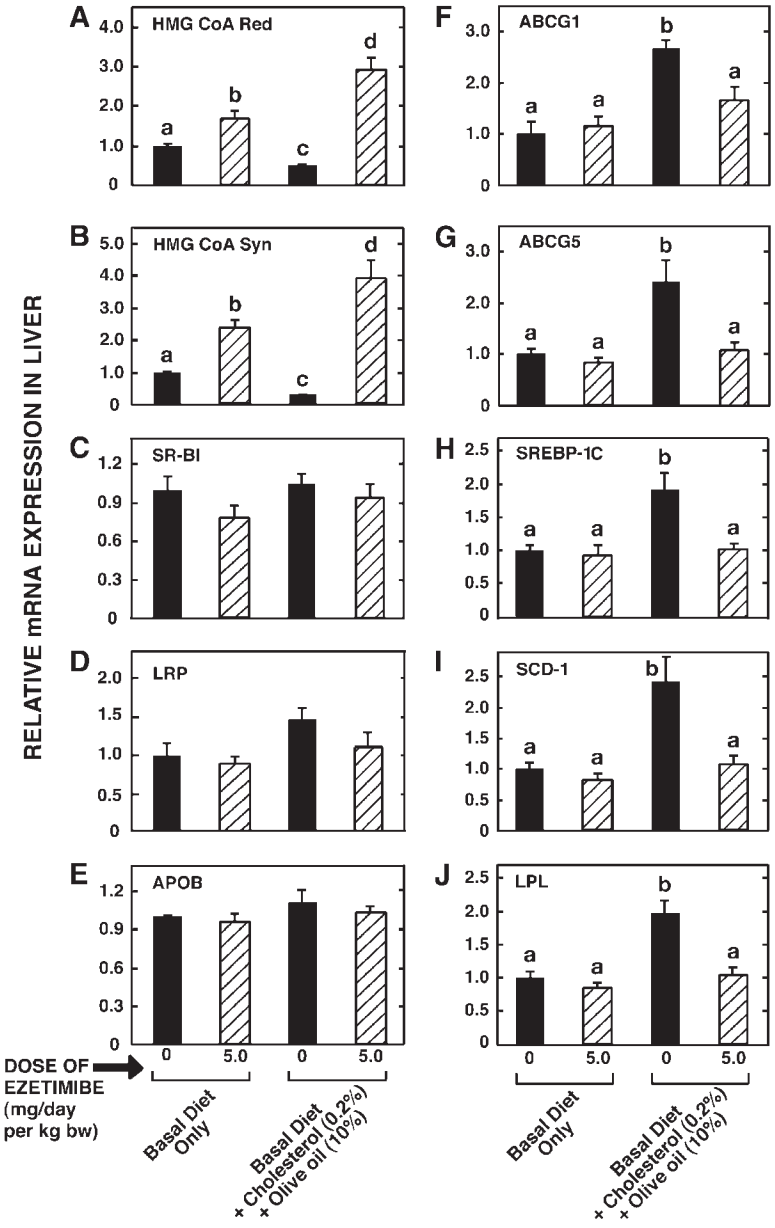

Fig. 7. Relative level of expression of mRNA for multiple proteins involved in cholesterol metabolism in the livers of LDLR$^{-/-}$ mice fed a basal or lipid-rich diet with or without ezetimibe. Aliquots of liver from the same mice used in the study described in Figs. 3, 6 were used for real-time quantitative PCR analysis. Values represent means ± SEM of data from seven animals in each group. ABCG1, adenosine triphosphate binding cassette transporter G1; apoB, apolipoprotein B; bw, body weight; HMG CoA Red, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMG CoA Syn, 3-hydroxy-3-methylglutaryl coenzyme A synthase; LRP, LDLR-related protein; SCD-1, stearoyl-coenzyme A desaturase-1; SR-BI, scavenger receptor class B type 1; SREBP-1C, sterol regulatory element binding protein-1C. Bars denoted by different letters are significantly different ($P < 0.05$).

secretion in this model (17). The marked hypercholesterolemia that developed was characterized by a massive accumulation of apoB/apoE-rich particles in the VLDL and LDL fractions (Fig. 4A, B). Ezetimibe essentially prevented these profound shifts in plasma lipoprotein composition. The increase in plasma apoB content occurred in the face of a nearly constant relative mRNA level for APOB in the liver (Fig. 7E). This is consistent with other findings that apoB synthesis does not regulate the rate of hepatic nascent VLDL secretion (36, 37). Hepatic mRNA levels for both SR-BI (Fig. 7C) and LRP (Fig. 7D) also did not change with either the type of diet or ezetimibe treatment. This was in contrast to the pronounced changes in the relative mRNA levels for HMG-CoA reductase (Fig. 7A) and HMG-CoA synthase (Fig. 7B), which showed that, by inhibiting sterol absorption, ezetimibe elicited a compensatory increase in hepatic cholesterol synthesis, even in the mice given the lipid-rich diet.

The potency of ezetimibe in preventing the flow of excess chylomicron cholesterol to the liver was equally apparent from the fact that the cholesterol-mediated increase in mRNA levels for several LXR-regulated proteins, in particular ABCG5/8, seen with the lipid-rich diet did not occur when the same diet contained ezetimibe. Together, then, these data demonstrate that the cascade of changes in intrahepatic cholesterol metabolism and the resultant increase in plasma LDL-C levels that ensue from the absorption of excess cholesterol are preventable by imposing a pharmacologic block on the uptake of cholesterol across the brush border membrane of the enterocyte. The fact that this can be achieved in the absence of any hepatic LDLR activity clearly suggests a major effect of diminished chylomicron cholesterol delivery to the liver on the rate of cholesterol secretion in VLDL and, hence, on LDL-C production. The triacylglycerol-lowering action of ezetimibe seen in these mice has been reported previ-

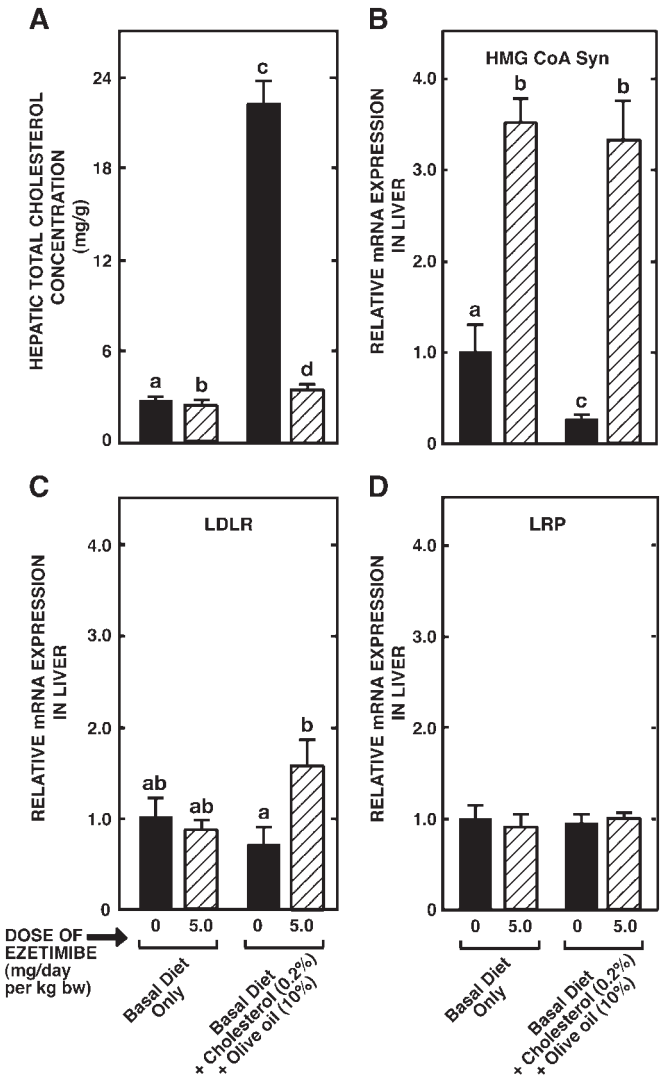

Fig. 8. Cholesterol concentration and relative level of expression of mRNA for several proteins in the livers of LDLR$^{+/+}$ mice fed a basal or lipid-rich diet with or without ezetimibe. Female LDLR$^{+/+}$ mice (129/SvJae) were fed ad libitum for 16–21 days either the basal diet with or without ezetimibe (5 mg/day/kg) or the basal diet enriched with both cholesterol (0.2%, w/w) and olive oil (10%, w/w) with or without ezetimibe (5 mg/day/kg). Hepatic cholesterol concentration (A) and the relative level of expression of mRNA for HMG-CoA synthase (HMG CoA Syn; B), LDLR (C), and LRP (D) were determined as described in Materials and Methods. Values represent means ± SEM of data from four to six animals in each group. bw, body weight. Bars denoted by different letters are significantly different ($P < 0.05$).

ously in hamsters (38). Given that ezetimibe does not block fat absorption (39), its action in decreasing hepatic and plasma triacylglycerol concentrations is likely a secondary effect of diminished cholesterol delivery and accumulation in the liver.

The present studies also investigated whether ezetimibe treatment increased the rate of receptor-mediated clearance of LDL by the liver. This was done indirectly by determining the relative mRNA levels for the LDLR in the livers of 129/SvJae mice given the same low- and high-lipid diets with or without ezetimibe that were fed to the LDLR$^{-/-}$ mice. The effects of ezetimibe on LDLR mRNA levels were clearly more discernible in the groups of mice fed the lipid-rich diet (Fig. 8C). Although feeding this diet alone caused only a slight reduction in the LDLR mRNA level, concurrent treatment with ezetimibe clearly increased it. The magnitude of change in the hepatic LDL clearance rate associated with this increase in mRNA level cannot be determined from these data. However, earlier studies in hamsters fed various lipid-enriched diets showed that changes in hepatic mRNA levels for the LDLR faithfully reflected changes in LDLR protein as well as LDL clearance rates (20). The combined data from the LDLR$^{-/-}$ mice and their LDLR$^{+/+}$ counterparts thus demonstrate that although the major mechanism for the decreased plasma LDL-C levels with ezetimibe is diminished hepatic lipoprotein secretion, an increase in receptor-mediated clearance of LDL by the liver also likely accounts for part of the cholesterol-lowering action of this drug. These findings are also fully consistent with studies by other investigators showing that ezetimibe treatment essentially prevents the development of atherosclerosis in apoE-deficient mice fed an atherogenic diet (40).

The second main conclusion relates to the changes in bile acid metabolism found when ezetimibe was added to the basal and lipid-rich diets. In hyperlipidemic patients given 10 mg of ezetimibe daily, intestinal cholesterol absorption was inhibited on average by 54%, but there was no statistically significant change in bile acid synthesis, as measured by fecal bile acid excretion (12). In LDLR$^{-/-}$ mice fed the basal diet, there was a trend toward a lower rate of bile acid synthesis as the dose of ezetimibe was increased (Fig. 1C). At the maximal dose of 10 mg/day/kg, cholesterol absorption was inhibited by 92% and bile acid excretion was 18% lower than in mice fed the basal diet alone. Although this reduction was trivial compared with the 4.7-fold increase in fecal neutral sterol excretion seen at this dose of ezetimibe (Fig. 1B), it was nevertheless a signal that the rate of conversion of cholesterol to bile acids decreased when there was a nearly complete block in cholesterol absorption. At the highest dose of ezetimibe, the concentration of cholesterol in the liver was 19% less than it was in untreated mice (Fig. 2C). This occurred despite a 4.9-fold increase in hepatic cholesterol synthesis (Fig. 2B). Although ezetimibe treatment did not affect bile acid pool size, it did increase consistently, to a modest degree, the proportion of cholic acid in the pool relative to that of muricholic acid. In other mouse models, cholic acid enrichment of the bile acid pool increases the level of cholesterol absorption (24, 41). Thus, the finding that ezetimibe inhibited cholesterol absorption in a dose-related manner in the face of a more cholic acid-rich pool clearly attests to the potency of this drug in blocking the facilitated uptake of sterols by the enterocytes. These data are fully consistent with those reported for ezetimibe-treated Niemann-Pick C1-like 1 (NPC1L1$^{+/+}$) and NPC1L1$^{-/-}$ mice fed a cholic acid-enriched diet (42).

The feeding of the cholesterol/olive oil-rich diet resulted in a modest but significant increase in the rate of bile acid synthesis (fecal bile acid excretion) and a paral-

lel increase in the relative mRNA level for CYP7A1, but not for any of the other major enzymes involved in bile acid synthesis (Fig. 6A–E). These increases were not seen when ezetimibe was given with the lipid-rich diet. Presumably, by blocking the delivery of much of the excess dietary cholesterol to the liver, there was no signal for the LXR-mediated induction of the primary pathway of bile acid synthesis initiated by CYP7A1. The observation that the relative mRNA level for CYP3A11 in the liver was unaffected by ezetimibe irrespective of the type of diet it was added to is an important confirmation of earlier reports that ezetimibe does not affect this CYP drug-metabolizing system (43). In summary, bile acid metabolism is not directly affected by ezetimibe but, at least in mice, may manifest some changes of a secondary nature depending on the dose of the drug and the type of diet that it is administered with.

The third main conclusion relates to the question of whether the marked stimulation of hepatic cholesterol synthesis stemming from the inhibition of cholesterol absorption affects biliary lipid composition, in particular the absolute and relative levels of cholesterol in the bile. In the mice fed the basal diet with ezetimibe at a dose of 5 mg/day/kg, both the absolute concentration (Fig. 5A) and molar ratio (Fig. 5D) of cholesterol in the bile remained unchanged in the face of a 4.6-fold increase in cholesterol synthesis by the liver (Fig. 2B). In contrast, feeding the lipid-rich diet alone significantly increased the level of biliary cholesterol (Fig. 5A, D). This occurred in parallel with an almost 6-fold increase in hepatic cholesterol concentration (Fig. 3A). When this increase was blocked with ezetimibe, biliary cholesterol levels were no different from those in mice given the basal diet alone. These findings thus demonstrate that, at least in this species, the inhibition of cholesterol absorption by ezetimibe reverses diet-related increases in biliary cholesterol levels. Similar results have been reported for apolipoprotein E*3-Leiden transgenic mice fed a high-cholesterol diet containing plant stanol esters (44). Previous studies in humans given various agents that inhibit cholesterol absorption found no adverse effects on biliary cholesterol saturation (45–48).

The final point relates to the pathophysiologic relevance of these data to the cause and treatment of atherosclerosis in humans. Data from a number of sources make the compelling argument that the optimal LDL-C concentration for humans is in the range of 50–70 mg/dl (49). This is approximately half the estimated average LDL-C level in American adults. Other studies show that in apparently healthy adult subjects the total cholesterol concentration in the liver averages from 4 to more than 5 mg/g tissue (50, 51). Such values, which are approximately double those typically found in animal models maintained on diets with a low cholesterol and triacylglycerol content, are not unexpected given that in individuals consuming a Western diet hundreds of milligrams of chylomicron cholesterol are delivered to the liver daily. The present data show that imposing a pharmacologic block on this process at the level of the brush border membrane of the enterocyte diminishes the rate of LDL-C production, prevents the downregulation of hepatic LDLR expression, and maintains the plasma LDL-C concentration in a normal range.

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