Inhibition of cholesterol absorption by SCH 58053 in the mouse is not mediated via changes in the expression of mRNA for ABCA1, ABCG5, or ABCG8 in the enterocyte

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Abstract **Intestinal cholesterol absorption is a major determinant of plasma low density lipoprotein-cholesterol (LDL-C) concentrations. Ezetimibe (SCH 58235) and its analogs SCH 48461 and SCH 58053 are novel potent inhibitors of cholesterol absorption whose mechanism of action is unknown. These studies investigated the effect of SCH 58053 on cholesterol metabolism in female 129/Sv mice. In mice fed a low cholesterol rodent diet containing SCH 58053, cholesterol absorption was reduced by 46% and fecal neutral sterol excretion was increased 67%, but biliary lipid composition and bile acid synthesis, pool size, and pool composition were unchanged. When the dietary cholesterol content was increased either 10- or 50-fold, those animals given SCH 58053 manifested lower hepatic and biliary cholesterol concentrations than did their untreated controls. Cholesterol feeding increased the relative mRNA level for adenosine triphosphate-binding cassette transporter A1 (ABCA1), ABC transporter G5 (ABCG5), and ABC transporter G8 (ABCG8) in the jejunum, and of ABCG5 and ABCG8 in the liver, but the magnitude of this increase was generally less if the mice were given SCH 58053. We conclude that the inhibition of cholesterol absorption effected by this new class of agents is not mediated via changes in either the size or composition of the intestinal bile acid pool, or the level of mRNA expression of proteins that facilitate cholesterol efflux from the enterocyte, but rather may involve disruption of the uptake of luminal sterol across the microvillus membrane.**—Repa, J. J., J, M. Dietschy, and S. D. Turley. **Inhibition of cholesterol absorption by SCH 58053 in the mouse is not mediated via changes in either the intestinal bile acid pool or the expression of mRNA for ABCA1, ABCG5, or ABCG8 in the enterocyte.** ***J. Lipid Res.*** **2002.** 43: **1864–1874.**

Supplementary key words liver • small intestine • bile acid • cholesterol efflux • cholesterol synthesis • fecal neutral sterol

Elevated plasma levels of LDL cholesterol (LDL-C) constitute a major risk factor for the development of atherosclerosis (1). The cholesterol carried in LDL, like all the other cholesterol in the body, is derived from de novo synthesis and absorption from the diet (2). In humans, the plasma LDL-C concentration often correlates positively with the level of intestinal cholesterol absorption (3, 4). This association is also seen in various primate models that have been identified as being hypo- or hyperresponsive to a dietary cholesterol challenge (5–8). The wide individual variation in cholesterol absorption seen within most species, including humans (9), has been well characterized in different strains of mice (10–12). Recent studies in this species demonstrate that such variability results from the interplay of multiple genes (12, 13).

The major cellular and biochemical steps involved in the translocation of cholesterol from the intestinal lumen to the lymph have been described in detail (14, 15). The efficiency with which cholesterol is absorbed can be changed dramatically by manipulating many of these steps. It is well documented, for example, that shifts in either the size or composition of the intestinal bile acid pool, or in the amount and species of biliary phospholipid entering the lumen, can result in profound changes in the level of cholesterol absorption (16–19). Deleting the gene responsible for apoB synthesis in the intestine or inhibiting the activity of acylCoA:cholesterol acyltransferase (ACAT) can also result in dramatic changes in the amount of cholesterol reaching the lymph from the intestinal lumen (16, 20, 21).

Although the rate limiting step in cholesterol absorp-

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Abbreviations: ABCA1, adenosine triphosphate-binding cassette transporter A1; ABCB4, ABC transporter B4 (MDR2/3); ABCB11, ABC transporter B11 (SPGP/BSEP); ABCG5, ABC transporter G5; ABCG8, ABC transporter G8; ACAT-2, acyl CoA:cholesterol acyltransferase-2; DPS, digitonin-precipitable sterols; HMG-CoA red, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HMG-CoA syn, 3-hydroxy-3-methylglutaryl coenzyme A synthase.

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tion remains unknown, several recent studies have provided new insights into the mechanism(s) that may ultimately dictate how much cholesterol gets absorbed. One group of studies describes the search for a sterol permease in the microvillus membrane of the enterocyte that facilitates the uptake of cholesterol and potentially other sterols after their release from mixed micelles (22, 23). Initially, there was some evidence that scavenger receptor class B, type 1 (SR-BI) might be the putative sterol transporter (24). However, in mice lacking SR-BI, sterol absorption is not reduced (25, 26). If it can be demonstrated unequivocally that the uptake of cholesterol by the enterocyte is protein facilitated, then clearly such a transporter would make an attractive target for pharmacologic intervention.

The other key discovery relates to the role that ABC transporter G5 (ABCG5) and ABC transporter G8 (ABCG8) play in preventing or impeding the absorption of plant sterols and stanols (27, 28). Mutations in the genes for these two proteins result in sitosterolemia, a rare autosomal recessive disorder characterized by hyperabsorption of sitosterol and other plant sterols (29, 30). Sitosterolemic individuals also absorb cholesterol more efficiently and are often hypercholesterolemic, implying a role of ABCG5 and ABCG8 in dictating the efficiency of cholesterol absorption.

The quest to learn more about the role of specific proteins in regulating the flux of sterols into and out of the enterocyte is timely given the recent development of a new class of novel, selective, and potent cholesterol absorption inhibitors. Ezetimibe (SCH 58235) and an analog, SCH 48461, inhibit cholesterol absorption at very low doses and exert a marked hypocholesterolemic effect in humans (31–34) and an array of different animal models (26, 35–40). Ezetimibe is glucuronidated in the enterocyte during its first pass. Both ezetimibe and its glucuronide are circulated enterohepatically, repeatedly delivering the agent back to the site of action on the luminal surface of the enterocyte (41). The molecular mechanism by which this agent inhibits cholesterol absorption is, however, unknown.

In the present studies, we used the 129/Sv mouse, a strain distinguished by its inherently high levels of cholesterol absorption (10–12), to learn more about how another one of the ezetimibe analogs, SCH 58053, mediates it cholesterol lowering effect. The data show that this agent does not change the physicochemical nature of the intraluminal environment, nor does it increase the expression of proteins that drive sterol efflux from the enterocyte. We speculate that, instead, it mediates its inhibitory action at the level of a putative sterol permease that facilitates the movement of cholesterol into the intestinal cell.

MATERIALS AND METHODS

Animals and diets

The female 129/Sv mice used in these studies were generated in our own colony from 129/SvEvBrd-Hprt$^{b\text{-}m2}$ breeding stock as described earlier (11). All experiments with mice used this strain except one study that utilized female LDL-receptor (LDLR) knockout mice. Those mice were of a mixed 129/Sv:C57BL/6 background, and were also bred in our own colony. All mice were housed either as groups or individually in plastic colony cages containing wood shavings in a temperature controlled room (22°C) with light cycling. At the time of study, the mice were about 13- to 20-weeks-old. They had access to drinking water at all times and were fed ad libitum a pelleted cereal-based rodent diet (Wayne Lab Blox, No. 8604; Harlan Teklad, Madison, WI). This formulation (basal diet) had an inherent cholesterol content of 0.02% (w/w) and a fatty acid composition as described elsewhere (20). The meal form of this diet was used to prepare the experimental diets which, in some of the studies, also contained additional cholesterol (final levels of either 0.20 or 1.00% w/w). These diets were prepared without or with SCH 58053 at a level of 0.021% (w/w). Based on their daily food consumption of approximately 165 g diet/kg body weight, this dietary level of SCH 58053 provided the mice with a dose of ~35 mg/day/kg body weight. The experimental diets were fed from 10 to 23 days, depending on the types of measurements that were made. All experiments were performed toward the end of the 12-h dark phase of the lighting cycle, and all animals were in the fed state at the time of study. Experiments were approved by the Institutional Animal Care and Research Advisory Committee.

SCH 58053

The SCH 58053 used in these studies was supplied by the Schering-Plough Research Institute (Kenilworth, NJ). SCH 58053 ((+)-7-(4-chlorophenyl)-2-(4fluorophenyl)-7-hydroxy-3(*R*)-(4-hydroxyphenyl)-2-azaspiro[3.5]nonan-1-one) is an analog of ezetimibe (SCH 58235) ((-)-1-(4-fluorophenyl)-(3*R*)-[3-(4-fluorophenyl)-(3*S*)-hydroxypropyl]-(4*S*)-(4-hydroxyphenyl)-2-azetidinone). **Figure 1** shows the respective structures of SCH 58053 (A) and SCH 58235 (B).

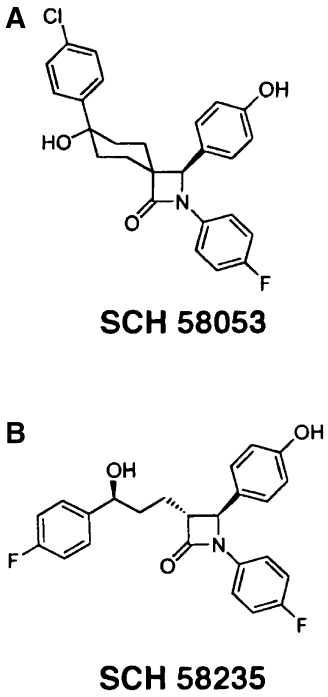

Fig. 1. Structure of SCH 58053, an analog of ezetimibe (SCH 58235). The complete chemical names for both compounds are given in Materials and Methods.

Sterol synthesis in liver and extrahepatic organs

The rate of sterol synthesis in all major organs was measured in vivo as described (42). Mice were given an ip injection of 40 mCi of [^{3}H]water (NEN Life Science Products, Boston, MA) and after 1 h were anesthetized and exsanguinated. Aliquots of liver, the entire small intestine, and the remaining carcass were saponified and their content of radiolabeled digitonin-precipitable sterols (DPS) was measured. The rate of sterol synthesis in each organ was expressed as the nmol of [^{3}H]water incorporated into DPS/h/g of tissue, while whole animal synthesis was calculated as μmol of [^{3}H]water incorporated/h/100 g body weight.

Intestinal cholesterol and lipid absorption

Cholesterol absorption was measured by a fecal dual-isotope ratio method. Mice were dosed ig with a mixture of 2 μCi [5,6-^{3}H]sitostanol (American Radiolabeled Chemicals, Inc., St. Louis, MO) and 1 μCi [4-^{14}C]cholesterol (NEN Life Science Products, Boston, MA). They were then housed individually in fresh cages and stools were collected over the following 3 days. Aliquots of stool and the dosing mixture were extracted, and the ratio of ^{14}C to ^{3}H in each was determined. The percent cholesterol absorption was calculated from these data as described (42). To determine the level of total lipid absorption, the lipid content of the diet and stools was determined gravimetrically. These data, together with the amount of diet consumed and stool excreted (both expressed as g/day/100 g body weight), were used to calculate the fraction of lipid consumed that was absorbed.

Fecal bile acid and neutral sterol excretion

Stools collected from individually housed mice over 3 days were dried, weighed, and ground to a fine powder. A 1-g aliquot of this material was used to determine total bile acid content by an enzymatic method previously described (42). A second 1-g aliquot was used to quantitate the amounts of cholesterol, coprostanol, epicoprostanol, and cholestanone by GC as described in detail elsewhere (42). The excretion rates of both bile acids and neutral sterols were expressed as μmol/day/100 g body weight.

Bile acid pool size and composition

Pool size was determined as the total bile acid content of the small intestine, gallbladder, and liver, which were extracted in ethanol in the presence of an internal standard ([24-^{14}C]taurocholic acid, NEN Life Science Products) and analyzed by high performance liquid chromatography (HPLC) (42). Bile acids were detected by measurement of the refractive index and identified by comparison to authentic standards. Pool size was expressed as μmol/100 g body weight.

Biliary lipid composition

Gallbladder bile was harvested from mice in the fed state. The gallbladder with contents was carefully excised and placed in a small microfuge tube (0.5 ml capacity). The gallbladder was punctured with a 23 gauge needle and the tube was then centrifuged at 2,000 rpm for 5 min in a tabletop centrifuge (Sorvall RT7), Dupont Co., Newtown, CT) fitted with a swinging bucket rotor (RTH-750). An aliquot of bile (10 μl to 20 μl) was then extracted in 1.0 ml of methanol containing 50 μg of stigmastanol. The respective concentrations of bile acid, phospholipids, and cholesterol in the methanolic extract were determined as described (42), and these data were used to calculate the molar percent of cholesterol in the bile.

Relative cholesterol and bile acid concentrations in liquid phase of intestinal contents

The proximal half of the small intestine was cut into four sections. The contents of each section were removed by gentle finger stripping and combined in a single microfuge tube The tubes were centrifuged at 4,000 rpm for 15 min at room temperature in a tabletop centrifuge (Sorvall RT7, Dupont, Newtown, CT). A 25 μl aliquot of the resulting supernatant was extracted in 1 ml of methanol. The absolute concentrations of cholesterol and bile acid in each extract were measured as described for the bile samples.

Plasma and tissue cholesterol levels

Plasma and tissue total cholesterol concentrations were determined by enzymatic or gas chromatographic methods as described (42). The latter used stigmastanol as an internal standard. In the studies with mice fed cholesterol enriched diets, the cholesterol concentration was measured in the plasma and liver, but not in the small intestine because the entire jejunal mucosa from each mouse was needed for the mRNA analyses as described below.

RNA analysis

Following exsanguination of the mice under ether anesthesia, aliquots of liver and the entire jejunal mucosa from each animal were frozen in liquid nitrogen. Total RNA was prepared from these tissues, and mRNA was further purified using oligo-dT resin from individual livers or a pool composed of equal amounts of jejunal RNA from the mice of each group. Five micrograms of poly(A)$^+$ RNA was fractionated by electrophoresis, transferred to membrane, and subjected to Northern analysis using the ^{32}P-labeled probes indicated. The amount of radioactivity in each band was quantified by phosphorimager, normalized to the signal generated by β-actin, and mathematically adjusted to establish a unit of 1 for the group fed the basal diet (0.02% w/w cholesterol) without SCH 58053 (43). In the mouse, two major RNA transcripts are detected for ABCG5 (2.4 and 3.3 kb) and ABCG8 (2.5 and 3.9 kb) (27).

RNA levels were also measured for jejunum using a quantitative real-time PCR assay. 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 (PerkinElmer Life Sciences) was used to design the following primers: *cyclophilin* forward 5′-TGGAGAGCACCAAGACAGACA, reverse 5′-TGCCGGAGTCGACAATGAT; adenosine triphosphate-binding cassette transporter A1 (*ABCA1*) forward 5′-CGTTTCCGGGAAGTGTCCTA, reverse 5′-GCTAGAGATGACAAGGAGGATGGA; *ABCG5* forward 5′-TGGATCCAACACCTCTATGCTAAA, reverse 5′-GGCAGGTTTTCTCGATGAACTG; *ABCG8* forward 5′-TGCCCACCTTCCACATGTC, reverse 5′-ATGAAGCCGGCAGTAAGGTAGA.

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 reaction contained (final volume of 20 μl): 50 ng of reverse-transcribed RNA, a 150 nM concentration of each primer, and 10 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems). All analyses were performed in triplicate and relative RNA levels were determined by the comparative Ct method (User Bulletin No. 2, PerkinElmer Life Sciences).

Analysis of data

All data are reported as the mean ± 1 SEM for the specified number of individual animals. Differences between mean values were tested for statistical significance ($P < 0.05$) by the two-tailed Student's *t*-test. In the case of the mRNA data, a two-way ANOVA was performed with dietary cholesterol level and SCH 58053 treatment as variables.

RESULTS

As shown in **Fig. 2**, mice fed a basal rodent diet without added cholesterol and given SCH 58053 at a dose of 35 mg/day/kg body weight manifested a 46% reduction in intestinal cholesterol absorption (Fig. 2A) and a 61% increase in the rate of fecal neutral sterol excretion (Fig. 2B) but no detectable change in total lipid absorption (Fig. 2C). There was no effect of this agent on fecal bile acid excretion (Fig. 2D), pool size (Fig. 2E) or pool composition (Fig. 2F). From these data, it is thus apparent that SCH 58053 does not affect the rate of bile acid synthesis. The rates of cholesterol synthesis in various organs in matching groups of mice are shown in **Fig. 3**. In the group given SCH 58053, there was a 2.9-fold increase in hepatic sterol synthesis. (Fig. 3A), a modest but nonsignificant stimulation of intestinal synthesis (Fig. 3B), and no change in the amount of cholesterol synthesized in the periphery (Fig. 3C). Hence, most of the increase in whole animal sterol synthesis (Fig. 3D) was attributable to the liver.

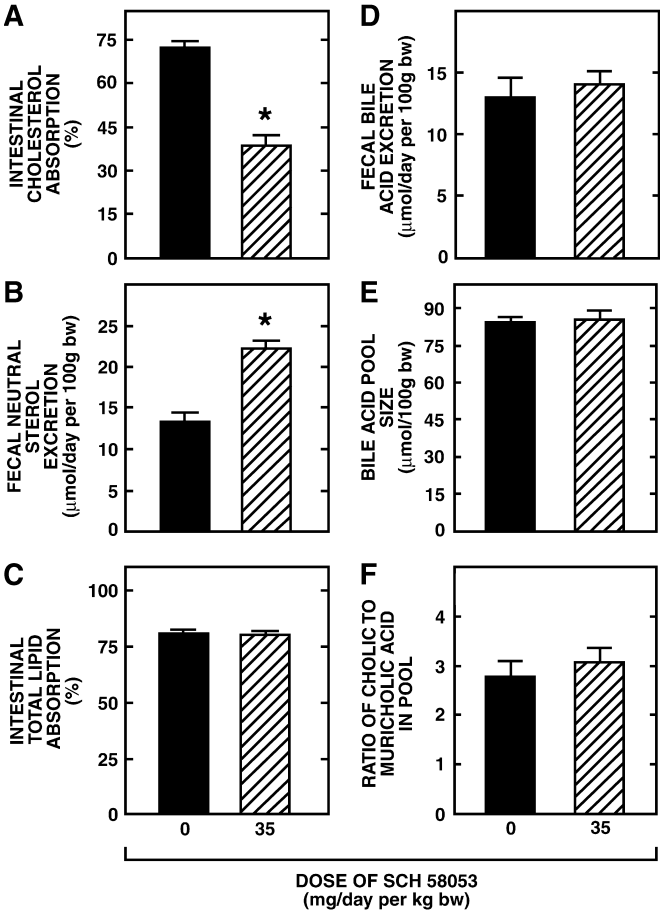

Fig. 2. Parameters of intestinal cholesterol and bile acid metabolism in mice fed a low cholesterol diet without and with SCH 58053. Female 129/Sv mice were fed ad libitum for 12 days a low cholesterol cereal-based rodent diet (cholesterol level of 0.02% w/w) either alone or containing SCH 58053 at a level of 0.021% (w/w) which provided an approximate daily intake of SCH 58053 of 35 mg/day/kg body weight. Intestinal cholesterol absorption was measured by a fecal dual-isotope ratio method as described in Materials and Methods. Intestinal total lipid absorption was measured in the same animals. Separate groups of mice were used for the measurement of the rates of fecal bile acid and neutral sterol excretion, and bile acid pool size and composition. Values represent the mean ± 1 SEM of data from eight animals in each group. *Denotes that the value for the group given SCH 58053 is significantly different from that for matching untreated animals ($P < 0.05$).

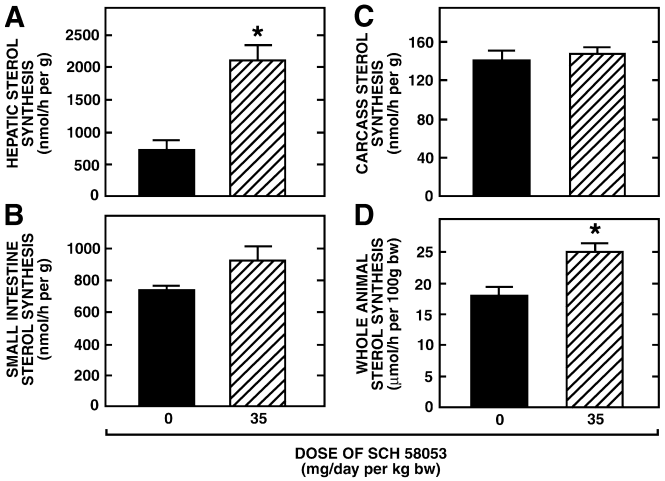

Fig. 3. Rates of sterol synthesis in various organs of mice fed a low cholesterol diet without and with SCH 58053. Female 129/Sv mice were fed ad libitum for 12 days the same diets that were used in the studies described in the legend for Fig. 2. The rates of sterol synthesis in the liver, small intestine and remaining carcass were determined in vivo using [^{3}H]water as described in Materials and Methods. The rate of sterol synthesis in these three tissues is expressed as the nmol of [^{3}H]water incorporated into sterols/h/g wet weight of tissue. Whole animal sterol synthesis represents the rate of synthesis in all tissues combined, and is expressed as the μmol of [^{3}H]water incorporated into sterols/h/100 g body weight. Values represent the mean ± 1 SEM of data from six animals in each group. *Denotes that the value for the group given SCH 58053 is significantly different from that for matching untreated animals ($P < 0.05$).

Another study addressed the question of whether the reduction in cholesterol absorption might be due to a disruption by SCH 58053 of the micellar solubilization of cholesterol in the proximal small intestine. The ratio of the absolute concentration of cholesterol to that of bile acid in the liquid phase of the contents of the proximal small intestine of mice given SCH 58053 (0.036 ± 0.008, n = 7) was not different from that in matching untreated mice (0.036 ± 0.007, n = 8).

The next series of experiments examined the impact of raising the dietary cholesterol intake of the mice either 10- or 50-fold, without and with SCH 58053 treatment, on various parameters of sterol metabolism. The results of several consecutive experiments were combined. The mice were fed their respective diets for an average of 23 days before study. Irrespective of which dietary regimen they were fed, all the mice gained weight to the same extent over this period. As shown in **Fig. 4A**, liver weight relative to body weight did not vary significantly either as a function of the dietary cholesterol level, or the presence or absence of SCH 58053 in the diet. There were, however, striking differences in hepatic cholesterol concentration depending both on how much cholesterol was fed,

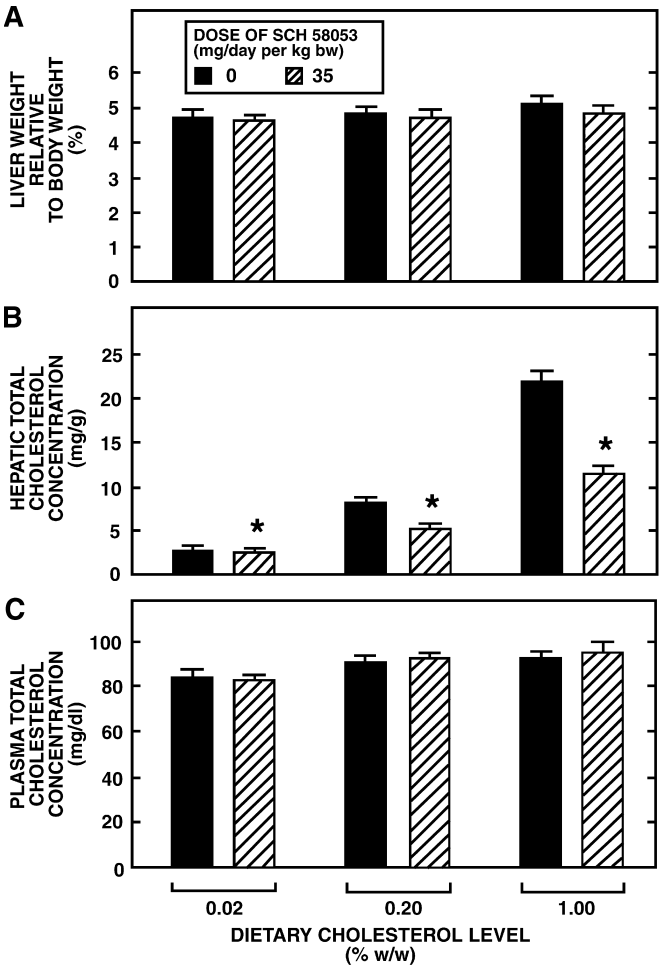

Fig. 4. Effect of cholesterol feeding without and with SCH 58053 on relative liver weight and cholesterol concentrations in the liver and plasma of mice. Female 129/Sv mice were fed ad libitum for an average of 23 days a cereal-based rodent diet containing cholesterol at levels of either 0.02, 0.20, or 1.00% (w/w) without or with SCH 58053 at a level of 0.021% (w/w). At this level the approximate daily intake of SCH 58053 was 35 mg/day/kg body weight. Hepatic and plasma total cholesterol concentrations were determined as described in Materials and Methods. Values represent the mean ± 1 SEM of data from 12 to 18 animals (relative liver weight), 10 to 13 animals (hepatic cholesterol concentration), and seven to 18 animals (plasma cholesterol concentration). *Denotes that the value for the animals given SCH 58053 is significantly different from the matching group fed the same amount of dietary cholesterol but without SCH 58053 ($P < 0.05$).

and on whether SCH 58053 was added to the diet (Fig. 4B). Thus, while raising the cholesterol content of the diet 10- or 50-fold in the absence of SCH 58053 increased hepatic cholesterol levels by 3.2- and 8.5-fold respectively, the concurrent feeding of SCH 58053 reduced the degree of cholesterol accumulation by about half in both cases. The plasma total cholesterol concentration did not vary significantly amongst the six groups of mice (Fig. 4C). However, in female LDLR deficient mice, SCH 58053 treatment did effect a significant lowering of plasma cholesterol concentrations. As shown in **Fig. 5**, this reduction was seen in mice given the basal diet or a diet with added cholesterol (1% w/w).

The lipid composition of gallbladder bile obtained from many of the mice used in the study described in Fig. 4 is shown in **Fig. 6**. In mice given just the cholesterol enriched diets, biliary cholesterol concentration increased as the dietary cholesterol level was raised (Fig. 6A). However, this increase was largely prevented if SCH 58053 was added to the diet. The absolute concentrations of phospholipid (Fig. 6B) and bile acid (Fig. 6C) did not vary significantly either as a function of the dietary cholesterol level, or the presence or absence of SCH 58053 in the diet. Thus, while there was no change in the relative cholesterol content of the bile when the agent was given to mice fed only the basal diet (Fig. 6D), in the cholesterol-fed groups SCH 58053 treatment was very effective in blocking the rise in the level of biliary cholesterol saturation. These findings closely parallel those described for hepatic cholesterol concentrations in Fig. 4B.

The relative mRNA levels for a number of proteins involved in the maintenance of cellular cholesterol homeostasis were determined in the jejunal mucosa (**Fig. 7**) and liver (**Fig. 8**) of many of the mice that were used in the studies described in Figs. 4 and 6. The values for the relative mRNA levels of ABCA1, ABCG5 and ABCG8 in the jejunum are shown in Fig. 7A, B, and C, respectively. Two

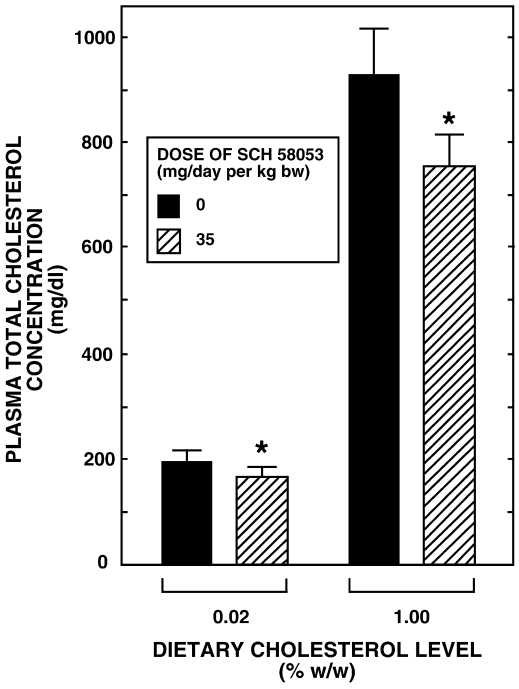

Fig. 5. Plasma total cholesterol concentrations in LDL-receptor (LDLR)-deficient mice fed low and high cholesterol diets without and with SCH 58053. Female LDLR$^{-/-}$ mice were fed ad libitum for 21 days a rodent diet containing cholesterol at levels of either 0.02 or 1.00% (w/w) without or with SCH 58053 at a level of 0.021% (w/w) which corresponded to a dose of 35 mg/day/kg body weight. Plasma total cholesterol concentrations were then determined as described in Materials and Methods. Values represent the mean ± 1 SEM of data from seven animals in each group. *Denotes that the value for the animals given SCH 58053 is significantly different from the matching group fed the same amount of dietary cholesterol but without SCH 58053 ($P < 0.05$).

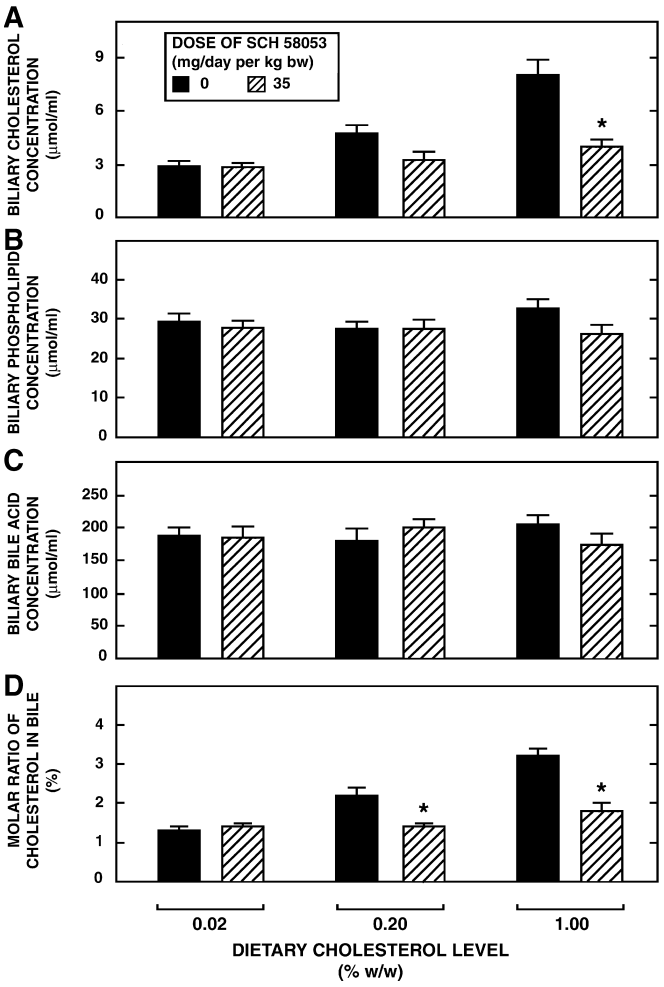

Fig. 6. Effect of cholesterol feeding without and with SCH 58053 on biliary lipid composition in mice. Gallbladder bile was harvested from many of the mice that were used in the study described in Fig. 4. The mice were not fasted before bile collection. Biliary lipid concentrations were determined as described in Materials and Methods. These were used to calculate the molar ratio of cholesterol in each bile sample. Values represent the mean ± 1 SEM of data from 11 to 13 animals given the diet with 0.02% (w/w) cholesterol, and from four to six animals given the diets with either 0.20 or 1.00% (w/w) cholesterol. *Denotes that the value for the animals given SCH 58053 is significantly different from the matching group fed the same amount of dietary cholesterol but without SCH 58053 ($P < 0.05$).

findings are noteworthy: first, in the mice fed the basal diet (0.02% w/w cholesterol) and given SCH 58053, the mRNA levels for all three of these proteins were consistently lower than they were in matching mice fed the basal diet alone. This was confirmed in a second independent study in which the duration of feeding of these same diets was only 5 days (data not shown.) Second, cholesterol feeding clearly raised the level of mRNA for all three proteins in the enterocyte, although the magnitude of the increase tended to be less if SCH 58053 was also present in the diet. With respect to the data for ABCG5 and ABCG8, another incidental finding was that the two RNA transcripts for each protein were similarly regulated.

In mice fed the basal diet, the relative mRNA levels for 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase) (Fig. 7D) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA red) (Fig. 7E) in the enterocyte were both increased about 40% when SCH 58053 was included in the diet. This increase, which was found in two independent experiments, is fully consistent with the modest stimulatory effect that this agent had on the rate of intestinal sterol synthesis (Fig. 3C). In the mice

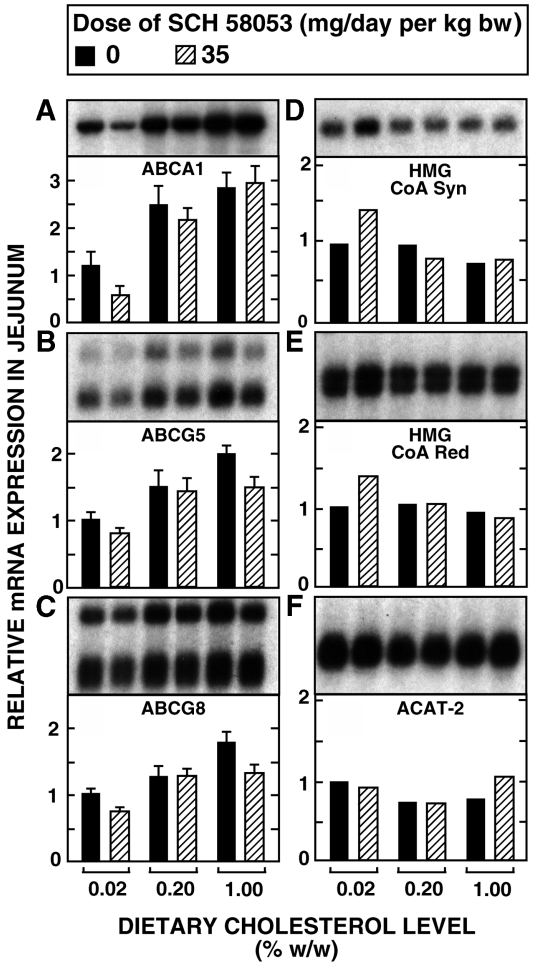

Fig. 7. Effect of cholesterol feeding without and with SCH 58053 on the level of expression of mRNA for various proteins involved in intestinal sterol metabolism in mice. Jejunal mucosa was removed from the mice used in the study described in Fig. 4. Five micrograms of poly(A)$^+$ RNA prepared from pooled RNA from individual mucosa samples were subjected to electrophoresis and Northern blot analysis using the ^{32}P-labeled probes indicated. The amount of radioactivity in each band was quantified by phosphorimager, normalized to the signal generated by β-actin, and mathematically adjusted to establish a unit of one for the group fed 0.02% (w/w) cholesterol without SCH 58053. The blots are shown in the upper compartment of each panel. The histograms in the lower compartment of D, E, and F indicate the relative amount of mRNA found by Northern analysis. Relative mRNA levels for ABCA1, ABC transporter G5 (ABCG5), and ABC transporter G8 (ABCG8) were also determined by quantitative real-time PCR using the ABI Prism 7000 System. The histograms for A, B, and C reflect the results expressed as the mean ± 1 SEM of real-time PCR analysis performed on samples from individual animals, and two-way ANOVA reveals a significant effect of dietary cholesterol ($P < 0.05$) and SCH 58053 ($P < 0.05$) with no significant interaction between these two variables.

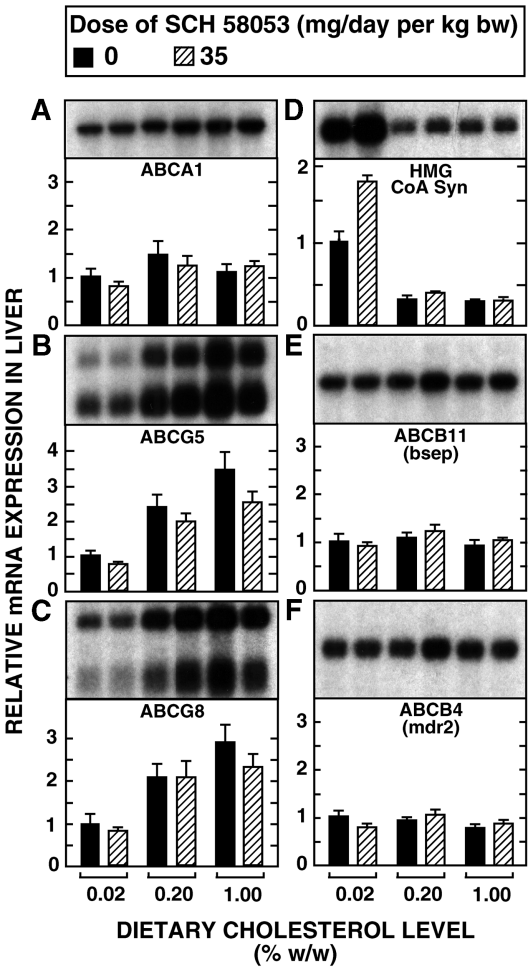

Fig. 8. Effect of cholesterol feeding without and with SCH 58053 on the level of expression of mRNA for various proteins involved in hepatic sterol metabolism in mice. Liver tissue from some of the mice that were used in the study described in Fig. 4 were used for the determination of the mRNA levels for several proteins by Northern analysis. The liver mRNA analyses were performed on tissue from individual animals. A representative blot for each respective protein is shown in the upper compartment of each panel. The histograms in the lower compartment indicate the relative amount of mRNA found for that protein. The values represent the mean ± 1 SEM of data from six animals in each group. A two-way ANOVA was performed on all data. In the case of ABCA1 (A), ABC transporter B11 (SPGP/BSEP) (ABCB11) (E), and ABC transporter B4 (MDR2/3) (ABCB4) (F) there was no significant effect of either diet or SCH 58053. However, for ABCG5 (B), ABCG8 (C), and 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA syn) (D), a significant ($P < 0.05$) effect of both diet and SCH 58053 was found.

fed the cholesterol enriched diets without or with SCH 58053, the mRNA levels for both of these proteins were about the same as they were in the animals given the basal diet alone. The relative mRNA level for acyl CoA:cholesterol acyltransferase-2 (ACAT-2) in the jejunal mucosa (Fig. 7F) did not vary consistently either as a function of the dietary cholesterol level, or the absence or presence of SCH 58053 in the diet.

In the liver, the relative mRNA levels for six proteins were determined in tissue from individual mice. As shown in Fig. 8A, the relative level of mRNA for ABCA1 was about the same in all six groups. In contrast, in the case of ABCG5 (Fig. 8B) and ABCG8 (Fig. 8C), the relative level of mRNA increased as the dietary cholesterol was raised, irrespective of whether SCH 58053 was also present in the diet. However, the magnitude of the increase was almost always noticeably less in the mice fed cholesterol together with this agent than it was in matching animals given the cholesterol enriched diets alone.

In mice given the basal diet without SCH 58053, the mRNA level for HMG-CoA syn (Fig. 8D) was 75% higher than it was in matching controls not given the agent. This finding is consistent with the marked stimulation of hepatic cholesterol synthesis seen when SCH 58053 was given to mice fed the basal diet alone (Fig. 3A). The relative mRNA level in the liver for both ABC transporter B11 (SPGP/BSEP) (ABCB11) (Fig. 8E) and ABC transporter B4 (MDR2/3) (ABCB4) (Fig. 8F) did not vary either as a function of dietary cholesterol level, or the presence or absence of SCH 58053 in the diet.

DISCUSSION

The use of cholesterol absorption inhibitors for treating hypercholesterolemia has a long history, with several classes of compounds having been developed (16, 44). Although they all exert some degree of LDL-C lowering, generally these agents, which usually need to be taken more than once daily and in gram quantities, are significantly less efficacious than the statins. The development of ezetimibe provides, for the first time, a class of absorption inhibitor that is not only specific, but which also consistently lower LDL-C levels in humans by about 18% with a single daily dose of only 10 mg (31). The cholesterol lowering potency of ezetimibe and one of its analogs, SCH 48461, is also well documented in a number of animal models (26, 35–40). Given the potential that this new class of hypolipemic agent has, both as a monotherapy and for use in combination with statins and other types of agents (31, 38), the full elucidation of its molecular mechanism of action is warranted.

The present studies represent the first detailed investigation of another ezetimibe analog, SCH 58053, in the mouse. Although it is likely that SCH 58053 and ezetimibe work through identical mechanisms, the lower potency of the analog could possibly be an indication that this is not the case. Female 129/Sv mice were chosen specifically for these studies because of their inherently high level of cholesterol absorption (10, 45). At a dose of 35 mg/day/kg body weight, SCH 58053 induced almost a 50% reduction in cholesterol absorption in mice fed the basal rodent diet. While this dose is significantly greater than the dose of ezetimibe needed to effect a similar degree of inhibition in this species (26, 40), it is only a fraction of the dose of other agents like surfomer (AOMA; α-olefin maleic acid) that is required to produce a comparable degree of inhibition in species like the hamster (20). Although effective at much lower doses, SCH 58053 induced changes

in cholesterol metabolism that were similar in character to those documented in the past for other classes of inhibitors. Thus, there was a marked compensatory increase in cholesterol synthesis in the liver, but not in the peripheral organs, and an accelerated loss of cholesterol in the feces with little or no change in the rate of conversion of cholesterol to bile acids (44, 46–48).

The data for SCH 58053 presented here yield several important new insights into the likely mechanism of action of this class of absorption inhibitor. Unlike agents such as sitostanol, sucrose polyester, or surfomer, which act at the level of the intraluminal phase of cholesterol absorption (6, 44, 49), this is apparently not the case with SCH 58053. This follows from several observations, the main one being that the level of absorption fell by almost half in the face of a normal bile acid pool size and composition. It is well documented that cholesterol absorption in mice is exquisitely sensitive, not only to pool size, but also to the relative proportions of cholic and muricholic acid in the pool (18, 19). There was also no change in the absolute concentration of phospholipid in the gallbladder bile of mice given SCH 58053, suggesting that the diminished absorption of cholesterol was not the result of reduced biliary phospholipid secretion into the lumen, as it is in the case of mice lacking ABCB4 (17). The finding that the ratio of the concentration of cholesterol to bile acid in the liquid phase of the contents of the proximal small bowel was unchanged by SCH 58053 is a further indication that this agent does not disrupt the intraluminal phase of sterol absorption. It should be noted here that in humans there are about 3 g of bile acid in the intestinal pool, and yet it takes only 10 mg of ezetimibe to effect a 54% reduction in cholesterol absorption (34, 50, 51). Together then, these data make it apparent that the inhibitory action of SCH 58053 and ezetimibe is mediated either at the step involving translocation of sterol across the plasma membrane of the enterocyte, or at the level of an intracellular event that either facilitates the eventual delivery of cholesterol into the lymph or, alternatively, effluxes it back into the lumen.

The last of these possibilities can probably be discounted based on the relative levels of mRNA for ABCA1, ABCG5, and ABCG8 in the jejunum. Thus, if the diminished level of cholesterol absorption seen in the mice fed the basal diet with SCH 58053 was due to accelerated efflux, then an increase in the mRNA for these proteins might be expected. Instead, the relative mRNA level for all three transporters was consistently lower in the mice given SCH 58053 than in those fed the basal diet alone. While these studies do not rule out the possibility that the protein levels or activities of these ABC transporters are altered by SCH 580053, it is currently difficult to make such measurements for these particular proteins. Although the cellular cholesterol content could not be determined in these same jejunal preparations, this was apparently reduced in mice fed the basal diet with the agent because sterol synthesis tended to be stimulated in these cells. This was evident from the modest, but consistent rise in the relative mRNA levels for both HMG-CoA syn and HMG-CoA red, and in the rate of incorporation of [^{3}H]water into sterols. Together, these data for the mice given the basal diet without or with SCH 58053 suggest that this agent does not inhibit cholesterol absorption by driving the efflux of sterol from the enterocyte. Instead, the block occurs at some other step in the absorption process, and the resultant reduction in the cholesterol content of the enterocyte leads to a contraction in the level of expression of ABCA1, ABCG5, and ABCG8.

This conclusion is supported by the mRNA data for the mice fed the cholesterol enriched diets. Thus, as previously reported, driving more cholesterol into the enterocyte raised the level of mRNA for ABCA1, ABCG5, and ABCG8 (27, 52). However, the presence of SCH 58053 in the high cholesterol diets generally lessened the extent of this increase for all three proteins. It should be emphasized here that, although jejunal cellular cholesterol concentration was not determined, more cholesterol must have been taken up by the enterocytes in the mice fed the cholesterol enriched diets because the dose of SCH 58053 used did not completely block cholesterol absorption. Moreover, there was significantly more cholesterol in the livers of the cholesterol fed mice, although the level of accumulation was appreciably less in the animals also given SCH 58053.

The finding that SCH 58053 did not act at the level of cellular cholesterol efflux, raised several other possibilities, one of which was that the agent suppressed ACAT-2, the major cholesterol esterifying enzyme in the enterocyte (53). In the past, other compounds with inhibitory activity toward intestinal cholesterol esterification were often found to effect marked cholesterol lowering in a number of cholesterol-fed animal models (16). However, the present observation that SCH 58053 had no effect on the mRNA level for ACAT-2 in the jejunum, irrespective of whether the mice were fed a low or high cholesterol diet, suggests that this agent does not block cholesterol esterification in the enterocyte. This finding is consistent with other studies involving another ezetimibe analog, SCH 48461, in various animal models and in HepG2 and CaCO-2 cells (35).

While SCH 58053 could potentially alter the expression of a protein(s) that acts distally to the esterification step, autoradiographic studies in the rat with the parent compound, ezetimibe (SCH 58235), show that it localizes throughout the intestinal villi, with the highest concentration in the villus tip (41). One interpretation of these data is that ezetimibe is binding to a membrane protein(s) that is involved in the uptake of sterols. Several attempts have been made to demonstrate the existence of such a protein (22, 23). However, as yet a specific sterol permease has not been identified. Although earlier in vitro studies suggested that the scavenger receptor SR-BI might play such a role (24), intestinal cholesterol absorption does not decrease in mice lacking SR-BI (25, 26). Furthermore, the dose of ezetimibe required to inhibit diet-induced hepatic cholesterol accumulation in SR-BI deficient mice is similar to that needed in SR-BI$^{+/+}$ mice (26). Clearly, further clarification of the process by which sterols are taken up by

the enterocyte will likely yield a clearer insight into the mechanism of action of this new class of absorption inhibitor.

Irrespective of the molecular mechanism by which these inhibitors block cholesterol absorption, there are three major points from the present studies regarding their impact on hepatic cholesterol homeostasis, biliary cholesterol secretion, and their LDL-C lowering action that warrant emphasis. First, cholesterol feeding resulted in a significant increase in the relative mRNA level for both ABCG5 and ABCG8, but not ABCA1, in the hepatocyte. However, just as was the case in the jejunal cells, the magnitude of the increase was blunted if the mice were also given SCH 58053. These differences in the level of mRNA expression for these two proteins correlated well with the changes in hepatic cholesterol concentration. Thus, since the dose of SCH 58053 used did not fully block cholesterol absorption, hepatic cholesterol levels did increase above those seen in mice fed the basal diet, but to a lesser extent than they did in the mice fed the high cholesterol diets without SCH 58053. There was, nevertheless, a sufficient increase in hepatic cholesterol content in the face of SCH 58053 treatment to keep the rate of cholesterol synthesis in the livers of those mice suppressed below the rates seen in animals on the basal diets, as judged by the hepatic mRNA levels for HMG-CoA syn.

The second point pertains to the changes in biliary lipid composition that occurred in response to cholesterol feeding and SCH 58053 treatment. Most important was the fact that in the mice fed the basal diet, biliary cholesterol levels, both absolute and relative, remained unchanged in the face of the 2.9-fold compensatory increase in hepatic cholesterol synthesis. Cholesterol feeding raised the absolute and relative cholesterol content of the bile. However, these increases were almost completely prevented by the addition of SCH 58053 to the diets. This protective effect of the agent on the level of biliary cholesterol saturation was not articulated through changes in the level of expression of ABCB11 and ABCB4, which respectively regulate biliary bile acid and phospholipid secretion (54), but merely reflected the delivery of less cholesterol to the liver from the intestine. The favorable effect that this new type of agent has on biliary lipid composition is consistent with what has been found in the past for other classes of absorption inhibitors in humans (46, 49).

The last point concerns the finding that SCH 58053 treatment did not change the plasma total cholesterol concentration in normal 129/Sv mice fed either the basal or cholesterol enriched diets, but did have a significant cholesterol lowering effect in LDLR deficient mice fed the same diets. Normal mice exhibit a very high turnover of cholesterol in the plasma pool, such that plasma cholesterol concentrations often remain low and constant in the face of manipulations that dramatically alter whole animal sterol balance (55). In mice, as in other species, the plasma LDL-C concentration is dictated largely by the balance between the rate of LDL-C production and the receptor mediated clearance of LDL from the circulation (56). In LDLR deficient mice, where circulating LDL-C levels are essentially a function of the rate of production (57), plasma LDL-C concentrations will change in response to manipulations that alter the rate of hepatic VLDL-C secretion, and hence the rate of LDL-C production (58). Given that all of the cholesterol absorbed from the small intestine is initially cleared by the liver, a sustained reduction in the level of cholesterol absorption, while partially compensated for by an increase in hepatic cholesterol synthesis, will potentially lower intrahepatic cholesterol content, reducing the rate of hepatic VLDL secretion and thereby decrease LDL-C production. Consistent with this concept, newly published studies have now shown that in humans with homozygous familial hypercholesterolemia, the administration of ezetimibe also produced a significant reduction in plasma LDL-C levels (33). Thus, the ability of this new class of absorption inhibitors to reduce the rate of LDL-C production, as well as to increase the level of hepatic LDLR activity, explains why these compounds will effectively lower the plasma LDL-C concentration when used either alone or with statins.

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