Inhibiting intestinal NPC1L1 activity prevents diet-induced increase in biliary cholesterol in Golden Syrian hamsters

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The liver plays a central role in the maintenance of sterol balance across the whole body because not only is it the organ that receives most of the cholesterol absorbed by the small intestine, but also it is the site for the degradation and excretion of cholesterol through the bile (40). The liver also actively synthesizes cholesterol but the rate at which it does so varies widely depending on numerous factors and, in particular, the amount of cholesterol being delivered to it from the small intestine (40). It is well documented that dietary or pharmacological manipulation of the enterohepatic flux of either cholesterol or bile acids can potentially cause marked changes in the rate at which the liver synthesizes cholesterol, converts cholesterol to bile acids, incorporates cholesterol into very low-density lipoproteins, esterifies and stores cholesterol, or secretes unesterified cholesterol directly into bile (18, 22, 40).

Such changes in the intrahepatic handling of cholesterol can, in turn, lead to clinically significant shifts in the circulating low-density lipoprotein-cholesterol (LDL-C) concentration and in the degree of biliary cholesterol saturation (15, 43, 48).

The intestinal absorption of cholesterol has long been a key target in the management of dyslipidemia primarily because of the substantial quantity of cholesterol that reaches the liver daily from the small bowel (5, 35). In the average adult consuming a typical Western diet, ~1,200–1,700 mg of cholesterol enters the lumen of the small intestine each day (18). Approximately 300–500 mg of this cholesterol comes from the diet and the remainder is derived largely from bile (19). Given that in humans fractional cholesterol absorption averages ~50%, the amount of intestinally derived cholesterol delivered to the liver throughout the day amounts to hundreds of milligrams (8, 34). This occurs through what is essentially a triphasic process that begins with a series of events within the lumen that culminate in the micellar solubilization of cholesterol and its movement up to the brush border membrane of the absorptive enterocyte (41). The second phase entails the transport of cholesterol, as well as noncholesterol sterols, across this limiting membrane facilitated by Niemann-Pick C1-Like 1 (NPC1L1) (1, 13). Within the enterocyte, cholesterol is esterified and subsequently incorporated, along with other lipids and apolipoprotein B48, into nascent chylomicrons. These latter processes together constitute the third phase of sterol absorption. Although the cellular and molecular events involved in each of these phases are generally well defined, there are nevertheless significant gaps in our knowledge of how cholesterol movement within the enterocyte is facilitated (49). Furthermore, although NPC1L1 clearly plays a fundamental role in sterol absorption, there is currently not unanimous agreement that it is this protein that facilitates the uptake of sterols across the brush border membrane (38).

Although the primary focus of research on the molecular control of cholesterol absorption has been to find more effective ways of modifying the plasma lipoprotein composition to lessen an individual’s risk of developing atherosclerosis, the question of how changes in chylomicron cholesterol uptake by the liver might also alter the rate of biliary cholesterol secretion, and thus a person’s predisposition to cholesterol gallstone formation, has not been as widely investigated. Most of the studies that have been carried out in animal models such as the baboon, squirrel monkey, Golden Syrian hamster, mouse, and prairie dog, as well as in humans, have shown that increases in dietary cholesterol intake result in a rise in biliary cholesterol content (2, 3, 14, 21, 24, 27, 28, 50, 52). However, the extent
to which this happens varies markedly depending on the type of model and the dietary formulation that were utilized. Comparatively, much less is known about the converse situation involving the use of inhibitors that diminish the amount of cholesterol reaching the liver from the small bowel. Studies with agents that interfere with the intraluminal phase of absorption such as β-sitosterol, sucrose polyester (a nonabsorbable fat substitute), and surfomer (AOMA, a copolymer of maleic acid and an 18-carbon α-olefin) all showed either no change or a modest reduction in biliary cholesterol saturation (6, 10, 11). The impact of agents that act at the intracellular phase of sterol absorption has not been widely studied. However, the focus of attention has now shifted to ezetimibe, an entirely new class of inhibitors that, in milligram doses, markedly reduces cholesterol absorption by inhibiting NPC1L1 activity in the brush border membrane (17). The dose of ezetimibe needed to block cholesterol absorption varies between species because of wide differences in the affinity with which ezetimibe binds to NPC1L1 (20). In the LDL receptor knockout mouse fed a lipid-rich diet, the degree of increase in biliary cholesterol content was blunted substantially by the addition of ezetimibe to the diet (29). Similar findings have been recently reported for mice harboring constitutive hepatic liver X receptor activity and for gallstone-susceptible C57L mice fed a lithogenic diet (45). Ezetimibe has also been found to have beneficial effects in a murine model of cholecystosteatosis (26). There are also now newly published data showing that ezetimibe treatment lowered the degree of biliary cholesterol saturation in small groups of gallstone patients and overweight subjects without gallstones (51).

In the present studies the Golden Syrian hamster has been utilized as an alternate model to determine the impact of ezetimibe treatment on biliary lipid composition during periods of low and high dietary cholesterol intake and to delineate the concomitant biochemical and molecular changes in sterol metabolism within the small intestine and liver. Previous studies have established that this small animal has features of cholesterol metabolism that more closely resemble those seen in the human and nonhuman primate than do species like the mouse and rat (44). Utilizing this hamster model, the data demonstrate that ezetimibe, by blocking the transport of excess chylomicron cholesterol to the liver, was highly effective in preventing an otherwise pathological increase in hepatic cholesterol storage and biliary cholesterol content. The data also show for the first time that the ezetimibe-induced changes in the expression of multiple genes involved in cholesterol handling by the enterocyte are quickly reversed when treatment is withdrawn.

MATERIALS AND METHODS

Animals and diets. Female and male Golden Syrian hamsters (Mesocricetus auratus) were obtained from either Charles River Laboratories, Wilmington, MA, or, for one experiment, from Harlan Sprague Dawley, Indianapolis, IN, in the weight range of 100–110 g. They were housed individually in plastic cages containing wood shavings and maintained in a room with alternating 12-h periods of light and darkness. The hamsters were adapted to these conditions for 2 wk, during which time they were fed a pelleted form of a cereal-based rodent diet (Wayne Lab Blox no. 8604; Harlan Teklad, Madison, WI). This was defined as the basal diet. It had cholesterol content of 0.02% (wt/wt) and a total lipid content of 4–5% (wt/wt). In some studies the meal form of the basal diet was enriched with cholesterol (1.0% wt/wt) and safflower oil (10% wt/wt). In the initial ezetimibe dose-response experiments, the hamsters were fed the basal diet containing only ezetimibe at levels that provided approximate doses of 0, 0.5, 1.0, and 2.0 mg·day⁻¹·kg body wt⁻¹ (based on the consumption of 73 g of diet·day⁻¹·kg body wt⁻¹) (44). In the studies with the lipid-rich diet, the ezetimibe was provided in the diet at a single dose of 1.0 mg·day⁻¹·kg body wt⁻¹. This dose was shown by other investigators to normalize combined dyslipidemia in obese, hyperinsulinemic male hamsters (47). Depending partly on the objective of the study, the hamsters were fed their respective experimental diet ad libitum for either 10, 21, or 34 days. All animals were studied in the fed state toward the end of the dark phase of their light cycle. All studies were approved by the Institutional Animal Care and Use Committee of The University of Texas Southwestern Medical Center.

Intestinal cholesterol absorption, fecal bile acid and neutral sterol excretion, and biliary lipid composition. The details of the methods used for measuring these parameters are described previously (32). Intestinal cholesterol absorption was determined as a fractional value (percentage) by a fecal dual-isotope ratio method (32). For tissue, plasma, and bile harvesting, the hamsters were anesthetized and exsanguinated from the abdominal aorta. The liver was exposed and the gallbladder accessed. Bile was taken from the gallbladder via a 1-ml plastic syringe fitted with a 25-gage, 5/8-in. needle. The bile was then transferred to a microfuge tube, after which aliquots were diluted in methanol for lipid composition analysis. The molar ratio of cholesterol in gallbladder bile was expressed as a percentage value. The rate of fecal bile acid excretion was taken as a measure of the rate of bile acid synthesis (44).

mRNA isolation and qRT-PCR gene expression analysis. Hamsters were anesthetized with isoflurane (Baxter Healthcare, Deerfield, IL) and exsanguinated into heparinized syringes via the abdominal aorta. Small intestines were removed, flushed with ice-cold PBS, then cut longitudinally. The intestinal mucosae were removed by gentle scraping, immediately frozen in liquid nitrogen, and stored at −85°C. Total RNA was isolated from tissue samples by use of RNA STAT-60 (Tel-Test, Friendswood, TX). RNA concentrations were determined by absorbance at 260 nm. Quantitative RT-PCR (qRT-PCR) was performed by using an Applied Biosystems Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA) as described previously (25). Briefly, total RNA was treated with DNase I (RNase-free) (Roche Diagnostics, Indianapolis, IN) and reverse transcribed with random hexamers by use of SuperScript II (Invitrogen, Carlsbad, CA) to generate cDNA.

Primers for each gene were obtained from the literature (16) or were designed with PrimerExpress software (PerkinElmer Life and Analytical Sciences, Boston, MA) and validated by analysis of template titration and dissociation curves (46) (see Table 1 for primer sequences). The gene sequence for hamster ACAT2 was not yet available, so a partial cDNA was cloned by reverse transcription-PCR using hamster RNA as template and the following primers: forward, 5'-ATCCTATTGAGTGAGGCAGG; reverse, 5'-CCCAAGCGAACAGGCAGAGA. These cloning primers were designed according to regions of highest sequence similarity between the human (NM_003578), mouse (NM_146064), and rat (NM_135732) ACAT2 genes. The resulting 871-bp product was sequenced and found to share 89% nucleotide identity with the mouse ACAT2 mRNA (NM_146064), and this novel sequence was subjected to the Primer Express algorithm to identify qRT-PCR primers for measuring hamster ACAT2 mRNA levels.

Each qRT-PCR reaction contained (final volume of 10 µl): 25 ng of reverse-transcribed RNA, each primer at 150 nM, and 5 µl of 2X SYBRgreen PCR master mix (Applied Biosystems), and each sample was analyzed in triplicate. Data from the PCR reactions were analyzed by the comparative cycle number determined at threshold (Ct) method (User Bulletin no. 2, PerkinElmer Life and Analytical Sciences) with cyclophilin as the invariant control gene. In most cases, mRNA levels in each experiment are expressed relative to those
wt
bile acid excretion remained unchanged (Fig. 1, 82% and fecal neutral sterol excretion increased 5.3-fold but values were tested for statistical significance (29).

Commercial laboratory. Hepatic triacylglycerol concentrations were determined as described (29). Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured by a commercial laboratory. Hepatic triacylglycerol concentrations were determined as described (29).

Statistical analysis of data. All data are reported as means ± SE for the specified number of individual animals. Differences between mean values were tested for statistical significance (P < 0.05) by an unpaired, two-tailed Student’s t-test. All statistical analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, CA).

RESULTS

Accelerated fecal loss of neutral but not acidic sterols accompanies ezetimibe-induced block of cholesterol absorption. The data in Fig. 1 illustrate the magnitude of change in several parameters of cholesterol metabolism in female hamsters fed a basal diet containing ezetimibe at various levels, the highest of which provided a dose of ~2 mg·day⁻¹·kg body wt⁻¹. At this dose, fractional cholesterol absorption fell by 82% and fecal neutral sterol excretion increased 5.3-fold but bile acid excretion remained unchanged (Fig. 1, A, B, and C, respectively). In addition, there was also a 2.3- and 2.8-fold increase in sterol synthesis in the small intestine (Fig. 1D) and liver (Fig. 1E), respectively. The plasma total cholesterol concentration remained unchanged across all doses of ezetimibe (Fig. 1F), as did the plasma activities of both ALT and AST. At ezetimibe doses of 0, 0.5, 1.0, and 2.0 mg·day⁻¹·kg body wt⁻¹, the plasma ALT activities (units/l) were 96 ± 13, 77 ± 11, 137 ± 30, and 100 ± 14, respectively, and the corresponding AST activities (units/l) were 48 ± 12, 33 ± 2, 45 ± 9, and 49 ± 10, respectively. The marked compensatory increase in sterol synthesis in the small intestine and liver seen with ezetimibe treatment was not found in other regions of the gastrointestinal tract, as demonstrated by both the rate of incorporation of [³H]water into sterols (Fig. 1G) and the relative mRNA level for 3-hydroxy-3-methylglutaryl-CoA reductase (ACAT2, acyl CoA:cholesterol acyltransferase 2).

Table 1. Quantitative real-time PCR primers used to measure hamster RNA levels

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>NCBI Accession Number or Reference</th>
</tr>
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<tbody>
<tr>
<td>NPC1L1</td>
<td>5'-GGGCGGAACAGCCAGGTTTCCCTAGCA-3'</td>
<td>DQ897680, Ref. 16</td>
</tr>
<tr>
<td>ABCA1</td>
<td>5'-GTCCTGGTGGGAGATAACCAAGTG-3'</td>
<td>Ref. 16</td>
</tr>
<tr>
<td>ABCG5</td>
<td>5'-GTATGAGGCGATCTTAAATTGCGG-3'</td>
<td>Ref. 16</td>
</tr>
<tr>
<td>ABCG8</td>
<td>5'-GCTGAGATGTTAGACGTCCTGGCAGG-3'</td>
<td>Ref. 16</td>
</tr>
<tr>
<td>HMG-CoA synthase</td>
<td>5'-CCATGACGTGGATTGGGGCC-3'</td>
<td>L00326</td>
</tr>
<tr>
<td>HMG-CoA reductase</td>
<td>5'-ACACATGATGATGATGATGATGATG-3'</td>
<td>X00494</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>5'-GCAATACGTCGCTATGATGATGATGATGATG-3'</td>
<td>X17105</td>
</tr>
<tr>
<td>ACAT2</td>
<td>5'-GGTGCCGATGTGATGATGATGATGATGATG-3'</td>
<td>See MATERIALS AND METHODS</td>
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obtained for the wild-type mice fed the basal diet and reflect means ± SE. Otherwise, levels are expressed relative to the group with the least abundance of mRNA.

Tissue cholesterol synthesis. The rate of cholesterol synthesis in the liver and entire small intestine was measured in vivo using [³H]water as described earlier and calculated as the nanomoles of [³H]water incorporated in digitonin-precipitable sterols per hour per gram wet weight of tissue (32).

Cholesterol concentration in plasma and liver and liver plasma enzyme activities. Plasma total cholesterol concentration was measured by an enzymatic and colorimetric assay using a reagent mixture from Roche Diagnostics (Indianapolis, IN; catalog no. 11875523). Aliquots of liver tissue were digested in ethanolic potassium hydroxide, and the total cholesterol concentration was determined by gas chromatography (32). Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured by a commercial laboratory. Hepatic triacylglycerol concentrations were determined as described (29).

Expression of mRNA for NPC1L1 is highly localized in the small intestine in hamsters. The data in Fig. 1 prompted us to compare the level of expression of mRNA for NPC1L1, the target of ezetimibe (17), in the liver, gallbladder, and various regions of the gastrointestinal tract of hamsters fed the basal diet alone. As shown in Fig. 2, the abundance of mRNA for NPC1L1 in the small intestine far exceeded that seen in other regions of the gastrointestinal tract, as demonstrated by both the rate of incorporation of [³H]water into sterols (Fig. 1G) and the relative mRNA level for 3-hydroxy-3-methylglutaryl-CoA synthase (Fig. 1H).

Response of the small intestine to acute withdrawal of ezetimibe treatment. The main objective of this experiment was to determine how the small intestine responded when ezetimibe treatment was suddenly discontinued. In evaluating the data shown in Fig. 3, it should be emphasized that in the case of the group of hamsters subjected to treatment withdrawal, they had already been off ezetimibe for 3 days at the time they, and the other groups of hamsters, were dosed with labeled sterols for the measurement of fractional cholesterol absorption. Hence, although the mRNA data for the hamsters taken off ezetimibe reflect measurements made 6 days after drug treatment had stopped, the fractional absorption data reflect what was happening in the small intestine over the fourth, fifth, and sixth days following cessation of treatment. The data in Fig. 3A demonstrate that within this short time frame, the marked inhibition of cholesterol absorption by ezetimibe had largely been reversed. Similarly, the relative mRNA level for several
proteins, which either fell (ABCG5 and G8) or increased (HMG CoA synthase and reductase) during treatment, had returned to normal values within 6 days of ezetimibe withdrawal (Fig. 3B).

Lipid composition of gallbladder bile was unchanged in hamsters given ezetimibe with a low-cholesterol, low-fat diet. In many of the hamsters used in the preceding studies, gallbladder bile was harvested. The absolute concentrations of cholesterol, phospholipid, and bile acid in the bile from hamsters fed the basal diets containing ezetimibe at doses up to and including 2 mg·day⁻¹·kg body wt⁻¹ (A–F). In the second experiment, the rate of sterol synthesis in the liver and different regions of the gastrointestinal tract was measured in hamsters that were fed for 34 days a low-cholesterol chow diet containing ezetimibe at doses of either 0 or 2 mg·day⁻¹·kg body wt⁻¹ (G). The relative mRNA level for 3-hydroxy-3-methylglutaryl (HMG) CoA synthase in the same tissues was determined in separate matching groups of hamsters (H). Here, each value was expressed relative to the mRNA level in the colon of hamsters not given ezetimibe, which was arbitrarily set at 1.0. All values are means ± SE of data from 5–7 animals in each group. *Significantly different from corresponding value for matching group not given ezetimibe, P < 0.05.

Diet-induced cholesterol accumulation in liver and plasma was prevented by ezetimibe. Western diets typically contain substantial amounts of triacylglycerol and cholesterol, particularly relative to endogenous synthesis rates. Thus, to examine the effect of this drug in a more relevant situation, female hamsters were fed for 28 days a diet containing cholesterol (1% wt/wt) and safflower oil (10% wt/wt) without or with ezetimibe at a dose of just 1 mg·day⁻¹·kg body wt⁻¹. The livers depicted
in Fig. 5A are representative of what was found in hamsters fed the lipid-rich diet without ezetimibe (left) or with ezetimibe (right). The lipid-rich diet alone resulted in massive accumulation of cholesterol in both the liver (Fig. 5B) and plasma (Fig. 5C). The livers from the untreated hamsters had a total cholesterol concentration of 99 ± 4 mg/g and, relative to body weight, were 50% larger than the livers of hamsters given the lipid-rich diet with ezetimibe. The total cholesterol concentration in the livers from the treated animals averaged 2.5 ± 0.2 mg/g. The pale appearance of the livers from the untreated animals presumably reflected the accumulation of cholesteryl esters because hepatic total triacylglycerol concentrations averaged ~5–6 mg/g irrespective of whether the lipid-rich diet contained ezetimibe.

Changes in hepatic relative mRNA levels for multiple proteins induced by lipid-rich diet were attenuated by ezetimibe. The relative mRNA level for multiple proteins was determined in the livers of the hamsters used in the study described in Fig. 5 and these are shown in Fig. 6. The mRNA for ACAT2 was essentially the same in all four experimental groups. In contrast, the level of mRNA expression for ABCA1, ABCG5, and ABCG8 was significantly elevated whereas that for HMG CoA synthase and HMG CoA reductase was markedly reduced in hamsters given the lipid-rich diet with ezetimibe. These changes in message levels either did not occur or were far less pronounced in the hamsters given ezetimibe with the high-fat, high-cholesterol diet.

Diet-induced increase in biliary cholesterol content was prevented by ezetimibe. In the study with the female hamsters fed the lipid-rich diet (Figs. 5 and 6) the lipid composition of gallbladder bile was also determined (data not shown). The cholesterol, safflower oil-enriched diet caused only a modest rise in the molar ratio of cholesterol in the bile (1.4 ± 0.2 vs. 0.8 ± 0.2% in matching hamsters given the basal diet alone). In the group fed the lipid-rich diet with ezetimibe, the molar ratio of cholesterol was 0.9 ± 0.1%. However, it is known that the male of this species responds much more dramatically to dietary lipids (3). Consequently, these studies were repeated in male hamsters given the diets enriched with cholesterol and safflower oil. As shown by the data in Fig. 7A, this dietary formulation did raise the cholesterol concentration in the gallbladder bile 5.2-fold. There was also a 2.4-fold increase in the phospholipid concentration (Fig. 7B) but no change in the bile acid concentration (Fig. 7C). Thus, although ezetimibe treatment alone did not change the molar ratio of cholesterol in the bile, the lipid-rich diet alone did increase this ratio 4.6-fold (Fig. 7D). When this diet was given with ezetimibe, the increase in biliary cholesterol saturation was greatly diminished. Thus increasing the flow of cholesterol into the liver of the male animal markedly increased the rate of secretion of sterol by the liver into bile and this effect was nearly completely obviated by the administration of ezetimibe.

DISCUSSION

The discovery and development of the novel sterol absorption inhibitor, ezetimibe, has provided not only an effective new strategy for the management of primary hypercholesterolemia in the general population (5), but also a treatment for sitosterolemia, a rare disorder resulting from mutations in the NPC1 gene that encodes a protein involved in intracellular cholesterol transport (7). Those studies showed that the sooner in life mice deficient innpc1 received daily treatment with ezetimibe, the better was the improvement in their plasma liver enzyme levels. This benefit correlated directly with the degree of reduction in hepatic cholesterol accumulation that resulted from the ezetimibe-induced inhibition of intestinal cholesterol absorption and the diminished delivery of chylomicron cholesterol to the liver. The efficacy of ezetimibe in treating liver dysfunction in a murine model of Niemann-Pick type C (NPC) disease, a multisystem disorder resulting from mutations in the NPC1 gene that encodes a protein involved in intracellular cholesterol transport (7).
dysfunction in humans with NPC disease has yet to be determined. The underlying objective of the present studies was to investigate whether ezetimibe might also be potentially useful in the prevention and treatment of yet another disorder of cholesterol metabolism, cholelithiasis. The premise for doing so stems from the results of studies performed in several animal models, including primates, and to some extent in humans, which together show that biliary cholesterol content is determined in part by the rate of hepatic chylomicron cholesterol clearance (2, 3, 14, 21, 24, 27, 28, 50, 52).

Two interrelated points regarding the design of the present studies warrant emphasis here. One concerns the selection of the Golden Syrian hamster as the model for these experiments, and the other has to do with the types of dietary formulations to which ezetimibe was added. Golden Syrian hamsters manifest significant gender differences in some aspects of cholesterol metabolism such as the basal rate of cholesterol synthesis and the level of cholesteryl ester in the liver (33). However, both male and female hamsters normally produce bile that has a very low degree of cholesterol saturation, with the molar ratio of cholesterol usually being only \(\sim 1\%\) or less (3, 43). This is also the case with the dog (4, 23), which was the first species in which biliary cholesterol content was measured following treatment with ezetimibe.1 This comparison between the hamster and dog is emphasized here because of the finding that biliary cholesterol content increased in dogs given ezetimibe for 1 mo. Despite this, treatment of dogs with ezetimibe at a very high dose (300 mg·day\(^{-1}\)·kg body wt\(^{-1}\)) for 1 yr did not result in gallstone formation or any other adverse hepatobiliary effects. It should be noted that these findings were made in dogs maintained on a low-cholesterol, low-fat commercial canine diet (personal communication from Harry R. Davis at the Schering-Plough Institute). Thus the second point to be made here concerns the type of diet that is used when cholesterol modifying agents like ezetimibe are being tested in animal models. Ideally, such studies should be conducted in animals given both their conventional regimens, as well as formulations that more closely mimic the diets typically consumed by humans. The Golden Syrian hamster was chosen as a model for the present studies, not only because it normally produces bile with a low degree of cholesterol saturation as dogs do but, moreover, because it is a species in which profound changes in biliary cholesterol saturation can be induced by specific dietary formulations (3, 43). There are also a number of inherent similarities in several aspects of cholesterol metabolism between humans and the Golden Syrian hamster that made this species an appropriate model for these studies (44).

Four conclusions can be drawn from the initial set of experiments with hamsters given ezetimibe at different doses while being fed a basal, low-cholesterol, low-fat diet. The first is that, although there were dramatic increases in intestinal and hepatic sterol synthesis (Fig. 1, D and E), and fecal neutral sterol excretion (Fig. 1B), there were no consistent changes in biliary lipid composition (Fig. 4, A–D). There was a trend toward higher absolute cholesterol concentrations in the bile of the hamsters given ezetimibe, but this was not dose related (Fig. 4A). It should be emphasized that these effects of ezetimibe on sterol balance are not unique to this class of absorption inhibitor. For example, in hamsters given diets containing surflurer (\(\omega\)-olefin maleic acid), a polymeric nonabsorbable agent that inhibits cholesterol absorption by making the surface environment of the brush border membranes of the enterocytes more hydrophilic, there is a marked stimulation of intestinal and hepatic sterol synthesis (39, 42), just as there is with ezetimibe. This is accompanied by a modest reduction in the relative cholesterol content of the bile (39), an effect also reported for a small number of human subjects given surflurer (11). Taken together, these findings demonstrate that the marked compensatory increase in cholesterol synthesis in the liver that occurs when cholesterol absorption is inhibited does not drive biliary cholesterol secretion.

The second conclusion concerns the lack of change in plasma total cholesterol concentrations (Fig. 1F) under circum-

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1 Information contained in the animal pharmacology section of the package insert for Zetia (ezetimibe) tablets.
stances in which there was a profound reduction in intestinal cholesterol absorption (Fig. 1A) and a markedly accelerated rate of fecal neutral sterol excretion (Fig. 1B). Presumably, in the ezetimibe-treated hamsters, the compensatory increase in intestinal and hepatic sterol synthesis (Fig. 1, D and E) fully balanced the additional loss of sterol from the body, so that there was no detectable impact on circulating plasma cholesterol levels in the face of a major reduction in cholesterol absorption. The lowest dose of ezetimibe given to the hamsters (0.5 mg·day⁻¹·kg body wt⁻¹) corresponds to about four times the dose typically prescribed to humans (10 mg once daily). In subjects with a very low dietary cholesterol intake, this dose of ezetimibe reduced fractional cholesterol absorption by 58% and increased fecal neutral sterol excretion by 81% (9). There was also a 72% increase in whole body cholesterol synthesis. Despite this, plasma LDL-C levels in these subjects fell, on average, by 17%. Thus, in these individuals, the compensatory increase in cholesterol synthesis, which presumably reflected increases mainly by the liver and small bowel, did not fully balance the increased loss of sterol from the body, thus leading to a fall in circulating LDL-C levels. Although no effect of ezetimibe on plasma cholesterol levels was seen in hamsters fed the basal diet, there was clearly a marked reduction when dietary cholesterol intake was raised (Fig. 5C).

The third conclusion relates to the changes in the relative level of expression of mRNA for a number of key proteins involved in regulating sterol homeostasis in the enterocyte during, and following cessation of treatment with ezetimibe. It is particularly noteworthy that the relative mRNA level for NPC1L1 remained almost unchanged, irrespective of whether the hamsters were on or off ezetimibe treatment (Fig. 3B). This was also true for the transcript level for ABCA1. In contrast, the mRNA level for ABCG5 and G8 fell significantly when ezetimibe was given but returned to baseline values after withdrawal of treatment. Clearly, the reduced level of mRNA for ABCG5 and G8 reflected an ezetimibe-induced deficit of cholesterol within the enterocyte, a condition that also triggered a marked induction of local cholesterol synthesis as evidenced not only by the increased mRNA levels for HMG CoA synthase and HMG CoA reductase but also by direct measurements of the rate of small bowel sterol synthesis in vivo (Fig. 1, D and G). Although these data clearly attest the impact of blocking cholesterol uptake into the enterocyte at the brush border membrane, they also demonstrate that once ezetimibe treatment ceases there is a rapid return toward normal levels of cholesterol absorption (Fig. 3A) and the pathways that govern cholesterol homeostasis in the enterocyte (Fig. 3B).

Fig. 5. Appearance of livers and hepatic and plasma total cholesterol concentrations in female hamsters fed a lipid-rich diet without or with ezetimibe. Female Golden Syrian hamsters were fed for 28 days either a basal diet alone or the same diet enriched with cholesterol and safflower oil without or with ezetimibe at a dose of 1.0 mg·day⁻¹·kg body wt⁻¹. The livers depicted (A) are representative of what was found in other hamsters fed the lipid-rich diet without ezetimibe (left) or with ezetimibe (right). Hepatic and plasma cholesterol levels (B and C, respectively) were measured as described in MATERIALS AND METHODS. Values are means ± SE of data from 6 or 7 animals in each group. *Significantly different from corresponding value for group fed basal diet without ezetimibe, P < 0.05. ‡Significantly different from value for group fed lipid-rich diet without ezetimibe, P < 0.05.
The fourth conclusion concerns the relative expression of NPC1L1 mRNA in the liver and extrahepatic organs of the Golden Syrian hamster compared with what has been reported for other animal models (1, 36) and humans (1, 12). Although such comparative data remain limited and are based on mRNA rather than protein or activity measurements, it appears that in humans there is a much greater relative expression of mRNA for NPC1L1 in liver compared with other tissues than is the case in the few animal species in which this has been investigated. On the basis of the present data, the Golden Syrian hamster appears to be more like the rat, mouse, and miniature pig in that the dominant site of expression of NPC1L1 message is the small bowel, with comparatively little being present in the liver (Fig. 2; Refs. 1, 36). In the hamster, NPC1L1 mRNA was detected in the gallbladder at levels higher than those seen in the liver but still trivial compared with the level of expression in the small intestine. The pathophysiological significance of NPC1L1 in the liver of humans is not well understood, but studies with hepatocytes from African Green monkeys reveal that the protein localizes in the canalicular membrane (54). Furthermore, other data from a transgenic mouse model show that hepatic overexpression of human NPC1L1 in the liver resulted in a dramatic decrease in biliary cholesterol concentration and, moreover, that ezetimibe treatment of these mice essentially normalized biliary cholesterol concentration (37). These findings have been interpreted to mean that in humans NPC1L1 may function in regulating the amount of cholesterol entering the bile (53).

Taken together, these various observations raise the clinically important question of what the long-term impact of using ezetimibe, either in combination with statins or in some cases as monotherapy, for managing hypercholesterolemia might be on biliary cholesterol content and an individual’s predisposition to cholesterol gallstone formation. The results of the present studies with ezetimibe treatment of hamsters fed a cholesterol- and fat-enriched diet (Fig. 5–7) make two points that bear directly on this question. One is that by placing a major block on absorption at the level of cholesterol uptake by the enterocyte, the dramatic and adverse changes in intrahepatic cholesterol metabolism and biliary cholesterol content associated with consumption of the high-cholesterol, high-fat diet are greatly attenuated (Fig. 5–7). The other is that although there was a detectable increase in the cholesterol concentration in the bile when hamsters fed the low-cholesterol basal diet were given ezetimibe, the corresponding increase associated...
with feeding the lipid-rich diet alone was far greater (Fig. 7A). More important, however, was the finding that ezetimibe treatment, given concurrently with this diet, resulted in a reduction in the absolute (Fig. 7A) and relative (Fig. 7D) cholesterol content of the bile to values at or near those seen in matching hamsters given the basal diet alone. Herein lies the potential relevance of these data in the hamster to the question of long-term ezetimibe treatment and biliary cholesterol levels in humans. Based on our current understanding of all the factors that can impact the level of biliary cholesterol saturation, it seems that any increase in biliary cholesterol levels that might arise from an effect of ezetimibe directly on the activity of NPC1L1 on the canalicular surface of hepatocytes would be more than offset by the marked reduction in the inflow of chylomicron cholesterol to the liver and the potential that this has to drive up biliary cholesterol content. Added to this is the point that ezetimibe is most widely used as an add-on to statins, which are known to reduce biliary cholesterol content (38). That aside, the present data, together with the results of a recent study demonstrating a favorable effect of short-term ezetimibe treatment on biliary cholesterol levels in humans (51), suggest that a clinical trial designed to investigate the potential of ezetimibe for reducing biliary cholesterol saturation in populations with a predisposition to cholelithiasis is now warranted.

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