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Effect of Ezetimibe on the Prevention and Dissolution of Cholesterol Gallstones¹

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Abstract

Background & Aims—Cholesterol cholelithiasis is one of the most prevalent and most costly digestive diseases in developed countries and its incidence has markedly increased in Asian countries due to the adoption of Western-type dietary habits. Because animal experiments showed that high efficiency of intestinal cholesterol absorption contributes to gallstone formation, we explored whether the potent cholesterol absorption inhibitor ezetimibe could prevent gallstones and promote gallstone dissolution in mice and reduce biliary cholesterol content in humans.

Methods—Male gallstone-susceptible C57L mice were fed a lithogenic diet and concomitantly administered with ezetimibe at 0, 0.8, 4 or 8 mg/kg/day for 8 or 12 weeks. Gallbladder biles and gallstones were examined by microscopy. Gallbladder emptying in response to CCK-8 was measured gravimetrically. Biliary lipid outputs were analyzed by physical-chemical methods. Cholesterol absorption efficiency was determined by fecal dual-isotope ratio and mass balance methods. Lipid changes in gallbladder biles of gallstone patients vs. overweight subjects without gallstones were examined before (day 0) and at 30 days after ezetimibe treatment (20 mg/day).

Results—Ezetimibe prevented gallstones by effectively reducing intestinal cholesterol absorption and biliary cholesterol secretion, and protected gallbladder motility function by desaturating bile in mice. Treatment with ezetimibe promoted the dissolution of gallstones by forming an abundance of unsaturated micelles. Furthermore, ezetimibe significantly reduced biliary cholesterol saturation and retarded cholesterol crystallization in biles of patients with gallstones.

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Conclusions—Ezetimibe is a novel approach to reduce biliary cholesterol content and a promising strategy for preventing or treating cholesterol gallstones by inhibiting intestinal cholesterol absorption.

Keywords

bile; bile flow; bile salt; biliary secretion; cholesterol absorption; chylomicron; *Lith* gene; nutrition

INTRODUCTION

Cholesterol gallstone formation represents a failure of biliary cholesterol homeostasis in which the physical-chemical balance of cholesterol solubility in bile is disturbed.^{1,2} Lithogenic bile is mainly caused by persistent hepatic hypersecretion of biliary cholesterol, which has both hepatic and small intestinal components. Obviously, in a person receiving no dietary cholesterol, all biliary cholesterol could be derived mainly from *de novo* synthesis. However, the contribution of *de novo* cholesterol synthesis to biliary cholesterol secretion is small, possibly less than 15%.^{3,4} The small intestine is a unique organ providing dietary and re-absorbed biliary cholesterol to the body.⁵

Epidemiological investigations have demonstrated that cholesterol cholelithiasis is prevalent in cultures consuming a “Western” diet consisting of high total calories, cholesterol, saturated fatty acids, refined carbohydrates, proteins, and salt, as well as low fibers, and its incidence in North and South American as well as European populations is significantly higher than that in Asian and African populations.^{6,7} Several clinical studies have found an association between the increased incidence of cholesterol gallstones in China and a “westernization” of the traditional Chinese diet.^{8–10} In Japan, cholesterol cholelithiasis once was rare, but over the past 40 years with the adoption of Western-type dietary habits, the incidence has increased markedly.^{11,12} Because biliary cholesterol hypersecretion is an important prerequisite for cholesterol gallstone formation,^{1,2} it is reasonable to assume that by inhibiting cholesterol absorption and/or hepatic uptake of chylomicron remnants, biliary cholesterol secretion and saturation could be significantly reduced. More recently, we observed that there is a significant and positive correlation between the efficiency of intestinal cholesterol absorption and the prevalence of cholesterol gallstone formation in 15 strains of inbred mice,⁵ suggesting that high efficiency of intestinal cholesterol absorption and high dietary cholesterol are two independent risk factors for cholesterol gallstone formation.

In this study, we investigated whether the potent cholesterol absorption inhibitor ezetimibe could prevent the formation of cholesterol gallstones, and facilitate the dissolution of gallstones in gallstone-susceptible C57L mice carrying *Lith1* and *Lith2* genes. Also, we studied whether ezetimibe may act as a potent biliary cholesterol-desaturating agent in patients with gallstones.

MATERIALS AND METHODS

Animals and diets

Male C57L/J mice, 6–8 weeks old, were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were provided free access to water and normal rodent chow containing trace (<0.02%) cholesterol (Harlan Teklad F6 Rodent Diet 8664, Madison, WI). For gallstone prevention studies, mice were divided into five groups (n=20 per group) fed 8 or 12 weeks with a lithogenic diet (1% or 2% cholesterol plus 0.5% cholic acid and 15% butter fat) supplemented with ezetimibe (Merck/Schering-Plough Pharmaceuticals, North Wales, PA) in doses of 0, 0.8, 4, or 8 mg/kg/day. We previously observed that at 12 weeks on 2% cholesterol plus 0.5% cholic acid and 15% butter fat, 100% of C57L mice formed cholesterol gallstones.¹³ For gallstone dissolution experiments, additional groups (n=20 per group) of mice that have

formed cholesterol gallstones due to the 12-week feeding of 2% cholesterol plus 0.5% cholic acid and 15% butter fat, were fed the chow diet supplemented with ezetimibe in doses of 0, 0.8, 4, or 8 mg/kg/day for 8 weeks. All procedures were in accordance with current National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee of Harvard University (Boston, MA).

Human study design

To observe whether ezetimibe reduces bile cholesterol content and saturation in humans, we studied patients with gallstones (n=7; 40.3±14.5 years old; 1 male and 6 females) and overweighted subjects without gallstones (n=5; 38.2±13.6 years old; 2 males and 3 females). All participants were examined by ultrasonography, which confirmed the existence of gallstones in the gallbladder. All subjects took 20 mg/day of ezetimibe daily for 30 days. During the study period, daily dietary cholesterol intake was approximately 500 mg. Gallbladder bile samples were collected endoscopically before (day 0) and at day 30 after medication. In brief, after an overnight fast and during a routine upper endoscopy, the tip of the endoscope was advanced to the second portion of the duodenum and the ampulla of Vater was visualized. Bile samples were harvested by stimulating gallbladder emptying using an intraduodenal infusion of 30 ml of an 80 g/L amino acid solution.¹⁴ A 20- to 30-ml sample of duodenal bile was obtained from each subject. Bile samples were frozen and stored at -20°C for further lipid analyses. Cholesterol saturation indexes (CSI) were determined after biliary cholesterol, phospholipid and bile salt concentrations, as well as bile salt species were measured.^{5,15} Detection times of plate-like cholesterol monohydrate crystals were investigated in these bile samples according to published methods.¹⁶ These studies were approved by the Human Subjects Committees at The Medica Sur Clinic and Foundation (Mexico City, Mexico), as conforming to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from all participants before entry.

Microscopic studies of gallbladder biles and gallstones

At 8 or 12 weeks on the lithogenic diet supplemented with various doses of ezetimibe, a cholecystectomy was performed in overnight fasted mice (n=20 per group). Fresh gallbladder bile were examined for mucin gel, liquid crystals, plate-like cholesterol monohydrate crystals, sandy stones, and real gallstones, all of which were defined according to previously established criteria.¹⁷

Determination of biliary lipid outputs and bile salt pool sizes

The first hour collection of hepatic biles in additional groups of mice (n=5 per group) were used for biliary lipid secretion studies as described previously.¹⁸

Lipid analyses

Biliary cholesterol, phospholipid, total and individual bile salts, bile cholesterol, cholesterol content in chow and gallstones, and fecal neutral steroids were determined as described previously.¹⁶⁻²⁰ The CSI values in gallbladder and hepatic biles were calculated from the critical tables.¹⁵

Measurement of intestinal cholesterol absorption by fecal dual-isotope ratio method

Nonfasted and nonanesthetized mice (n=10 per group) were given by gavage an intragastric bolus of 150 µl of medium-chain triglyceride containing 1 µCi of [¹⁴C]cholesterol and 2 µCi of [³H]sitostanol. The ratios of the two radiolabels in the fecal extracts and in the dosing mixture were used for calculating the percent cholesterol absorption.¹⁹

Cholesterol balance analysis

Because high dietary cholesterol exerts the effect of the radioisotope dilution on specific activity of cholesterol in the upper small intestine, we used cholesterol balance analysis¹⁹ to determine cholesterol absorption efficiency in mice challenged to the lithogenic diet. Mice (n=5 per group) were fed the lithogenic diet supplemented with various doses of ezetimibe for 7 days. After cholecystectomy was performed and the common bile duct was cannulated, hepatic bile was collected for the first hour of biliary secretion. Percent cholesterol absorption was calculated as described previously.¹⁹

Quantitative real-time PCR assay

Total RNA was extracted from fresh jejunum tissues of mice (n=4 per group) using RNeasy Midi (Qiagen, Valencia, CA). Primer Real-time PCR assays of *Npc1l1*, *Abcg5* and *Abcg8* for all samples were performed in triplicate.²⁰ To obtain a normalized target value, the target amount was divided by the endogenous reference amount of rodent *Gapdh* as the invariant control.

Gallbladder contraction study

To explore whether ezetimibe has a protective effect on gallbladder motility function, we measured gallbladder contraction in response to exogenously administered sulfated CCK octapeptide (CCK-8) in mice (n=5 per group) fed the lithogenic diet supplemented with varying doses of ezetimibe for 8 weeks according to published methods.²¹ Gallbladder contractile function was determined by comparing the areas under the curves of bile flow rates and bile salt outputs, respectively, in the collected samples with mixed gallbladder and hepatic biles.

Statistical method

All data are expressed as means±SD. Statistically significant differences among groups of mice were assessed by Student's *t*-test, Mann-Whitney U-tests, or Chi-square tests. If the F-value was significant, comparisons among groups of mice were further analyzed by a multiple comparison test. Analyses were performed with a *SuperANOVA* software (Abacus Concepts, Berkeley, CA). Statistical significance was defined as a two-tailed probability of less than 0.05.

RESULTS

Effect of ezetimibe on biliary cholesterol secretion in mice and humans

At 8 weeks on the lithogenic diet, administration of ezetimibe to mice resulted in a clear dose-dependent decrease in biliary cholesterol outputs during the first hour of biliary secretion of interrupted enterohepatic circulation (Figure 1A). Similarly, biliary phospholipid outputs were significantly reduced in a dose-dependent fashion (Figure 1B). However, hepatic outputs of biliary bile salts varied slightly and were not significantly different among four groups of mice (Figure 1C). We also observed a dose-dependent decrease in cholesterol/phospholipid ratios and cholesterol/bile salts ratios, indicating that bile cholesterol saturation was significantly reduced by ezetimibe.

As shown in Table 1, at day 30 after ezetimibe treatment, plasma concentrations of total cholesterol in overweight subjects without gallstones and LDL cholesterol in gallstone patients were significantly reduced. Other parameters of plasma lipids were decreased slightly and did not reach significant statistical differences. Furthermore, mole percent cholesterol and cholesterol saturation index were significantly reduced in patients with gallstones, and to some extent, in overweighted subjects without gallstones. As a result, crystal detection times were significantly retarded in patients with gallstones and overweight subjects without gallstones.

Mole percent phospholipid and bile salts in gallbladder biles were not influenced in humans treated with ezetimibe at 20 mg/day.

Prevention of cholesterol gallstones by ezetimibe

Administration of ezetimibe induced a significant dose-dependent inhibition of intestinal cholesterol absorption in chow-fed mice as measured by the fecal dual-isotope ratio method (Figure 1D). Compared with the chow diet, cholesterol absorption efficiency was significantly increased in mice fed the lithogenic diet, because of the dietary cholic acid as discussed elsewhere.^{22,23} However, there was a significant dose-dependent inhibitory effect on intestinal cholesterol absorption by ezetimibe in mice challenged to the lithogenic diet, as measured by the mass balance method (Table 2). As expected, the lithogenic diet significantly increased mole percent cholesterol in gallbladder biles.¹⁷ In contrast, mole percent cholesterol in biles was gradually decreased with increasing doses of ezetimibe. Thus, the CSI values of pooled gallbladder biles were obviously reduced from 1.56 to 0.37 by ezetimibe (Table 3). As a result, there was a dose-dependent decrease in gallstone prevalence rates from 80% to 10% in mice treated with ezetimibe from 0 to 4 mg/kg/day (Figure 1E). Of special note is that no gallstones were found in mice treated with the highest dose of ezetimibe at 8 mg/kg/day. This protective effect of ezetimibe was also observed in mice fed the lithogenic diet with even higher (2%) amounts of cholesterol for a longer (12 weeks) period.

Physical-chemical analysis of gallbladder and hepatic biles

Analyses of individual bile salt species by HPLC revealed that all bile salts in gallbladder and hepatic biles of mice were taurine conjugated with a similar distribution of bile salt compositions. In mice challenged to the lithogenic diet, taurocholate (58.7–70.1%) was the major bile salt of biliary pool, followed by taurodeoxycholate (6.9–17.6%) and taurochenodeoxycholate (7.8–14.0%). There was a low concentration in tauro- β -muricholate (2.9–6.4%), tauro- ω -muricholate (1.7–4.1%) and tauroursodeoxycholate (2.4–4.0%). Hydrophobicity indexes of bile salts in biles were essentially similar (–0.01 to +0.10) among four groups of mice. These results indicate that ezetimibe does not influence bile salt species in bile. The relative lipid compositions of pooled gallbladder biles from mice fed the lithogenic diet supplemented with ezetimibe at 0 or 0.8 mg/kg/day were located in the central three-phase area denoted Region C (Figure 1F). These biles were composed of solid cholesterol monohydrate crystals, liquid crystals and saturated micelles.¹⁶ Furthermore, with an increase in doses of ezetimibe, the relative lipid compositions of pooled gallbladder biles progressively shifted downward and to the left of the phase diagrams. These alterations were caused by a significant reduction in cholesterol content (Table 3). Consequently, administration of ezetimibe at 8 mg/kg/day resulted in the biliary lipid compositions plotted within the one-phase micellar zone. By phase analysis, these biles were composed of one phase, namely micellar bile, exactly as was observed experimentally in model biles.¹⁶ It indicates that the highest dose of ezetimibe can successfully prevent the formation of cholesterol gallstones in mice challenged to high dietary cholesterol.

Dissolution of cholesterol gallstones by ezetimibe

At 12 weeks on the lithogenic diet containing 2% cholesterol, 100% of male C57L mice formed cholesterol gallstones, with stone size being 0.72 ± 0.33 mm. After the diet was changed to the normal rodent chow diet, gallstones were still present in 85% of mice (Figure 2A), with stone diameters in 0.63 ± 0.28 mm. This indicates that spontaneous dissolution of gallstones did not occur after the lithogenic diet was replaced with the chow diet for 8 weeks (Figure 2B). When the chow diet was added with ezetimibe at 0.8 mg/kg/day, some mucin gel, solid and liquid crystals, sandy stones, and tiny real gallstones (0.27 ± 0.14 mm in diameter) were observed in 5% of mice. Although preexisting cholesterol gallstones were completely dissolved by

ezetimibe at 4 mg/kg/day, an abundance of liquid crystals and cholesterol monohydrate crystals were still detected in biles (Figure 2B). In contrast, in mice treated with ezetimibe at 8 mg/kg/day, gallbladder biles were transparent and their examination by polarizing light microscopy revealed no solid cholesterol crystals or gallstones (Figure 2B). It indicated that treatment with the highest dose of ezetimibe for 8 weeks resulted in complete gallstone dissolution rates of 100%. Compared with the chow diet, the CSI values, cholesterol/phospholipid ratios, and mole percent cholesterol of pooled gallbladder biles were decreased markedly by ezetimibe in a dose-dependent manner (Table 4). In addition, no differences in bile salt species and the hydrophobicity indexes were found in biles of these mice. Figure 2C plots the relative lipid compositions of pooled gallbladder biles before (day 0) and at week 8 after ezetimibe treatment on a taurocholate-rich bile phase diagram. The lipid compositions of pooled gallbladder biles with preexisting cholesterol gallstones in chow-fed mice still fell in the central three-phase zone. In contrast, the relative lipid compositions of biles from mice fed the highest dose of ezetimibe were located on the one-phase micellar zone in which bile is composed of unsaturated micelles.¹⁶

Amelioration of gallbladder contraction by ezetimibe

Under lithogenic diet feeding conditions, gallbladder sizes were significantly increased compared with the chow diet (Figure 3A). In the lithogenic state, gallbladder sizes were still enlarged in mice treated even with a low (0.8 mg/kg/day) dose of ezetimibe (Figures 3A and 3B). However, these lithogenic effects on gallbladder dynamics were fully blocked by ezetimibe in doses of higher than or equal to 4 mg/kg/day. Because CCK-8 stimulated gallbladder emptying and concentrated gallbladder bile was released, biliary bile salt output and bile flow rate were increased sharply and significantly only in mice treated with the highest (8 mg/kg/day) dose of ezetimibe. In contrast, these parameters were decreased when the doses of ezetimibe were reduced from 4 to 0 mg/kg/day. These alterations suggested that during lithogenesis, gallbladder motility function was impaired completely in mice fed the lithogenic diet and partially in mice treated with lower doses (≤ 4 mg/kg/day) of ezetimibe. In contrast, the administration of CCK-8 induces complete gallbladder emptying in mice treated with ezetimibe at 8 mg/kg/day (Figures 3C and 3D).

Effect of ezetimibe on gene expression of intestinal sterol transporters

As shown in Figure 4, the data are expressed relative to the mRNA levels of intestinal lipid transporters in mice fed the lithogenic diet and receiving no ezetimibe, and their relative expression levels are set at 1. Treatment of ezetimibe at 8 mg/kg/day resulted in significant decreases in expression levels of the intestinal *Abcg5*, *Abcg8* and *Acat2* genes in mice on the lithogenic diet. It is highly likely that these changes are a secondary phenomenon due to reduced amounts of the absorbed cholesterol entering the enterocyte in ezetimibe-treated mice. Moreover, ezetimibe treatment significantly reduced expression levels of *Npc1l1* in the small intestine.

DISCUSSION

Cholesterol cholelithiasis is a multifactorial disease influenced by a complex interaction of genetic and environmental factors.^{1,2} In mouse studies, targeted deletion of the acyl-CoA:cholesterol acyltransferase gene 2 (*Acat2*) results in the lack of cholesterol ester synthesis in the small intestine. This causes a marked reduction in intestinal cholesterol absorption and complete resistance to diet-induced cholesterol gallstones.²⁴ Furthermore, absence of expression of intestinal APO-B48, but not APO-B100, reduces biliary cholesterol secretion and cholelithogenesis, possibly by decreasing intestinal absorption and hepatic bioavailability.²⁵ Reduced gallstone prevalence in lithogenic diet-fed apolipoprotein E knockout mice may be explained by decreased availability of chylomicron-derived cholesterol in the liver for

biliary secretion.²⁶ These studies^{24–26} support the notion that high dietary cholesterol through the chylomicron pathway could provide an important source of excess cholesterol molecules for secretion into bile, thereby inducing cholesterol-supersaturated bile and enhancing cholelithogenesis. Since ezetimibe significantly suppresses cholesterol absorption from the small intestine via the NPC1L1 pathway,²⁷ possibly a transporter-facilitated mechanism,²⁸ this should diminish the cholesterol content of the liver, which in turn decreases bioavailability of cholesterol for biliary secretion. Indeed, we observed that ezetimibe induces a significant dose-dependent reduction in intestinal cholesterol absorption efficiency, coupled with a significant dose-dependent decrease in biliary cholesterol outputs and gallstone prevalence rates. In particular, even under high dietary cholesterol loads, cholesterol gallstones can be prevented in C57L mice treated with the highest dose of ezetimibe at 8 mg/kg/day.

We observed that although ezetimibe primarily reduces cholesterol content and to some extent phospholipid content but not in bile salt content in gallbladder bile, all crystallization pathways and phase boundaries on the bile phase diagram are essentially similar, regardless of whether mice are treated with or without ezetimibe. Of special note is that the relative lipid compositions of gallbladder biles from mice treated with varying doses of ezetimibe become appreciably less saturated with cholesterol and also contain relatively lower mole percent phospholipid. As a result, in company with increased doses of ezetimibe, the relative lipid compositions of pooled gallbladder biles are progressively shifted down and to the left of the phase diagram, as well as entering the one-phase micellar zone when the dose of ezetimibe is the highest at 8 mg/kg/day (see Figure 2C). The micellar zone contains an abundance of unsaturated micelles but never solid cholesterol crystals or liquid crystals.¹⁶ Under these circumstances, the micellar cholesterol solubility is dramatically increased in gallbladder bile. Thus, the cholesterol molecules could be transferred from the cholesterol monohydrate surface into unsaturated micelles so that gallstones become smaller and eventually dissolved. This provides an excellent physical-chemical explanation for the absence of solid cholesterol crystal formation in mice treated with the highest dose of ezetimibe (8 mg/kg/day) at week 8 of gallstone dissolution treatment.

In humans, the gallbladder is another key player in gallstone pathogenesis because enlarged fasting gallbladder volume, together with impaired postprandial and interdigestive gallbladder emptying, are frequent and distinctive features in gallstone patients.^{29,30} This type of “gallbladder stasis” provides time for nucleation of cholesterol crystals and their aggregation into macroscopic stones.^{1,2,30} It has become increasingly evident that the gallbladder epithelium could actively modify the lipid compositions of bile by secretion and absorption of lipids and water.^{31,32} Cholesterol-supersaturated bile facilitates gallbladder absorption of cholesterol and enhances the accumulation of excess cholesterol in the gallbladder wall.^{1,2} Because gallbladder absorptive cells apparently cannot assemble lipoproteins for lipid transport into plasma, the absorbed cholesterol is converted to cholesteryl ester and stored in the mucosa and lamina propria. Hence, excess cholesterol in smooth muscle cells could stiffen sarcolemmal membranes and decouple the G-protein-mediated signal transduction that usually ensues when CCK binds to its receptor, thereby further paralyzing gallbladder contractile function and consequently impairing gallbladder emptying function. In contrast, these lithogenic effects on gallbladder motility function can be totally blocked by ezetimibe in the highest dose at 8 mg/kg/day, mostly attributable to decreased biliary cholesterol content in bile.³³

The hydrophilic bile acid, ursodeoxycholic acid (UDCA) has been recommended as first-line pharmacological therapy in a subgroup of symptomatic patients with small, radiolucent cholesterol gallstones,^{2,34} and its long-term administration has been shown to promote the dissolution of cholesterol gallstones and to prevent the recurrence of gallstones after extracorporeal shock wave lithotripsy.³⁵ Despite major improvements in the treatment of

cholesterol gallstones, optimal use of UDCA is not always achieved in clinical practice, because of failure to titrate the dose adequately. On the basis of animal studies, the natural trihydroxy hydrophilic bile acid of rodents, β -muricholic acid has recently been proposed for the prevention and the treatment of cholesterol gallstones.¹³ It should be emphasized that the cholelitholytic mechanism of ezetimibe is different from that of hydrophilic bile acids such as β -muricholic acid and UDCA. These hydrophilic bile acids favor the formation of vesicles in bile so that the growth of liquid crystals on the cholesterol monohydrate surface and their subsequent dispersion might occur during gallstone dissolution. As a result, liquid crystalline dissolution allows the transport of a great amount of cholesterol from stones. Our results indicate, therefore, that ezetimibe and hydrophilic bile acids promote the dissolution of cholesterol gallstones by two distinct mechanisms via the formation of an unsaturated micelle and a liquid crystalline mesophase, respectively.

Of note is that the *NPC1L1* gene is expressed in livers of humans, but not in mice. More recently, Temel et al.³⁶ found that mice transgenic for a human *NPC1L1* gene display an increase in biliary cholesterol concentrations. This suggests that inhibition of NPC1L1 in the liver by ezetimibe may rescue biliary cholesterol secretion and increase CSI values in bile. However, in this study we observed that at day 30 after the medication, ezetimibe at 20 mg/day can significantly reduce cholesterol concentrations and CSI values of gallbladder bile in patients with gallstones, mostly attributable to its inhibitory effect on intestinal cholesterol absorption.^{27,28} As a result, cholesterol crystallization is retarded so that detection time of cholesterol monohydrate crystals is significantly delayed. Recent research on molecular transporters responsible for biliary lipid secretion suggests that the secretion efficiency of biliary cholesterol is most likely determined by the net effect between efflux and influx of cholesterol molecules across the canalicular membrane of the hepatocyte, which could be regulated by the ABCG5/G8-dependent and independent pathways as well as the NPC1L1 pathway.³⁷ One possible reason for our results is that because biliary cholesterol secretion is a unique path for excretion of cholesterol from the body in humans and animals, hepatic ABCG5/G8 may play a stronger role in the regulation of biliary cholesterol secretion than NPC1L1. Another possible explanation is that in the gut-liver axis, the intestinal NPC1L1 plays a significant role in providing dietary and reabsorbed biliary cholesterol to the body and the inhibition of its functions by ezetimibe significantly reduces cholesterol absorption. So, the bio-availability of cholesterol from intestinal sources for biliary secretion is decreased markedly. In contrast, the blockage of the hepatic NPC1L1 by ezetimibe has a weak effect on biliary cholesterol secretion and CSI values, as we found in this study. Nevertheless, we are performing clinical studies to observe whether gallstones may completely dissolve after treating patients with ezetimibe for a longer period. To evaluate treatment time, response rate and overall cost-benefit analysis, a more detailed, long-term human study is requested as well. Furthermore, on the basis of our mouse study, it is highly likely that cholesterol gallstones could be dissolved faster by combination therapy of ezetimibe and UDCA in patients. Again, further studies are required to confirm this hypothesis.

Overall, our results show that ezetimibe prevents cholesterol gallstones through inhibiting intestinal cholesterol absorption so that biliary cholesterol secretion is reduced, and gallbladder motility function is preserved by desaturating bile in gallstone-susceptible C57L mice challenged to the lithogenic diet. Also, ezetimibe promotes the dissolution of cholesterol gallstones through a greater capacity to form an abundance of unsaturated micelles. Therefore, we conclude that ezetimibe is a novel and potential cholelitholytic agent for preventing or treating cholesterol gallstone disease. Our findings suggest a novel strategy for the prevention of cholesterol gallstones by inhibiting intestinal cholesterol absorption in humans.

Abbreviations

ABC, ATP-binding cassette (transporter); CSI, cholesterol saturation index; NPC1L1, Niemann-Pick C1 like 1 (protein).

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Figure 1. Effect of ezetimibe on the prevention of cholesterol gallstones

Ezetimibe significantly reduces, in a dose-dependent fashion, hepatic outputs of (A) biliary cholesterol and (B) phospholipid, but not (C) bile salts. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, compared with mice fed the lithogenic diet and receiving no ezetimibe. (D) There is a clear dose-dependent reduction in intestinal cholesterol absorption efficiency from $50 \pm 6\%$ to $4 \pm 2\%$ in chow-fed mice, as measured by the fecal dual-isotope ratio method. (E) When doses of ezetimibe are increased from 0 to 4 mg/kg/day, gallstone prevalence rates are reduced from 80% to 10% in mice fed the lithogenic diet for 8 weeks. No gallstones are found in mice treated with ezetimibe at 8 mg/kg/day. (F) The relative lipid compositions of pooled gallbladder biles from mice fed the lithogenic diet and receiving no ezetimibe are located in the central three-phase zone, where biles are composed of solid cholesterol monohydrate crystals, liquid crystals, and saturated micelles at equilibrium.¹⁶ In contrast, administration of the highest dose (8 mg/kg/day) of ezetimibe results in the relative biliary lipid compositions of pooled gallbladder biles plotted in the one-phase micellar zone, even upon the lithogenic diet feeding for 8 weeks. By phase analysis, these biles are composed of unsaturated micelles at equilibrium.

¹⁶ A symbol ♦ represents relative lipid compositions of pooled gallbladder biles at 8 weeks on the lithogenic diet supplemented with ezetimibe at 0; ● 0.8; ▲ 4; and ■ 8 mg/kg/day.

Figure 2. Effect of ezetimibe on the dissolution of cholesterol gallstones

(A) For gallstone dissolution experiments, mice with the preexisting gallstones are fed the chow diet alone for 8 weeks, which does not result in a spontaneous dissolution of gallstones. In contrast, treatment with ezetimibe at 0.8 to 8 mg/kg/day induces rapid dissolution of gallstones. Gallstones are completely dissolved by the highest (8 mg/kg/day) dose of ezetimibe. (B) Representative photomicrographs of mucin gel, liquid crystals, cholesterol monohydrate crystals, and gallstones as observed in gallbladder biles at week 8 after ezetimibe treatment. All magnifications are $\times 800$, except for ezetimibe treatment at 0 and 0.8 mg/kg/day, which are $\times 400$, by polarizing light microscopy. (C) The relative lipid compositions of pooled gallbladder biles from mice fed 8 weeks with the chow diet supplemented with varying doses of ezetimibe

are plotted on a condensed phase diagram. Because of a 12-week feeding of the lithogenic diet, the relative lipid compositions of pooled gallbladder biles from mice that have formed cholesterol gallstones are located in the central three-phase zone. Although the lithogenic diet is replaced with the chow diet for 8 weeks, the relative biliary lipid compositions of biles are still in Region C, where at equilibrium the biles are composed of solid cholesterol crystals, liquid crystals, and saturated micelles.¹⁶ By feeding varying doses of ezetimibe, the relative lipid compositions of pooled gallbladder biles gradually shift down, and finally, enter the one-phase micellar zone. These alterations explain that gallstones are dissolved through an unsaturated micelle mechanism. A symbol * represents relative lipid compositions of pooled gallbladder biles from mice that have preexisting gallstones and before ezetimibe treatment; ♦ relative lipid compositions of pooled gallbladder biles at end of gallstone dissolution study at week 8 of feeding the chow diet only (control); ● 0.8; ▲ 4; and ■ 8 mg/kg/day of ezetimibe.

Figure 3.

Fasting gallbladder volumes (**A** and **B**) are significantly increased by the lithogenic diet compared with the chow diet. However, these lithogenic effects on gallbladder dynamics are totally blocked by ezetimibe in doses of higher than or equal to 4 mg/kg/day. * $P < 0.01$ and ** $P < 0.05$, compared with the chow group. (**C** and **D**) Because of gallbladder emptying and the release of a concentrated gallbladder bile, biliary bile salt output and bile flow are increased sharply and significantly in response to exogenously administered CCK-8 (as shown by arrows) in mice with bile fistulae. Gallbladder contractile function is completely impaired in mice fed the lithogenic diet for 8 weeks and partially in mice fed the lithogenic diet and concomitantly treated with ezetimibe at 0.8 or 4 mg/kg/day. In contrast, there is normal gallbladder contractile function in mice treated with the highest dose of ezetimibe at 8 mg/kg/day, even in challenge to the lithogenic diet. As shown in insets of Figures 3C and 3D, the areas under the curves of bile salt outputs and bile flow rates are significantly increased in a dose-dependent fashion in mice treated with ezetimibe. * $P < 0.01$ and ** $P < 0.05$, compared with mice fed the lithogenic diet and receiving no ezetimibe.

Figure 4.

Upon the lithogenic diet feeding, compared with mice receiving no ezetimibe, mice treated with ezetimibe at 8 mg/kg/day display significantly reduced expression levels of *Abcg5/g8* and *Acat2*, mostly attributable to secondary reaction in response to decreased amounts of the absorbed cholesterol. Also, ezetimibe treatment significantly reduces expression levels of *Npc1l1* in the small intestine. * $P < 0.01$, ** $P < 0.001$, and *** $P < 0.00001$, compared with mice fed the lithogenic diet and receiving no ezetimibe.

Table 1Plasma and biliary lipids before (day 0) and at day 30 after ezetimibe treatment in humans (20 mg/day)^a

Parameter	Overweight subjects without gallstones		Gallstone patients	
	Before	After	Before	After
BMI (kg/m ²)	31.5±3.8	31.4±3.4	27.0±2.8	27.1±2.3
<i>Plasma lipid concentrations</i>				
Total Ch (mg/dL)	220±41	168±29 ^b	223±32	193±26
LDL Ch (mg/dL)	144±53	99±36	145±26	115±23 ^b
HDL Ch (mg/dL)	44±13	37±13	45±11	45±11
TG (mg/dL)	164±88	160±104	166±64	165±76
<i>Biliary lipid compositions of gallbladder bile</i>				
Ch (mole%)	7.4±0.7	6.8±1.9	9.3±1.9	7.2±1.2 ^b
PL (mole%)	20.2±2.4	21.8±2.5	19.3±2.8	20.0±3.5
BS (mole%)	72.4±2.9	71.4±3.9	71.4±4.3	72.8±4.2
Ch/PL	0.37±0.03	0.31±0.08	0.48±0.05	0.37±0.06 ^c
Ch/BS	0.10±0.01	0.10±0.03	0.13±0.03	0.10±0.02
[TL] (g/dL)	5.3±0.4	5.0±0.9	5.5±0.7	5.3±0.8
CSI	1.2±0.1	1.0±0.2	1.6±0.2	1.3±0.2 ^b
CDT (days)	6.4±1.1	10.4±1.1 ^c	4.0±1.2	7.0±1.3 ^c

^aValues were determined from overweight subjects without gallstones (n=5) and gallstone patients (n=7).^bP<0.05^cP<0.01, compared with before ezetimibe treatment (paired *t* test).

Abbreviations: BMI, body mass index; TG, triglycerides; Ch, cholesterol; PL, phospholipids; BS, bile salts; [TL], total lipid concentrations; CSI, cholesterol saturation index; and CDT, crystal detection time.

Table 2
Cholesterol balance data in ezetimibe-treated mice in the lithogenic state

Ezetimibe (mg/kg/day)	Cholesterol intake (mg/day)	Biliary cholesterol (mg/day)	Steroid excretion (mg/day)	Absorbed cholesterol ^a (mg/day)	Cholesterol absorption ^b (%)
0	38.98±2.27	7.25±1.37	21.81±2.59	24.42±2.97	62.5±4.6
0.8	39.63±1.50	4.77±0.95 ^c	28.47±1.74 ^d	15.94±1.63 ^d	40.2±3.0 ^e
4	38.68±1.90	3.52±0.77 ^d	35.20±1.95 ^e	6.99±1.02 ^f	18.1±2.5 ^g
8	39.17±1.89	2.52±0.46 ^e	39.71±1.31 ^f	1.98±0.65 ^f	5.0±1.4 ^g

^a Absorbed cholesterol was determined by subtracting the daily fecal neutral steroid output from the daily cholesterol intake and the daily biliary cholesterol output as measured by the HPLC methods. 19,20

^b The percent cholesterol absorption was determined by the cholesterol balance analysis according to published methods. 19,20

^c P<0.05

^d P<0.01

^e P<0.001

^f P<0.0001

^g P<0.00001, compared with mice fed the lithogenic diet and receiving no ezetimibe.

Table 3
Biliary lipid compositions of gallbladder and hepatic biles after gallstone formation^a

Ezetimibe (mg/kg/day)	Cholesterol (Mole%)	Phospholipid (Mole%)	Bile Salts (Mole%)	Cholesterol Phospholipid Ratio	Cholesterol Bile Salts Ratio	Total Lipid Concentration (g/dL)	CSI
Pooled gallbladder biles							
0	9.36	16.72	73.92	0.56	0.13	9.42	1.56
0.8	6.68	16.21	77.11	0.41	0.09	9.23	1.16
4	5.30	16.22	78.48	0.33	0.07	9.88	0.92
8	2.12	16.14	81.73	0.13	0.03	10.44	0.37
Individual hepatic biles							
0	12.50±2.82	24.04±4.06	63.46±6.02	0.52±0.10	0.20±0.06	2.02±0.27	2.18±0.40
0.8	8.96±2.61	20.56±8.02	70.49±9.89	0.47±0.19	0.13±0.06	1.88±0.24	1.99±0.66
4	7.25±1.98 ^b	18.95±4.16 ^b	73.80±5.80	0.38±0.07 ^b	0.10±0.03 ^b	1.70±0.21	1.62±0.33
8	5.63±1.54 ^d	15.20±2.57 ^c	79.17±3.39	0.37±0.10 ^b	0.07±0.02 ^c	1.58±0.27	1.58±0.41 ^b

^a Values were determined from pooled gallbladder biles (n=20 per group) and five hepatic biles (the first hour of biliary secretion) per group.

^b P<0.05

^c P<0.01

^d P<0.001, compared with mice fed the lithogenic diet and receiving no ezetimibe.

Table 4
Biliary lipid compositions of pooled gallbladder biles after gallstone dissolution^a

(Ezetimibe mg/kg/day)	Cholesterol (Mole%)	Phospholipid (Mole%)	Bile Salts (Mole%)	Cholesterol Phospholipid Ratio	Cholesterol Bile Salts Ratio	Total Lipid Concentration (g/dL)	CSI
0	11.46	17.51	71.02	0.65	0.16	9.41	1.82
0.8	7.41	15.65	76.95	0.47	0.10	9.03	1.32
4	5.79	16.92	77.30	0.34	0.07	9.35	0.98
8	3.62	15.68	80.70	0.23	0.04	8.43	0.67

^aValues were determined from pooled gallbladder biles (n=20 per group).