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Augmented cholesterol absorption and sarcolemmal sterol enrichment slow small intestinal transit in mice, contributing to cholesterol cholelithogenesis.

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Non-technical Summary

Peristaltic function of the small intestine is compromised in humans and animal models of cholesterol gallstones. Using a lithogenic diet-fed mouse model (C57L/J), we show that slowing of small intestinal transit is due to excess absorption of cholesterol molecules from the upper small intestine followed by their integration into plasma membranes of smooth muscle cells. This, in turn, causes CCK signal-transduction decoupling, impairing muscle contraction. Blocking cholesterol absorption with ezetimibe (Zetia®), an inhibitor of intestinal sterol transport, prevents cholesterol enrichment of sarcolemmae and reverses the motility disorder. In most gallstone-prone humans, the small intestine is flooded constantly with an abundance of biliary cholesterol secreted by the liver. Its absorption slows small intestinal motility, and facilitates more biliary and dietary cholesterol to be absorbed and more 'secondary' bile salts to be synthesised by the anaerobic microflora of the gut, both of which amplify the cholelithogenic state.

Abstract

Cholesterol gallstones are associated with slow intestinal transit in humans as well as in animal models, but the molecular mechanism is unknown. We investigated in C57L/J mice whether the components of a lithogenic diet (LD; 1.0% cholesterol, 0.5% cholic acid, and 17% triglycerides), as well as distal intestinal infection with *Helicobacter hepaticus*, influenced small intestinal transit time. By quantifying the distribution of ^3H -sitostanol along the length of the small intestine following intraduodenal instillation, we found that the geometric centre (dimensionless) in both sexes was retarded significantly ($P < 0.05$) by the LD but not slowed further by helicobacter infection; (males, 9.4 ± 0.5 (uninfected), 9.6 ± 0.5 (infected) on LD) compared with (12.5 ± 0.4 and 11.4 ± 0.5 on chow). The effect of the LD was reproduced only by the binary combination of cholesterol and cholic acid. Treatment with ezetimibe sufficient to block intestinal cholesterol uptake caused small intestinal transit time to return to normal. We discovered that the LD induced cholesterol enrichment of the sarcolemmae of intestinal smooth muscle cells, known to produce hypomotility from signal-transduction decoupling of CCK, a physiological agonist for small intestinal propulsion in mice. In gallstone-prone humans, lithogenic bile carries large quantities of hepatic cholesterol into the upper small intestine continuously, thereby reproducing this effect. Intestinal hypomotility augments cholelithogenesis since it further increases cholesterol absorption, as well as the formation of deoxycholate, a pro-lithogenic secondary bile salt.

Abbreviations: CSI, cholesterol saturation index; C57L/J (C57L), inbred cholesterol gallstone-susceptible mouse strain from The Jackson Laboratory (JAX), Bar Harbor, ME; DCA, deoxycholic acid; LD, lithogenic diet; MCT, medium-chain triglycerides; Tukey HSD test, Tukey Honestly Significant Difference test; *Lith*, murine lithogenic gene; CCK, Cholecystokinin; NPC1L1, Niemann-Pick C1 Like-1; CCK-1R, CCK-1 Receptor.

Introduction

Cholesterol cholelithogenesis is characterized by cholesterol supersaturation of bile usually from hepatic hypersecretion of cholesterol, gallbladder hypomotility, and

rapid cholesterol nucleation and phase transitions in gallbladder bile (Paigen & Carey, 2002). Hypomotility of the small and possibly large intestines is also part of this ‘syndrome’ and may augment the lithogenic process from production of more secondary bile salts, especially deoxycholate, and increasing intestinal cholesterol absorption (Van Erpecum & Van Berge-Henegouwen, 1999; Dowling, 2000; Heaton, 2000). Two independent groups (Heaton *et al.*, 1993; Azzaroli *et al.*, 1999) estimated that whole gut transit times are nearly 30% longer in normal-weight women with cholesterol gallstones compared to non-stone formers. In addition, acromegalics treated with octreotide display prolonged intestinal transit times, elevated deoxycholate conjugates, and higher cholesterol saturation indices (CSIs) in gallbladder bile, plus rapid cholesterol crystallization (Dowling *et al.*, 1992; Veysey *et al.*, 2001; Thomas *et al.*, 2005), whereas cisapride, a prokinetic agent, counteracted this effect on small and large bowel transit (Veysey *et al.*, 2001). Furthermore, cholesterol-rich diets prolong small intestinal transit times in hamsters (Fan *et al.*, 2007) and ground squirrels (Xu *et al.*, 1996), and also lead to a two-fold increase in the cholesterol saturation of bile. This promotes cholesterol gallstone formation in these models, a trend reversible with the prokinetic agent, erythromycin (Xu *et al.*, 1998). However, neither in humans nor in animal models has the molecular mechanism for prolonged small intestinal transit in the lithogenic state been determined.

Most recent research on cholesterol gallstone pathogenesis has utilized inbred mice (Wang *et al.*, 1997; Wang *et al.*, 1999b; Lammert *et al.*, 2001; Paigen & Carey, 2002; Lyons & Wittenburg, 2006). The gallstone-prone C57L mouse (Khanuja *et al.*, 1995; Lammert *et al.*, 2001; Paigen & Carey, 2002; Lyons & Wittenburg, 2006))

develops gallstones most frequently when fed a lithogenic diet when infected with certain species of enterohepatic helicobacter strains (Maurer *et al.*, 2005) but not *H. pylori* (Maurer *et al.*, 2006). The standard lithogenic diet (LD) contains 1.0% cholesterol, 0.5% cholic acid, and 17% triglycerides and induces a significant increase in the proportion of biliary deoxycholate conjugates (from cholic acid) as the enlarged pool of cholate conjugates replaces the muricholates. This combination promotes cholesterol hyperabsorption, increases biliary cholesterol secretion, and induces a phase-change in bile together with cholesterol supersaturation and, eventually, cholesterol gallstone formation (Wang *et al.*, 1999b).

In this study, we reasoned that intestinal transit time may be affected by one or more components of the LD, or alternatively by the enterohepatic helicobacter infection. Because it was established earlier that excess cholesterol in the sarcolemmal membranes of smooth muscle cells in the gallbladder impaired its contractile function (Yu *et al.*, 1996; Yu *et al.*, 1998; Xiao *et al.*, 1999), we hypothesised that small intestinal transit in C57L mice might be slowed by cholesterol molecules absorbed from the LD and similarly integrated. We demonstrate here that the LD, and not *H. hepaticus* infection, significantly prolongs small intestinal transit times and that increased cholesterol absorption, with its intercalation into the phospholipid structure of intestinal sarcolemmal membranes, is the critical factor responsible for this phenomenon. Moreover, by ablating intestinal cholesterol absorption with ezetimibe (Zetia®), a drug that does so by inhibiting the NPC1L1 transporter, LD-induced slowing of small intestinal transit was abolished and sarcolemmal lipid composition returned to normal. As in the gallbladder of humans, cholesterol enrichment of sarcolemmae blocks signal-transduction with CCK

(Xiao *et al.*, 1999), which has been shown to be a physiological agonist for the caudal propulsive activity of the small intestine in mice (Wang *et al.*, 2004).

Materials and Methods

Animal sources and husbandry

All animal protocols were reviewed and approved by the Massachusetts Institute of Technology (MIT) and Harvard Medical School Standing Committees on Animals. Three- to five-week-old male and female C57L mice, free of helicobacter spp. infection, were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in polycarbonate micro-isolator cages under specific pathogen-free conditions (free of helicobacter spp., *Citrobacter rodentium*, salmonella spp., endoparasites, ectoparasites, and all known murine viral pathogens) in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Mouse rooms were maintained at constant temperature (22°C) and humidity (40-60%) on a 12:12-h regular light-dark cycle, and mice were fed food and water *ad libitum*.

Experimental diets

All animals were fed a standard chow diet (Purina 5001; PMI Feeds, Richmond, IN) until 8 weeks of age, after which they were maintained either on chow or changed to an experimental diet. The latter included a standard lithogenic diet (LD; a balanced mouse diet replete with vitamins and minerals plus 1.0% cholesterol, 0.5% cholic acid, and 17% triglycerides (Khanuja *et al.*, 1995); prepared in the “diet kitchen” of The Jackson Laboratory, Bar Harbor, ME), a high-cholesterol diet (Purina 5001 containing 1.0% cholesterol; Harlan Teklad, Madison, WI), a high-cholic acid diet (Purina 5001 containing 0.5% cholic acid; Harlan Teklad), and a high-cholesterol plus high-cholic acid diet (Purina 5001 containing 1.0% cholesterol plus 0.5% cholic acid; Harlan Teklad). In

addition, certain experimental diets (chow, high-cholesterol, and lithogenic) were supplemented with 60 mg/kg of ezetimibe (Zetia®, Schering-Plough, Kenilworth, NJ), a specific inhibitor of NPC1L1, the intestinal transporter controlling sterol absorption (Altmann *et al.*, 2004), to yield a dose of approximately 8 mg/kg body weight/day. This level of drug causes a tenfold decrease in intestinal cholesterol absorption in mice (Wang *et al.*, 2008). Mice were fed the LD for 8 days, 14 days, or 8 weeks; all other diets were fed for 8 weeks.

***Helicobacter hepaticus* infection**

Designated mice were infected with *Helicobacter hepaticus* (strain 3BI) with appropriate uninfected controls. *H. hepaticus* was grown under microaerobic conditions on blood agar plates at 37°C, as described (Maurer *et al.*, 2005; Maurer *et al.*, 2006). Four-week-old mice were gavaged orally with the organisms three times on alternative days during the first week, and re-dosed once at 8 and 12 weeks. The dose employed per inoculum was approximately 1.5×10^8 to 3.0×10^8 organisms.

Measurement of small intestinal transit times

Small intestinal transit times were measured by a validated method for mice (Wang *et al.*, 2001; Wang *et al.*, 2003; Wang *et al.*, 2004), with a modification that the latency period between instillation of the ^3H -sitostanol dissolved in Medium-Chain Triglycerides (MCT) and euthanasia, which was decreased from 30 to 20 minutes. On the first day, mice were weighed and anaesthetized using a vaporizer with 5% isoflurane in O_2 . For analgesia, a subcutaneous injection of flunixin (1 mg/kg) was administered pre-

operatively, post-operatively, and again on the following morning. Laparotomy was performed aseptically with a 1-cm midline incision on the upper abdomen. A polyethylene-10 catheter with 0.28 mm i.d. and 0.61 mm o.d., sterilized by ethylene oxide, was inserted into the duodenum 5 mm distal to the pylorus. The catheter, with its tip pointing away from the stomach, was secured with 6-0 polypropylene sutures. The other end of the catheter was externalized through the peritoneum, heat-sealed and implanted under the skin. The abdominal incision was closed in two layers with polypropylene sutures. Mice were allowed to recover for 48 hours in individual cages, receiving a paste of ground diet mixed with water and water *ad libitum*. Mice were then fasted for 20 hours with access to water only.

On the fourth day, mice were re-anaesthetized, and the implanted end of the duodenal catheter was re-exposed and cut open. Two μCi of ^3H -sitostanol, dissolved in 100 μL MCT, were injected. After being transferred to individual cages, mice regained consciousness within 1-3 minutes. Exactly 20 minutes after the ^3H -sitostanol injection, the animals were euthanized with CO_2 , the abdomen was opened, and ligatures were placed circumferentially around the intestine distal to the tip of the duodenal catheter as well as at the ileocecal junction. The stomach and small and large intestines were excised and removed. The small intestine was flash-frozen in liquid N_2 and cut into 20 equal segments, which were placed in vials containing 10 mL solvent consisting of chloroform : methanol (2:1, v/v), and stored at 4°C for 48 to 72 hours. The stomach, including the short duodenal segment containing the catheter, and the large intestine were placed in individual vials. The vials were then vortex-mixed to achieve a uniform solution of ^3H -sitostanol and centrifuged at 3000 rpm for 30 minutes. One-mL aliquots were transferred

into counting vials, the solvent was evaporated under reduced pressure at 25°C overnight, and 8 mL of scintillation fluid (Ecolite(+)TM, MP Biomedicals, Solon, OH) was added. Radioactivity was assayed using a scintillation counter. The stomach and large intestine, analyzed in the same manner, did not show any appreciable radioactivity above background.

Results for small intestinal transit were quantified by two methods. The percentage of total radioactivity in each segment was determined and plotted, producing a distribution of radioactivity across the full length of the small intestine. In addition, the geometric centre of radioactivity, a dimensionless number representing intestinal transit function, was calculated as the sum of the fraction of ³H-sitostanol per segment times the segment number (Miller *et al.*, 1981).

Preparation of proximal small intestine smooth muscle cell sarcolemmae and lipid analysis

A total of 36 male and 8 female C57L mice, four- and five-weeks old and free of helicobacter spp. infection, were obtained from The Jackson Laboratory (Bar Harbor, ME) and fed a standard chow diet. The mice were allowed to reach 8 weeks of age, at which time they were either maintained on chow or were fed one of two experimental diets for 14 days, the standard LD or LD+EZ, containing the maximum dose of ezetimibe (approx. 60 mg/kg diet). On day 14, each mouse was fasted for eight hours and then supplied with the same diet to stimulate overnight feeding. The following morning, the mouse was weighed and then euthanized in a bell jar with an overdose of isoflurane in O₂. A laparotomy was performed, and the entire small intestine from the pylorus to the

ileocecal junction was excised, and all connective and adipose tissues were removed. The small intestine was cut in half and the distal part (ileum to ileocecal junction) discarded. The proximal small intestine was placed in a Petri dish of cold PBS, prepared according to Sambrook et al (Sambrook *et al.*, 1989). A lateral incision was made along the length of the intestine to expose the inner mucosal layer. After emptying the intestine of its contents by gentle agitation in PBS, the tissue was weighed in a dry Petri dish. Tissues of two mice were pooled and placed in a 10 mL solution composed of 3 mM Na₂EDTA dihydrate and 0.05 mM dithiothreitol in PBS for 45 minutes at 4°C, according to Whitehead et al (Whitehead *et al.*, 1999). The wash solution was decanted, 10 mL of PBS was added, and the sample was vortex-mixed for 30 sec to dislodge the mucosa. The supernatant containing the mucosal layer was discarded and the remainder rinsed in another 10 mL of PBS. This tissue was shredded with an Ultra Turrax® T25 disperser employing two 15-second bursts at setting 5 (22,000 rpm) in 5 mL of 0.25 M sucrose buffer (Medium A), prepared according to Rybal'chenko et al (Rybal'chenko *et al.*, 1984). The tissue was further homogenized in a Potter-Elvehjem homogenizer with a polytetrafluoroethylene pestle (Chen *et al.*, 1999), and the homogenate placed in a thick-walled polycarbonate centrifuge tube and balanced. Preliminary centrifugation steps (Chen *et al.*, 1999), were performed in a Beckman L8-80M Ultracentrifuge using a Beckman 70.1 Ti rotor and Medium A as the buffered solvent for pellet reconstitution (Rybal'chenko *et al.*, 1984). Samples were separated over a stepwise sucrose density gradient (20%, 3 mL; 30%, 4 mL; 35%, 3 mL; 40%, 2 mL sucrose) at 100,000 × *g* (SW 40 Ti rotor) for 3 hours without braking during deceleration. Bands were separated, diluted in Medium A, and centrifuged at 150,000 × *g* (Type 70.1 Ti rotor) for 30 minutes.

The pellets were harvested, placed on ice and, depending on the pellet yield, reconstituted in 40-100 μ L of 0.25 M sucrose in 5 mM Tris-HCl. The reconstituted pellets were stored separately in four 1-mL Eppendorf tubes at -80°C until thawed for analysis.

Total Protein Content and Western Blotting

Total protein content of each pellet (four per sample) was determined by the standard procedure for the Pierce bicinchoninic acid (BCA) total protein assay (Thermo Fisher Scientific, Rockford, IL). Following the standard protocol for Western Blotting, the presence and relative yield of sarcolemmae in the reconstituted pellets was verified and normalized to total protein content. Caveolin-3(C-2) (Santa Cruz Biotechnology, Santa Cruz, CA) was employed as a primary antibody against Caveolin-3, a protein intrinsic to sarcolemmae. The blots were visualized using HRP chemiluminescence. The highest yield of sarcolemmae was found at the 20%-30% sucrose interface of the gradient, and as such, both electron microscopy and the subsequent lipid assays were performed on pellets from this interface only.

Electron Microscopy

Isolated sarcolemmal membranes from three samples were fixed briefly in 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M Tris-HCl buffer. They were then gently pelleted and embedded in 2% SeaPrep Agarose (Lonza, Rockland, ME) prepared in water, allowing the membranes to be equally distributed in the agarose, and then hardened. The solid agarose block was additionally fixed for 2 days in the same buffer, washed in water, fixed in 2% aqueous uranyl acetate, and then dehydrated through graded

alcohols and propylene oxide. The block containing membranes was embedded in LX112 resin, sectioned with an UltracutE ultramicrotome, and imaged with a JEOL 1400 electron microscope.

Extraction of Lipids and Analyses

Lipids from the aqueous membrane samples were extracted into 2:1 chloroform:methanol following the method of Folch et al (Folch *et al.*, 1957). This procedure removed all traces of inorganic phosphates left by the PBS washes following surgery. Using methods identical to those employed for gallbladder bile (see below), cholesterol and phospholipid contents were assayed and absolute concentrations were calculated.

Analysis of gallbladder bile

Immediately after euthanasia, mouse bile was obtained by puncture and aspiration from the gallbladder of euthanized mice. Total bile salts were assayed by the 3 α -hydroxysteroid dehydrogenase method (Turley & Dietschy, 1978). Biliary phospholipids were measured as inorganic phosphorus (Bartlett, 1959), and, after extraction in hexane, biliary cholesterol was quantified by HPLC (Duncan *et al.*, 1979; Vercaemst *et al.*, 1989). CSIs were calculated according to critical tables (Carey, 1978).

Statistical analyses

Prism 4.0 software (GraphPad, San Diego, CA) was utilized for statistical tests, including two-way ANOVA, one-way ANOVA with Tukey HSD or Bonferroni post-hoc tests, and

two-tailed *t*-tests. Data are presented as mean \pm SEM, and $P < 0.05$ is considered a significant difference.

Results

Influence of lithogenic diet and *Helicobacter hepaticus* infection on small intestinal transit times

Figure 1A displays the percent intestinal distribution of ^3H -sitostanol in uninfected mice on a chow diet, with radioactivity peaking in segment 13. These results are essentially similar to those of mice on the chow diet infected with *H. hepaticus* (Figure 1B). Figures 1C and 1D respectively, show that the distribution of radioactivity in uninfected or infected mice fed the LD is shifted toward proximal intestinal segments (i.e. slower transit times) compared to uninfected or infected mice on the chow diet (Figure 1A and 1B). Peak radioactivity occurs in segment 10 in uninfected mice, and in segment 8 in *H. hepaticus*-infected mice on the LD. There were no differences in mouse weight and small intestinal length among all four groups (data not displayed).

Figure 1E records the dimensionless geometric centre of radioactivity in each group. A two-way ANOVA revealed a significant main effect of diet ($F = 23.40$, $P < 0.0001$) with the geometric centres of radioactivity lower (i.e. slower) in uninfected (9.4 ± 0.5) and infected (9.6 ± 0.5) mice on the LD compared with uninfected (12.5 ± 0.4) and infected (11.4 ± 0.5) mice on the chow diet. In contrast, *H. hepaticus* infection shows no significant effect ($F = 0.77$, $P = 0.39$) on motility, nor is there any interaction of diet and infection status ($F = 1.89$, $P = 0.18$). These data confirm that the LD prolongs small intestinal transit times, but infection with *H. hepaticus* does not alter transit times significantly, nor does the combination of LD and *H. hepaticus* infection induce an additive effect compared with LD alone.

Induction and reversal of prolonged small intestinal transit times

Figure 2A describes the speed of onset of slowed intestinal transit for uninfected mice fed the LD and its reversal by replacing the LD with chow. A one-way ANOVA revealed a significant effect ($F = 8.33$, $P = 0.0001$), and *post-hoc* analysis with Tukey HSD tests indicated that small intestinal transit time is prolonged significantly ($P < 0.01$) at 8 days on the LD (10.3 ± 0.2) relative to mice on the chow diet (12.5 ± 0.4). This effect is even more pronounced at 14 days of LD feeding relative to the chow-fed mice (9.7 ± 0.2 , $P < 0.001$), although the 8- and 14-day LD groups are statistically equivalent ($P > 0.05$). Figure 2A reveals also that, after replacement of the LD with chow for 3 days, small intestinal transit times (11.9 ± 1.0) do not differ significantly from the chow-fed group (12.5 ± 0.4 , $P > 0.05$), but are significantly faster than the 14-day LD group. However, the difference in small intestinal transit times between 14-day LD followed by 3 days of chow is not statistically significant compared with the 8-day LD group. Likewise, after 6 days replacement of the LD for 14 days with chow, transit times (10.8 ± 0.8) are statistically equivalent to the chow-fed group. Comparison of the two chow replacement groups, i.e. at 3 and 6 days, revealed no significant differences between them. Taken together, these observations indicate that prolonged small intestinal transit induced by the LD is rapidly reversible by discontinuing the diet.

Time course of cholesterol supersaturation of gallbladder bile

Figure 2B displays CSI values calculated from results of lipid analyses of gallbladder bile in the same animals. This shows that bile is supersaturated with cholesterol (i.e. $CSI > 1$) at 8 days on the LD ($CSI = 1.2 \pm 0.05$), and more supersaturated at 14 days ($CSI = 1.5 \pm 0.2$). When chow was reinstated for 3 or 6 days following 14

days of LD feeding, bile became unsaturated ($CSI = 0.9 \pm 0.05$ and 0.5 ± 0.06 , respectively), corresponding to literature values for chow-fed mice (Wang *et al.*, 1997). These data indicate that cholesterol supersaturation of bile is induced by 8 days of LD feeding and that supersaturation is reversed within 3 days on the chow diet, corresponding to the time on each diet required for induction and reversal of slowed intestinal transit times (Fig. 2A).

Comparison of small intestinal transit times in male and female mice

In contrast to humans, most strains of female mice display a lower cholesterol gallstone prevalence on the LD than males (Paigen & Carey, 2002). We therefore tested whether the LD would produce the same effect in uninfected females as in uninfected males. Female mice ($n=8$) fed the LD for 8 weeks display significantly longer transit times than those fed the chow diet (9.7 ± 0.6 and 13.1 ± 0.6 , respectively), as determined by a two-tailed *t*-test ($P = 0.002$). Mouse weight and small intestinal length do not differ between the two groups (data not displayed). Furthermore, small intestinal transit times, as derived from the geometric centres, are statistically equivalent in females (13.1 ± 0.6) and males (12.5 ± 0.4 , viz. Figure 1E) on the chow diet ($P = 0.38$), as well as in females (9.7 ± 0.6 , data not displayed) and males (9.4 ± 0.5 , viz. Figure 1E) on the LD ($P = 0.65$).

Small intestinal transit times on diets containing high cholesterol, high cholic acid, and a combination of both

Figure 3A determines which component of the LD is responsible for retarding small intestinal motility, employing three additional chow-based diets studied in *H*.

hepaticus-free male mice. A one-way ANOVA revealed a significant effect ($F = 3.66$, $P = 0.02$), and *post-hoc* analysis with Tukey HSD tests showed that mice fed the 1.0% cholesterol diet (13.1 ± 0.4) or the 0.5% cholic acid diet (12.1 ± 0.6) for 8 weeks exhibit statistically equivalent small intestinal transit times; neither group differs significantly from mice maintained on the chow diet (12.5 ± 0.4). However, Figure 3A also shows that mice fed the 1.0% cholesterol plus 0.5% cholic acid diet for 8 weeks exhibit ($P < 0.05$) prolonged small intestinal transit times (9.7 ± 1.2) that is statistically significant compared with chow-fed mice, suggesting that the high triglyceride content (not studied individually) of the LD is not a critical component. Importantly, the combination of dietary cholesterol and cholic acid promotes cholesterol absorption in mice (Wang *et al.*, 1999a). Transit times in the 1.0% cholesterol plus 0.5% cholic acid group are significantly slower ($P < 0.05$) than those in the 1.0% cholesterol-fed group and the 0.5% cholic acid-fed group, and the difference between these groups is not statistically significant.

After 8 weeks on the 1.0% cholesterol diet, mice display equivalent body weights (34.4 ± 0.9 g) to mice on chow (35.7 ± 1.1 g). However, mice in the 0.5% cholic acid group are significantly lighter at the end of the experiment (29.7 ± 0.7 g; $P < 0.01$) than either the chow or 1.0% cholesterol groups ($P < 0.001$ and $P < 0.01$, respectively). Mice in the 1.0% cholesterol plus 0.5% cholic acid group are significantly lighter still (25.0 ± 0.7 g) at the end of the feeding than the chow-fed ($P < 0.001$), 1.0% cholesterol-fed ($P < 0.001$), and 0.5% cholic acid-fed ($P < 0.01$) groups. Small intestinal length did not differ between these groups (data not displayed).

Small intestinal transit times on LD and on diets containing ezetimibe

Figure 3B shows the results of blocking cholesterol absorption using ezetimibe (Zetia®, Schering-Plough) at 60 mg/kg of diet, on small intestinal transit times in uninfected male mice. A one-way ANOVA revealed no significant differences in the geometric centres ($F = 3.66$, $P = 0.61$) between mice fed 8 weeks with the chow diet (12.5 ± 0.4), chow plus ezetimibe (13.4 ± 0.5), 1.0% cholesterol plus ezetimibe (12.7 ± 0.7), and the LD plus ezetimibe (12.8 ± 0.3). Therefore, in contrast to mice on the LD alone, mice on the LD diet plus ezetimibe display completely normal small intestinal motility compared with the chow-fed group. Furthermore, Figure 3C demonstrates that the LD plus ezetimibe group exhibits significantly faster small intestinal transit times compared with mice fed the LD alone (12.8 ± 0.3 and 9.4 ± 0.5 , respectively; $P < 0.001$). This indicates that the slowing of small intestinal transit by the LD is reversed completely by blocking intestinal cholesterol absorption with ezetimibe. Small intestinal length was once again equivalent between ezetimibe-treated, LD, and chow diet groups.

Proximal small intestinal weights and lipid analyses of isolated sarcolemmae

The electron photomicrograph in Figure 4 is typical, showing that all three reconstituted pellets reveal vesicular plasma membranes at high yield, similar to those in smooth muscle cell sarcolemmae from rats (Kidwai, 1974). This, as well as heavy staining of Caveolin-3 in Western Blots (data not displayed), confirms the success of sarcolemmal isolation in the expected sucrose interface (see methods). The inset reveals the bilayered structure of the plasma membrane, where uranyl acetate has ‘stained’ the polar head groups of the constituent phospholipids of the membrane bilayer.

Figure 5A gives the weights of proximal small intestine for mice in the chow, LD, and LD + EZ groups. The weight of the latter (0.87 ± 0.02 g) is significantly higher ($P < 0.001$) than in chow-fed controls (0.71 ± 0.03 g) and LD groups (0.65 ± 0.04 g) most likely because of the high density (1.334 g/cm^3) of ezetimibe bound to NPC1C1 transporters (Patel *et al.*, 2011). Phospholipid concentration in the sarcolemmae was not significantly different between groups (data not displayed). However, as Figure 5B shows, the cholesterol concentration in the LD group ($0.25 \pm 0.04 \text{ } \mu\text{mol/mg protein}$) is significantly higher than in the chow-fed control ($0.16 \pm 0.01 \text{ } \mu\text{mol/mg protein}$, $P < 0.01$). Furthermore, the LD+EZ group ($0.14 \pm 0.03 \text{ } \mu\text{mol/mg protein}$) shows no change in cholesterol concentration when compared to the chow-fed controls, but a significant decrease is evident ($P < 0.01$) compared to the LD group.

Figure 5C provides the cholesterol to phospholipid (Ch/PL) ratios ($F = 15.54$, $P = 0.0002$), which increased significantly in the LD group (0.57 ± 0.03 , $P < 0.01$), whereas the difference in the LD+EZ group (0.38 ± 0.03) is not statistically significant when compared to the chow-fed controls (0.45 ± 0.02). Moreover, the Ch/PL ratio of the LD+EZ group is decreased significantly ($P < 0.001$) when compared to that of the LD group.

Discussion

We found here that feeding a standard LD, containing both cholesterol and cholic acid, to inbred mice with multiple *Lith* genes and a marked proclivity towards cholesterol gallstone formation (Paigen & Carey, 2002) significantly prolonged small intestinal transit times (Fig. 1A). This is known to promote cholesterol cholelithogenesis by at least two mechanisms. First, LD feeding in mice permits greater cholesterol absorption from the small intestine (Ponz de Leon *et al.*, 1982) and increases biliary cholesterol secretion (Wang *et al.*, 2004). Since elevated luminal cholesterol, as shown in the past (Xu *et al.*, 1996; Fan *et al.*, 2007), may result in slower small intestinal transit, this could engender a vicious cycle that further augments cholesterol absorption. Second, prolonged small intestinal transit increases deoxycholic acid (DCA) formation in the distal small and large intestines (Thomas *et al.*, 2005). DCA promotes cholesterol gallstone formation (Paigen & Carey, 2002) by augmenting cholesterol secretion into bile (Carulli *et al.*, 1984) and accelerating cholesterol crystallization in the gallbladder (Hussaini *et al.*, 1995; van Erpecum & Carey, 1997).

In searching for explanatory mechanisms, we considered our previously established findings that the LD infrequently produces cholesterol gallstones in the C57L strain of mice without concurrent infection with certain enterohepatic *Helicobacter* spp (Maurer *et al.*, 2005). From the present results (Fig. 1B, D) we can rule out altered small intestinal motility as a mechanism by which helicobacter infection influences cholelithogenesis. Prior to this study, there was a dearth of information on whether enterohepatic *Helicobacter* spp. infection influences intestinal transit (Fox, 2002); moreover, studies of gastrointestinal motility in patients infected with the more common

gastrotropic *H. pylori* have yielded inconclusive results (Pieramico *et al.*, 1993; Minocha *et al.*, 1994; Leontiadis *et al.*, 2004). It is much more likely that immune responses of the host gallbladder are the principal mechanism whereby enterohepatic helicobacter infection promotes cholesterol cholelithogenesis (Maurer *et al.*, 2007; Maurer *et al.*, 2009).

We then explored cholesterol absorption as a possible explanation for our results. It has been established that cholesterol hyperabsorbed into sarcolemmae of gallbladder smooth muscle has a profound effect on smooth muscle function (Yu *et al.*, 1996). Since cholesterol (and other sterol) absorption takes place primarily in the duodenum and proximal jejunum, we have now carried out similar studies on sarcolemmae from upper small intestinal smooth muscle, whose successful isolation was validated by electron microscopy (Fig. 4) and Caveolin-3 expression using Western blotting. We observed augmented cholesterol integration (Fig. 5B, C) into the sarcolemmae without a change in phospholipid content as has been found in lithogenic human gallbladder sarcolemmae (Yu *et al.*, 1996). Although the gallbladder does not incorporate absorbed cholesterol from bile into lipoproteins, the small intestine does. Therefore, our observation of sarcolemmal enrichment with free cholesterol suggests strongly that all cholesterol molecules after absorption are not immediately diverted into lipoprotein (i.e. chylomicron and nascent HDL) synthesis.

In the gallbladder, cholesterol-induced hypomotility is believed to occur through at least two intracellular mechanisms: cholesterol's inhibitory effect on action potentials in smooth muscle (Jennings *et al.*, 1999) and CCK-1R-mediated activation of phospholipase C, which, when gallbladder sarcolemmae become enriched in cholesterol,

leads to signal-transduction decoupling by CCK (Yu *et al.*, 1998; Xiao *et al.*, 1999). In the current work, we advance that the hyperabsorbed cholesterol has similar inhibitory effects on the contraction of proximal intestinal smooth muscle cells, resulting in the markedly prolonged small intestinal transit times that we observed. In this connection, the LD was previously found to have no effect on intestinal transit times in CCK-1R knockout mice (Wang *et al.*, 2004). The gene for this protein (previously CCK-A) is expressed, not only on smooth muscle cells of the gallbladder and stomach, but also of the small intestine (Lacourse *et al.*, 1997). Its function on the intestine is now known to be physiologically relevant since its motor action on small intestinal motility occurs with normal post-prandial levels of CCK (Wang *et al.*, 2004). Therefore, the absence of an effect of the LD on intestinal motility in CCK-1R knockout mice supports our hypothesis that cholesterol's inhibitory effect on intestinal motility occurs through a CCK-1R-dependent pathway (Yu *et al.*, 1998; Xiao *et al.*, 1999).

Prior to the present study, it was known that high levels of dietary cholesterol prolonged small intestinal transit times in both hamsters (Fan *et al.*, 2007) and ground squirrels (Xu *et al.*, 1996). It stands to reason that this implies a positive feedback loop to permit greater intestinal cholesterol absorption, which, in turn, further increases transit time. Nonetheless, in our attempt to mimic such experiments in mice, we did not observe any effect on small intestinal motility in animals fed a chow diet supplemented with a high cholesterol concentration (Figure 3A). This can be explained by the poor absorption efficiency of cholesterol from upper small intestinal mixed bile salt micelles due to the high levels (40-60%) of hydrophilic muricholates and ursodeoxycholate conjugates in the endogenous bile salt pool of mice, including the C57L strain (Wang *et al.*, 1997). In

contrast, in ground squirrels and hamsters, dietary cholesterol is more efficiently absorbed due to their more hydrophobic bile salt pools (Heuman, 1989; Xu *et al.*, 1996). Nonetheless, both the LD and the cholesterol plus cholic acid combination in a chow diet increase intestinal cholesterol absorption in mice (Wang *et al.*, 2004), and we observed prolonged small intestinal transit times in both of these groups (Fig. 2A and Fig. 3A).

When gastrointestinal motility is slowed, bile saturation with cholesterol is also augmented from increased DCA formation (Carulli *et al.*, 1984; Hussaini *et al.*, 1995; Shoda *et al.*, 1995) in addition to greater intestinal cholesterol absorption (Traber & Ostwald, 1978; Wang *et al.*, 2004). In the current model, DCA is the major catabolic product of the ingested cholic acid, which is a crucial component of the LD without which the mouse fails to absorb or secrete sufficient cholesterol to develop supersaturated bile (Wang *et al.*, 1999a). Prolonged intestinal transit enables greater DCA formation by increasing residence time of endogenous bile salts in the distal small and large intestines. In addition to elevated DCA predisposing to gallstone formation by augmenting hepatic cholesterol secretion, it also destabilizes cholesterol-rich biliary vesicles, leading to accelerated cholesterol crystallization (Stolk *et al.*, 1994). Despite cholate conjugates being the better promoters of cholesterol absorption (Wang *et al.*, 2003; Woollett *et al.*, 2004), DCA conjugates enhance micellar solubility of cholesterol in the intestine (Wang *et al.*, 2009). This solubilizing effect, however, is probably more important for absorption in animal models with relatively hydrophilic bile salt pools (Xu *et al.*, 1996; Wang *et al.*, 1997; Wang *et al.*, 1999b), such as the mouse, than in humans or other gallstone-prone animal model, such as hamsters (Fan *et al.*, 2007) and ground squirrels (Xu *et al.*, 1996). Slow gastrointestinal transit not only allows greater absorption of dietary cholesterol

from the upper small intestine in mice (Wang *et al.*, 2004), but also in humans (Ponz de Leon *et al.*, 1982).

The question invariably arises as to whether the altered composition of the bile salt pool from feeding cholic acid in the LD could itself influence small intestinal motility. Levels of DCA conjugates in bile increase markedly in mice fed the LD, despite efficient rehydroxylation of DCA to cholic acid in the murine liver (Wang *et al.*, 2003). DCA has been shown to prolong small intestinal transit in the rat (Brown *et al.*, 1990). Conversely, taurocholate, a bile salt of intermediate hydrophobic-hydrophilic balance, which also increases markedly in mice on the LD, accelerates intestinal transit in rats (Brown *et al.*, 1990), and gallstone patients to whom the hydrophilic bile acid, ursodeoxycholic acid is prescribed for gallstone dissolution, exhibit faster oro-ileal transit times (Colecchia *et al.*, 2006). Nonetheless, in this study we did not observe any acceleration or deceleration of small intestinal transit from feeding cholic acid alone (Fig. 3A), including the necessary accumulation of its catabolic product DCA, as evidenced also in previous work from this laboratory (Wang *et al.*, 2003).

Finally, we tested the effects of reversing the induction of the scenario for hyperabsorbed cholesterol molecules on gut hypomotility. It is known that cholesterol-induced alterations in gallbladder smooth muscle contractility are reversed when the sterol is extracted artificially (Yu *et al.*, 1996). This is consistent with our findings that slowed intestinal transit was reversed within 3 days when the LD was discontinued (Fig. 2A) or when ezetimibe and the LD were co-administered (Fig. 3B). However, in our studies with diets containing ezetimibe, we demonstrated conclusively the key role that hyperabsorbed cholesterol plays in gut hypomotility. Ezetimibe binds to the sterol

transport protein, Niemann-Pick C1 Like-1 (NPC1L1) on the apical plasma membranes of enterocytes (Garcia-Calvo *et al.*, 2005) and inhibits cholesterol absorption by blocking transport of cholesterol across brush border membranes (Altmann *et al.*, 2004). Predictably, NPC1L1-knockout mice are insensitive to ezetimibe and display dramatically lower cholesterol absorption rates that are not altered by dietary supplementation with bile acids (Altmann *et al.*, 2004). In our experiments, we found that small intestinal transit times did not differ significantly between the high-cholesterol and high-cholesterol-plus-ezetimibe diet groups (Fig. 3B), indicating that very little cholesterol absorption was taking place. In addition, the chow diet plus ezetimibe was well tolerated and did not produce a change in transit times compared to the chow diet group without ezetimibe (Fig. 3B), again consistent with the low baseline level of dietary cholesterol absorption in mice (Wang *et al.*, 2003). In concert with extensive work on gallbladder motility (Yu *et al.*, 1996), we have shown that sarcolemmae isolated from intestinal smooth muscle cells of mice fed the LD are enriched in cholesterol (Fig. 5B, C) and that this compositional change is blocked by ezetimibe (Fig. 5B). Consequently, when a maximum dose of ezetimibe (approx. 8 mg/kg BW/day) was administered in the LD, the gut is unaffected by the higher cholesterol content of the diet, and the sarcolemmal Ch/PL ratio decreases significantly into a range slightly lower than that of controls (Fig. 5C).

Our findings strongly implicate cholesterol as the likely dietary or endogenous molecule that when hyperabsorbed slows gut motility in cholelithogenic humans. Slowed small and large bowel transit time is well documented in cholesterol gallstone patients (Heaton *et al.*, 1993; Azzaroli *et al.*, 1999). We argue that this is likely to be caused by

compromised CCK-mediated caudad propulsion secondary to increased cholesterol absorption by the intestine and its integration into plasma membranes of smooth muscle cells. Nonetheless, the excess cholesterol is more likely to be derived from the liver in secreted bile than from the diet in humans, since the majority of cholesterol gallstone patients hypersecrete cholesterol continuously from the liver by as much as 2- to 4-fold compared to controls even after gallstones have formed (Shaffer & Small, 1977; Leiss & von Bergmann, 1985; Reuben *et al.*, 1985) but do not customarily ingest diets rich in cholesterol (Cuevas *et al.*, 2004; Méndez-Sánchez *et al.*, 2007).

As alluded to earlier, both DCA levels in bile and high biliary CSIs work together in augmenting lithogenicity and rapidly producing cholesterol gallstones. Earlier work has show that these can be decreased with acceleration of intestinal transit employing simple laxatives for example; conversely, both indices have been shown to increase when intestinal transit is decelerated, such as by exhibiting the opioid drug, loperamide (Marcus & Heaton, 1986). In animal studies, oral administration of the antibiotic erythromycin, which is a motilin receptor agonist, promotes small intestinal motility and has been shown to restore normal intestinal transit in cholesterol-fed ground squirrels (Xu *et al.*, 1998). As a result, biliary cholesterol saturation decreases and nucleation and cholesterol crystal formation are reduced in gallbladder bile (Xu *et al.*, 1998). Similarly, slow oro-ileal transit times in gallstone patients are accelerated by the hydrophilic bile acid ursodeoxycholic acid (Colecchia *et al.*, 2006). Our demonstration using ezetimibe to prevent and treat cholesterol-mediated small intestinal dysmotility is encouraging in this respect, since, in mice, the drug has been shown to diminish cholesterol saturation of bile and to prevent gallstones at the high dose employed in this study (Wang *et al.*, 2008;

Zúñiga *et al.*, 2008). However, no attention to the possible effects of ezetimibe on normalizing gut motility has ever been mentioned or tested till now. Although the metabolic fate of absorbed intestinal cholesterol is much more complicated in humans than in mice, there is ample reason to believe that manipulation of intestinal motility, such as by employing ezetimibe as a new modality for prokinesis, could become a plausible method for minimizing cholesterol gallstone formation in high-risk humans. Finally, if our findings in the inbred mouse are translatable to lithogenic humans, it will mean that small intestinal transit hypomotility must be added as a fourth canonical defect, together with cholesterol supersaturation, gallbladder hypomotility, and rapid nucleation plus phase transitions, in the cholesterol cholelithiasis syndrome.

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Author Contributions

Dr. Meimin Xie, then a post-doctoral research fellow, played a central role in the conception, design, and execution of most of the small intestinal transit time studies. She drafted the earlier versions of the paper, incorporating analysis and interpretation of motility data.

Mr. V.R. Kotecha took over where Dr. Xia left off and designed, performed, collected, interpreted as well as wrote-up the remaining motility studies, including most studies involving the cholesterol absorption blocking drug, ezetimibe's effects on small intestinal motility.

Mr. J.D. Andrade played a key role in working out the molecular mechanism for the intestinal hypomotility observed. This requires setting up *ab initio* a protocol for isolating the sarcolemmae of intestinal smooth muscle cells, proving their identity and purity, and carrying out all the studies in the paper involving alterations in cholesterol levels in these plasma membranes. He also wrote-up and interpreted these results and reworked the discussion.

Professors J. Fox and M. C. Carey are Principal Investigators at MIT and Brigham & Women's Hospital in whose laboratories these studies were done. Professor Fox conceived, designed, and interpreted all intellectual aspects of the *Helicobacter hepaticus* infection experiments. Professor Carey conceived, designed, and interpreted all other aspects of this study.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

FIGURE LEGENDS

Figure 1: *Influence of lithogenic diet and helicobacter infection on small intestinal transit times in male C57L mice. (A) - (D).* Small intestinal transit is plotted as percent distribution of a non-absorbed marker ^3H -sitostanol along the length of the small intestine 20 minutes following duodenal injection (Time 0). (A) Peak radioactivity on a chow diet occurs in segment 13 of uninfected mice as well as (B) in *H. hepaticus*-infected mice. On the lithogenic diet (LD), peak radioactivity is found (C) in segment 10 of uninfected mice and (D) in segment 8 of *H. hepaticus*-infected mice, indicating that LD feeding (E) slows intestinal transit irrespective of infection states. Small intestinal transit (A-D) plotted as dimensionless geometric centres [Σ (fraction of ^3H per segment x segment number)] of the radioactive marker for individual mice, with means \pm SEM plotted for each group. The geometric centres for all mice on the chow diet (12.5 ± 0.4 and 11.4 ± 0.5 , respectively) are significantly ($P < 0.05$) larger (i.e. intestinal transit is faster) than the geometric centres of the uninfected and infected groups fed the LD (9.6 ± 0.5 and 9.4 ± 0.5 , respectively, as determined by two-way ANOVA). No statistically significant differences in geometric centres exist between the two chow-fed or two LD-fed groups. Asterisks indicate P values of $\lambda < 0.05$.

Figure 2: *Effect of LD duration and reversal on small intestinal transit times and cholesterol saturation indices (CSIs) of gallbladder bile. (A).* Geometric centres of ^3H -sitostanol in uninfected male C57L mice fed chow, LD and LD followed by 3 and 6 days chow diet. Compared with the chow group, geometric centres are significantly reduced

after 8 and 14 days on the LD. In 14-day LD-fed mice and in mice returned to the chow diet for 3 or 6 days, the geometric centres become statistically equivalent to the chow diet group. These values are also significantly different from the 14-day LD-fed group. (B). The mean \pm S.D. cholesterol saturation indexes in the same C57L mice at 8 days of LD feeding is 1.2 ± 0.05 , and in mice fed the LD for 14 days is 1.5 ± 0.2 . When the mice are returned to the chow diet for three days, gallbladder bile becomes unsaturated with cholesterol (CSI = 0.9 ± 0.05 (B)), and after 6 days of chow feeding, bile becomes further unsaturated (CSI = 0.5 ± 0.06). Asterisks represent a *P* value < 0.05 .

Figure 3: *Influence of cholesterol and/or cholic acid and added ezetimibe on geometric centres with respect to intestinal transit times.* (A). Mice fed the 1.0% cholesterol plus 0.5% cholic acid diet display significantly prolonged small intestinal transit (9.7 ± 1.2) compared with the chow diet group (12.5 ± 0.4) and the 1.0% cholesterol diet group (13.1 ± 0.4). The small intestinal transit times of mice on either the 1.0% cholesterol or 0.5% cholic acid (12.1 ± 0.6) diet are equivalent to mice on the chow diet. (B). The addition of ezetimibe (60 mg/kg) to the chow diet does not alter small intestinal transit times (13.4 ± 0.5), nor does the addition of ezetimibe to the 1.0% cholesterol diet (12.7 ± 0.7). Moreover, small intestinal motility in the LD-plus-ezetimibe group (12.8 ± 0.4) is also statistically equivalent to the chow diet group. Panel (C) shows that small intestinal transit in the LD-plus-ezetimibe group is significantly faster than in mice fed the LD alone (12.8 ± 0.3 and 9.4 ± 0.5 , respectively; $P = 0.0002$) and is essentially the same as for mice fed chow alone (12.5 ± 0.4). This demonstrates that slowed small intestinal

transit times can be reversed completely by blocking intestinal cholesterol absorption.

Asterisks represent *P* values < 0.05; Ch = cholesterol, CA = cholic acid, EZ = ezetimibe.

Figure 4: *Electron photomicrograph of isolated sarcolemmae from upper small intestinal smooth muscle cells of male C57L mice.* The plasma membranes isolated from the small intestinal smooth muscle cells of mice show multiple vesicular structures (original magnification, $\times 2,000$). The inset displays the bilayer structure of uranyl acetate-stained membranes (original magnification, $\times 20,000$). A scale bar of 1 μm is shown at bottom right.

Figure 5 (A-C): *(A) Weight of identical length of the proximal small intestine (mucosa and smooth muscle) in male C57L mice.* The weight (g) of the LD-plus-ezetimibe group (0.87 ± 0.02 g) is increased significantly ($P < 0.001$) compared with both the chow (0.71 ± 0.03 g) and LD (0.65 ± 0.04 g) groups. The high density of ezetimibe (1.334 g/cm^3) could explain the weight differences, especially if most or all NTC1L1 transporters bound an ezetimibe molecule (Patel *et al.*, 2011), as expected for the dose employed and diminution in % cholesterol absorbed (Wang *et al.*, 2008). *(B) Cholesterol concentrations in smooth muscle sarcolemmae pooled from proximal small intestine of male C57L mice.* The proximal half of the small intestine from two 10-week-old mice fed standard chow, lithogenic diet (LD), or lithogenic diet with ezetimibe (LD+EZ) for 14 days prior to surgery was used for membrane isolation and lipid analysis. The cholesterol concentration (normalized for total protein content) in the LD group (0.25 ± 0.04 $\mu\text{mol/mg}$ protein) is significantly ($P < 0.01$) greater than in both LD-plus-ezetimibe (0.14

± 0.03 $\mu\text{mol/mg}$ protein) and chow (0.16 ± 0.01 $\mu\text{mol/mg}$ protein, $P < 0.01$) groups. (C) *Cholesterol to phospholipid ratios (Ch/PL) in smooth muscle sarcolemmae of the proximal small intestine of pooled male C57L mice.* The Ch/PL ratio in the LD group (0.57 ± 0.03) is significantly larger than in the LD-plus-ezetimibe (0.38 ± 0.03 , $P < 0.001$) and in the chow (0.45 ± 0.02 , $P < 0.01$) groups. Asterisks denote P values < 0.05 as listed above.