Cholic acid aids absorption, biliary secretion, and phase transitions of cholesterol in murine cholelithogenesis

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1Department of Medicine, Harvard Medical School, Gastroenterology Division, Brigham and Women’s Hospital and Harvard Digestive Diseases Center, Boston, Massachusetts 02115; and 2 Jackson Laboratory, Bar Harbor, Maine 04609

Wang, David Q.-H., Frank Lammert, David E. Cohen, Beverly Paigen, and Martin C. Carey. Cholic acid aids absorption, biliary secretion, and phase transitions of cholesterol in murine cholelithogenesis. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G751–G760, 1999.—Cholic acid is a critical component of the lithogenic diet in mice. To determine its pathogenetic roles, we fed chow or 1% cholesterol with or without 0.5% cholic acid to C57L/J male mice, which because of lith genes have 100% gallstone prevalence rates. After 1 yr on the diets, we measured bile flow, biliary lipid secretion rates, hepatic cholesterol and bile salt synthesis, and intestinal cholesterol absorption. After hepatic conjugation with taurine, cholate replaced most tauro-β-muricholate in bile. Dietary cholic acid plus cholesterol increased bile flow and biliary lipid secretion rates and reduced cholesterol 7α-hydroxylase activity significantly mostly via deoxycholic acid, cholate’s bacterial 7α-dehydroxylation product but did not downregulate cholate biosynthesis. Intestinal cholesterol absorption doubled, and biliary cholesterol crystallized as phase boundaries shifted. Feeding mice 1% cholesterol alone produced no lithogenic or homeostatic effects. We conclude that in mice, cholic acid promotes biliary cholesterol hypersecretion and cholelithogenesis by enhancing intestinal absorption, hepatic bioavailability, and phase separation of cholesterol in bile.

genetics; phase diagrams; bile salt species; bile flow; microscopy; mucin; nutrition; 3-hydroxy-3-methylglutaryl-coenzyme A reductase; cholesterol 7α hydroxylase

CHOLESTEROL GALLSTONES develop in a majority of mice carrying lith genes on feeding a lithogenic diet containing cholesterol and cholic acid (2, 21, 34, 39), whereby the bile salt (BS) pool is enriched with taurocholate (TC). In an experiment with a small number of mice, Tepperman and co-workers (34) as well as Fujino and colleagues (14) found that feeding 1% cholesterol alone for 4–8 mo did not induce cholesterol gallstone formation. This indicates that the role of cholic acid is essential in the murine gallstone model, but its mechanism is not well understood. In rats, Uchida and co-workers (36) observed that cholic acid is necessary for intestinal cholesterol absorption via marked increases in TC and taurodeoxycholate (TDC) in bile. Akiyoshi and colleagues (1) found that in diabetic mice, enhanced cholesterol absorption was induced by increased synthesis and secretion of endogenous TC and that this plays an important role in formation of cholesterol gallstones even on a chow diet. In contrast, others (11, 30) have suggested that addition of cholic acid or TC to chow or 2% cholesterol diets does not influence intestinal cholesterol absorption. Furthermore, there are contradictory studies in the literature concerning percent cholesterol absorption in healthy inbred mice fed chow, which has been reported to vary from 20 to 80% with small intraspecies variation (1, 17, 22, 33, 43). These findings suggest that the effect of cholic acid on cholesterol absorption and gallstone formation in the mouse should be reevaluated. Therefore, using gallstone-susceptible C57L/J mice (21), we explored the role of cholic acid on intestinal cholesterol absorption, hepatic cholesterol, and BS synthesis as well as cholesterol gallstone formation. We investigated whether long-term feeding of 1% cholesterol without cholic acid could induce the same biliary and hepatic enzymatic phenotypes as observed when mice were fed the lithogenic diet. Our findings suggest that, through forming TC, cholic acid has multiple effects at pathophysiological, biochemical, and physicochemical levels, all of which appear important in murine cholesterol gallstone formation.

MATERIALS AND METHODS

Chemicals. Intralipid (20%, wt/vol) was obtained from Pharmacia (Clayton, NC), and medium-chain triglyceride oil was from Mead Johnson (Evansville, IN). All radiochemicals were purchased from DuPont NEN (Boston, MA). The radiochemical purities of [1,2-3H]cholesterol and [4-14C]cholesterol were >97% as determined by HPLC analyses. The radiochemical purity of DL-[3-14C]hydroxy-3-methylglutaryl (HMG)-CoA was verified to be >97% by paper chromatography in butanol-glacial acetic acid-water (7:2:3, vol/vol/vol). The purity of DL-[5-3H]mevalonolactone, used as an internal standard, was 99% by TLC in toluene-acetone-acetic acid (20:10:1, vol/vol/vol). For HPLC analyses of BS species and cholesterol, all reagents were HPLC grade and obtained from Fisher Scientific (Fair Lawn, NJ). BS standards were obtained from Sigma Chemical (St. Louis, MO) and Calbiochem-Behring (San Diego, CA), with the exception of the taurine conjugates of β- and ω-muricholates: 3α,6β,7α-trihydroxy-5β-cholanoate (tauro-β-muricholate or T-β-MC) and 3α,6α,7β-trihydroxy-5β-cholanoate (tauro-ω-muricholate or T-ω-MC), respectively, which were provided generously by Tokyo Tanabe (Tokyo, Japan; courtesy of H. Sugata). Purity of individual BS by HPLC was >98% (8, 37). All other chemicals and solvents were American Chemical Society or reagent grade quality (Fisher Scientific, Medford, MA).

Animals and diets. Male C57L/J mice, 4–6 wk old, were bred at The Jackson Laboratory (Bar Harbor, ME) and were...
homozygous for susceptible lith alleles (21). All animals were maintained in a temperature-controlled room (22 ± 1°C) with regular 12:12-h day-night cycles (6 AM–6 PM). Mice were allowed to adapt to the environment for at least 2 wk before lithogenic diet feeding and were provided free access to water and normal mouse chow. Throughout the experimental periods, mice were fed Purina Laboratory Chow, which contains trace cholesterol (<0.02%) (Mouse Diet 1401, S. Hanky Road, St. Louis, MO) or a semisynthetic lithogenic diet (27) each 100 g, which contains 1 g cholesterol, 15 g butter fat, 2 g corn oil, 50 g sucrose, 20 g casein, and essential vitamins and minerals with or without 0.5 g cholic acid. Once mice reached 8 wk of age, they were divided into three groups (n = 20 each fed 1) Chow containing <0.02% cholesterol, 2) the semisynthetic diet containing 1% cholesterol, or 3) the semisynthetic diet containing 1% cholesterol and 0.5% cholic acid. All experiments were executed according to accepted criteria for the care and experimental use of laboratory animals, and euthanasia was consistent with recommendations of the American Veterinary Medical Association. All protocols were approved by the Institutional Animal Care and Use Committees of Harvard University and The Jackson Laboratory.

Collection of gallbladder biles and gallstones and microscopic studies. After 1 yr of feeding, surgery was performed on mice that were fasted overnight but had free access to water. Animals were weighed and anesthetized with an intraperitoneal injection of 35 mg/kg pentobarbital (Abbott Laboratories, North Chicago, IL). Surgery commenced at 9 AM and was performed under sterile conditions through an upper midline incision. After cholecystectomy, gallbladder volume was measured by weighing the whole gallbladder and equating gallbladder weight (including stones) with gallbladder volume. Gallbladders were then opened, and 5 µl of fresh gallbladder bile were examined for mucin gel, cholesterol crystals, liquid crystals, and gallstones (37–39). Liquid crystals and solid crystals as well as gallstones were defined according to previously established criteria (37–39). After pooled gallbladder biles were ultracentrifuged at 100,000 g for 30 min at 37°C and filtered through a preheated (37°C) Swinnex-GS filter assembly containing a 0.22 µm filter (Millipore Products Division, Bedford, MA), samples were frozen and stored at −20°C for further lipid analyses.

Cannulation of common bile duct and collection of hepatic biles. Additional groups of mice (n = 5 each) fed Chow (<0.02%), 1% cholesterol, or 1% cholesterol plus 0.5% cholic acid were used for biliary lipid secretion studies. In brief, the lower end of the common bile duct was ligated, and the common bile duct was cannulated below the entrance of the cystic duct via a PE-10 polyethylene catheter with an inside diameter of 0.28 mm and an outside diameter of 0.61 mm (Becton Dickinson Primary Care Diagnostics, Becton Dickinson, Sparks, MD). After successful catheterization and flow of fistula bile, the cystic duct was doubly ligated and cholecystectomy was performed. Hepatic bile was collected by gravity for the first hour. After fresh hepatic biles were examined by polarization light microscopy and their volumes determined by weighing (39), all samples were frozen and stored at −20°C for further lipid analyses. During surgery and hepatic bile collection, mouse body temperature was maintained at 37 ± 0.5°C with a heating lamp and monitored with a thermometer.

Determination of cholesterol absorption. Four groups of mice (n = 5 each) were fed either Chow containing <0.02% cholesterol, 1% cholesterol, 1% cholesterol plus 0.5% cholic acid, or 0.5% cholic acid for 2 days, which was sufficient to reach a TC steady state in bile (39). Cholesterol absorption was determined by the dual-isotope plasma ratio method described by Zilversmit and Hughes (46) and Turley et al. (35) with major modifications in methods for intravenous injection, intragastric administration, as well as lipid volumes and radiolabeled cholesterol given to each mouse. In brief, nonfasted mice were anesthetized lightly by intraperitoneal injection of 35 mg/kg pentobarbital. An 0.4-cm incision was made on the right or left side of the neck, and the jugular vein was exposed. Exactly 2.5 µCi of [3H]cholesterol dissolved completely in 100 µl of Intralipid was injected intravenously directly into the jugular vein using a 100-µl Hamilton syringe fitted with a 30-gauge needle and carried out over 1 min to prevent cardiac arrest. The incision was closed tightly with 3-0 silk sutures. After this procedure, a feeding needle with round tip (18 gauge, 50 mm in length) was inserted completely into the stomach of the mouse, and then by gavage the animal was given an intragastric dose of 1 µCi of [14C]cholesterol fully dissolved in 150 µl of medium-chain triglyceride oil. With this protocol, no problems were encountered with mice regurgitating gastric contents during and after the recovery period. After dosing, mice were returned to the animal room where they were free to eat chow or the appropriate semisynthetic diets for an additional 3 days. Mice were then anesthetized lightly as described and were bled from the heart into a microtube containing heparin (Elkins-Sinn, Cherry Hill, NJ) as anticoagulant. Aliquots of plasma were obtained by centrifugation at 10,000 g for 30 min at room temperature. To determine the proportions of [14C] and [3H]cholesterol doses remaining in plasma after 72 h, 100-µl plasma aliquots and the original dosing mixture were added directly to 10 ml EcoLite (ICN Biomedicals, Costa Mesa, CA). The vials were shaken vigorously for 10 min and counted in a liquid scintillation spectrometer (Beckman Instruments, San Ramon, CA). The ratio of the two radiolabels in plasma was used for calculating the percent cholesterol absorption using the following expression:

\[
\%\text{Ch absorption} = \frac{\text{percentage of ig dose} [14C] \text{Ch per ml plasma}}{\text{percentage of iv dose} [3H] \text{Ch per ml plasma}} \times 100
\]

where Ch is cholesterol, ig is intragastric, and iv is intravenous.

Measurement of activities of hepatic HMG-CoA reductase and cholesterol 7α-hydroxylase. Liver samples were obtained from nonfasted mice after 1 yr of feeding chow, 1% cholesterol, or 1% cholesterol plus 0.5% cholic acid. To minimize diurnal variations of hepatic enzyme activities, all procedures were performed between 8 and 9 AM. Microsomal activities of HMG-CoA reductase were determined by measuring the conversion rate of [14C]HMG-CoA to [14C]mevalonic acid using a radiochemical assay (10, 44). Products were quantified by liquid scintillation counting with [3H]mevalonolactone as internal standard. Protein concentration was determined by the assay of Bradford (10). Hepatic activities of cholesterol 7α-hydroxylase were determined by the HPLC-based assay system of Hylemon et al. (19).

Lipid analyses. Biliary phospholipids were determined as inorganic phosphorus by the method of Bartlett (3). Total BS and individual BS concentrations were measured by HPLC according to the methods of Rossi et al. (31). Bile cholesterol, as well as cholesterol content in chow and gallstones, were determined by HPLC (15, 39). Cholesterol saturation indexes (CSI) in gallbladder and hepatic biles were calculated from the critical tables (5). Relative lipid compositions of mouse gallbladder and hepatic biles were plotted on condensed
phase diagrams appropriate to their mean total lipid concentrations. The phase boundaries and crystallization pathways were extrapolated from model bile systems according to relative and total lipid concentrations developed for TC at 37°C (6, 37).

Statistical methods. Data are expressed as means ± SD. Differences among groups of mice fed chow, 1% cholesterol, 1% cholesterol plus 0.5% cholic acid, or 0.5% cholic acid were assessed for statistical significance by Student’s t-test. Statistical significance is defined as a two-tailed probability of <0.05.

RESULTS

Prevalence and characteristics of gallstones and mucus gel. After 1 yr of being fed chow or 1% cholesterol, both groups of mice had clear gallbladder biles and no mucus gel was detected. Moreover, they did not form either liquid crystals or solid cholesterol crystals or gallstones. In contrast, 100% of mice fed 1% cholesterol plus 0.5% cholic acid formed gallstones. The extracted sterols from these stones contained only cholesterol, which constituted >99% of stone weight. Most mice formed seven to nine gallstones. On average, the size of gallstones was 0.86 ± 0.26 mm in diameter. Gallbladders from all mice fed 1% cholesterol plus 0.5% cholic acid had layers of thick mucus gel (100%) containing liquid crystals (75%) (37–39).

Gallbladder volumes. At 1 yr, gallbladder volumes were similar between mice fed chow (26 ± 8 µl) and 1% cholesterol (23 ± 9 µl), and both were significantly (P < 0.01) smaller than those of mice (35 ± 12 µl) fed 1% cholesterol plus 0.5% cholic acid.

Lipid compositions of gallbladder and hepatic biles. Table 1 shows biliary lipid compositions of pooled gallbladder biles (n = 20) and individual hepatic biles (n = 5 each per group) at 1 yr. In general, biliary lipid compositions of gallbladder and hepatic biles were identical between mice fed chow or 1% cholesterol alone. There were no significant differences in total lipid concentration (gallbladder biles 6.6–8.7 g/dl and hepatic biles 1.3–1.7 g/dl) among the three groups of mice. Compared with mice fed chow or 1% cholesterol, mice fed 1% cholesterol plus 0.5% cholic acid had marked increases in mole percent cholesterol and lecithin but decreases in percent BS (Table 1). Moreover, in pooled gallbladder biles apparent CSI (Table 1) were similar between mice fed chow (CSI 0.52) and 1% cholesterol (CSI 0.64), and both were significantly lower (P < 0.05) than mice fed 1% cholesterol plus 0.5% cholic acid (CSI 1.74). Nevertheless, if an urso-correction (5) is carried out for the diminution in cholesterol solubility by T-µ-MC in the gallbladder biles, CSI values are 0.89 in chow and 1.14 in 1% cholesterol fed groups, respectively; whereas in mice fed 1% cholesterol plus 0.5% cholic acid, CSI increased to 1.85. Mean apparent CSI values of hepatic biles (Table 1) were not significantly different between chow (1.13 ± 0.26) and 1% cholesterol feeding (1.41 ± 0.17), but both were significantly (P < 0.05) lower than 1% cholesterol plus 0.5% cholic acid feeding (1.91 ± 0.45; Table 1).

Table 1. Biliary lipid compositions of pooled gallbladder and individual hepatic biles

<table>
<thead>
<tr>
<th>Diet</th>
<th>Mole %Ch</th>
<th>Mole %L</th>
<th>Mole %BS</th>
<th>L/(L + BS)</th>
<th>[TL], g/dl</th>
<th>CSI</th>
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<td></td>
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<td>Chow</td>
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<td>1% Ch + 0.5% CA</td>
<td>12.21</td>
<td>22.64</td>
<td>65.14</td>
<td>0.26</td>
<td>6.61</td>
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<td></td>
</tr>
<tr>
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<td>1.92</td>
<td>1.22</td>
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<td>Means ± SD</td>
<td>4.47 ± 0.90</td>
<td>16.52 ± 3.85</td>
<td>79.01 ± 4.07</td>
<td>0.17 ± 0.04</td>
<td>1.73 ± 0.20</td>
<td>1.13 ± 0.26</td>
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<td>1% Ch</td>
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<td>16.17</td>
<td>77.49</td>
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<td>1.83</td>
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<td>77.85</td>
<td>0.18</td>
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<td>Means ± SD</td>
<td>5.58 ± 0.67</td>
<td>16.22 ± 0.85</td>
<td>78.21 ± 0.72</td>
<td>0.17 ± 0.01</td>
<td>1.68 ± 0.11</td>
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<td>1% Ch + 0.5% CA</td>
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<td>68.32</td>
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<tr>
<td>Means ± SD</td>
<td>9.48 ± 1.85†</td>
<td>23.04 ± 2.21*</td>
<td>67.48 ± 1.76†</td>
<td>0.25 ± 0.02†</td>
<td>1.30 ± 0.12†</td>
<td>1.91 ± 0.45*</td>
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</table>

Values were determined from pooled gallbladder biles (n = 20/group) and individual hepatic biles (n = 5 each). Ch, cholesterol; L, lecithin; BS, bile salt; [TL], total lipid concentration; CSI, cholesterol saturation index; CA, cholic acid. *P < 0.05 and †P < 0.01, 1% Ch plus 0.5% cholic acid vs. chow or 1% Ch.
Table 1). An urso-correction factor is unavailable for such dilute biles (39).

Figure 1, top, shows on a condensed phase diagram according to average total lipid concentration (7.50 g/dl) of the biles (see Table 1). A one-phase micellar zone is enclosed by solid curved line. Above it, 2 solid and 2 dashed lines divide the phase diagram into regions A–E with different crystallization sequences (from Ref. 37). Relative lipid compositions of pooled gallbladder biles from mice fed chow and 1% cholesterol alone were located in the one-phase micellar zone. Lipid compositions of pooled gallbladder biles from mice fed 1% cholesterol plus 0.5% cholic acid plotted in central three-phase zone, where at equilibrium biles would be composed of cholesterol-saturated micelles, solid cholesterol crystals, and liquid crystals. Bottom: lipid compositions of individual hepatic biles (n = 5 each per group) are plotted on a condensed phase diagram for average total lipid composition (1.75 g/dl) of biles (see Table 1). This system exhibits the same physical states at equilibrium as for gallbladder biles, but all crystallization pathways are shifted to left with decreases in total lipid concentration and the one-phase micellar zone becomes smaller (37). These changes generate a new condensed phase diagram, with enlarged region E. Relative lipid compositions of essentially all hepatic biles locate in region E, where at theoretical equilibrium the biles would be composed of liquid crystals and saturated micelles but not solid cholesterol crystals. In this work, fresh hepatic biles of all mice were generally clear microscopically, suggesting their nonequilibrium nature. □, Chow; ▲, 1% cholesterol; and ●, 1% cholesterol plus cholic acid at 1 yr of feeding. See text for further details.

Fig. 1. Top: mean relative lipid compositions (moles per 100 moles) of pooled gallbladder biles (n = 20 each) plotted on a condensed phase diagram according to average total lipid concentration (7.50 g/dl) of the biles (see Table 1). One-phase micellar zone is enclosed by solid curved line. Above it, 2 solid and 2 dashed lines divide the phase diagram into regions A–E with different crystallization sequences (from Ref. 37). Relative lipid compositions of pooled gallbladder biles from mice fed chow and 1% cholesterol alone are located in the one-phase micellar zone. Lipid compositions of pooled gallbladder biles from mice fed 1% cholesterol plus 0.5% cholic acid plotted in central three-phase zone, where at equilibrium biles would be composed of cholesterol-saturated micelles, solid cholesterol crystals, and liquid crystals. Bottom: lipid compositions of individual hepatic biles (n = 5 each per group) are plotted on a condensed phase diagram for average total lipid composition (1.75 g/dl) of biles (see Table 1). This system exhibits the same physical states at equilibrium as for gallbladder biles, but all crystallization pathways are shifted to left with decreases in total lipid concentration and the one-phase micellar zone becomes smaller (37). These changes generate a new condensed phase diagram, with enlarged region E. Relative lipid compositions of essentially all hepatic biles locate in region E, where at theoretical equilibrium the biles would be composed of liquid crystals and saturated micelles but not solid cholesterol crystals. In this work, fresh hepatic biles of all mice were generally clear microscopically, suggesting their nonequilibrium nature. □, Chow; ▲, 1% cholesterol; and ●, 1% cholesterol plus cholic acid at 1 yr of feeding. See text for further details.

Table 1). An urso-correction factor is unavailable for such dilute biles (39).

Figure 1, top, shows on a condensed phase diagram the mean relative lipid compositions of pooled gallbladder biles (Table 1) at 1 yr of feeding mice different diets. The micellar phase boundary (6, 37) and cholesterol crystallization (regions A–E) pathways (37, 38) are appropriate for a mean total lipid concentration of 7.50 g/dl (see Table 1) with TC-rich bile. Relative compositions of pooled gallbladder biles from mice fed chow and 1% cholesterol plotted within the one-phase micellar zone, but the value of mice fed 1% cholesterol plus 0.5% cholic acid fell in a central three-phase area denoted region C. By previous phase analysis (6, 37), these biles are predicted to be composed of solid cholesterol crystals, liquid crystals, and saturated micelles at equilibrium. Figure 1, bottom, displays mean relative lipid compositions of individual hepatic biles plotted in a similar fashion and employing an appropriate micellar phase boundary (6, 37) and cholesterol crystallization pathways (37, 38) for dilute (1.75 g/dl) TC-rich bile (see Table 1). Relative lipid compositions of essentially all hepatic biles plotted within the crystallization pathway denoted region E (37), in which only liquid crystals occur at equilibrium but not solid cholesterol crystals. As we observed in both model (37) and human biles (38), when total lipid concentrations decrease, all physical states and crystallization pathways shift progressively to the left, i.e., to lower lecithin contents, and the micellar zone becomes smaller. With chow and 1% cholesterol feeding, relative lipid compositions of hepatic biles plotted in the high BS area of region E. However, hepatic biles from mice fed 1% cholesterol plus 0.5% cholic acid were shifted upward to the right, i.e., to high cholesterol and lecithin contents (P < 0.05; Table 1) but yet remained within region E. On microscopic examination (× 800 magnification) we detected small liquid crystals occasionally in fresh hepatic biles but never solid cholesterol crystals.

Figure 2 shows the distributions of BS species in pooled gallbladder biles (Fig. 2, top), as well as individual hepatic biles (Fig. 2, bottom) for mice on different diets. As we found in previous studies (39), all BS were taurine conjugated and all mice displayed similar
distributions of BS compositions between gallbladder and hepatic biles. In mice fed different diets for 1 yr. In both chow and 1% cholesterol (Ch) groups, taurocholate (TC) and tauro-β-muricholate (T-β-MC) were the predominant BS. In mice fed 1% cholesterol plus 0.5% cholic acid (CA), TC became the major BS replacing most T-β-MC. Hydrophobic BS [taurochenodeoxycholate (TCDC) and taurodeoxycholate (TDC)] increased appreciably when the diet contained 1% cholesterol plus 0.5% cholic acid. TUDC, tauroursodeoxycholate. T-ω-MC, tauro-ω-muricholate. See text for further description.

Bile flow and biliary lipid secretion rates. Figure 3 summarizes mean bile flow rates on the different diets at 1 yr for the first hour following interruption of the enterohepatic circulation. In mice fed 1% cholesterol alone, bile flow (285 ± 23 μl·h⁻¹·100 g body wt⁻¹) was similar to that in mice fed chow (310 ± 86 μl·h⁻¹·100 g body wt⁻¹) and both were significantly smaller (P < 0.05) than in mice fed 1% cholesterol plus 0.5% cholic acid (599 ± 105 μl·h⁻¹·100 g body wt⁻¹).

Figure 4 plots biliary cholesterol, lecithin, and BS secretion rates during the first hour of biliary washout in mice fed the different diets for 1 yr. In general, outputs of all three biliary lipids were similar between mice on chow and 1% cholesterol, but all outputs in mice fed 1% cholesterol plus 0.5% cholic acid were significantly higher (P < 0.05). The most marked changes were in cholesterol and lecithin outputs, which were 4.2 ± 1.4 μmol·h⁻¹·kg⁻¹ and 15.1 ± 4.2 μmol·h⁻¹·kg⁻¹ in mice fed chow and 4.7 ± 0.8 μmol·h⁻¹·kg⁻¹ and 13.7 ± 1.6 μmol·h⁻¹·kg⁻¹ in mice fed 1% cholesterol, both significantly (P < 0.05) lower than in mice fed 1% cholesterol plus 0.5% cholic acid.
(11.7 ± 2.2 µmol·h⁻¹·kg⁻¹ and 31.6 ± 8.4 µmol·h⁻¹·kg⁻¹). Differences in BS outputs were less marked (Fig. 4), although significantly lower (P < 0.05) in mice fed chow (75.9 ± 27.2 µmol·h⁻¹·kg⁻¹) and 1% cholesterol (66.0 ± 6.1 µmol·h⁻¹·kg⁻¹) compared with 1% cholesterol plus 0.5% cholic acid (90.8 ± 10.2 µmol·h⁻¹·kg⁻¹).

Influence of cholic acid feeding on intestinal cholesterol absorption. Percent cholesterol absorption, calculated from the plasma ratio of tracer [¹⁴C]- and [³⁵C]cholesterol after 3 days of dosing, is shown in Fig. 5. Normal mouse chow contains only <0.02% cholesterol, so that the addition of 1% cholesterol by weight to the diet markedly increased oral intake. However, there was no significant difference in the cholesterol absorption between mice on chow (34 ± 7%) and those fed 1% cholesterol (27 ± 9%). In contrast, dietary cholic acid significantly increased (P < 0.001) cholesterol absorption to 55 ± 5% in mice fed 1% cholesterol plus 0.5% cholic acid and to 63 ± 7% in mice fed 0.5% cholic acid without cholesterol compared with mice fed chow or 1% cholesterol alone.

Effects of feeding 1% cholesterol with or without 0.5% cholic acid on hepatic cholesterol and BS synthesis. Figure 6, top, shows the activities of hepatic HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. The values, although somewhat higher on the chow diet (33 ± 9 pmol·min⁻¹·mg⁻¹) compared with those fed 1% cholesterol (26 ± 8 pmol·min⁻¹·mg⁻¹) and 1% cholesterol plus 0.5% cholic acid (30 ± 6 pmol·min⁻¹·mg⁻¹), were not significantly different between the groups. In contrast, Fig. 6, bottom, shows that after 1yr of feeding 1% cholesterol plus 0.5% cholic acid, cholesterol 7α-hydroxylase activities decreased significantly (P < 0.05) to 1.8 ± 1.3 pmol·min⁻¹·mg⁻¹ compared with 5.2 ± 2.4 pmol·min⁻¹·mg⁻¹ with chow and 5.0 ± 2.9 pmol·min⁻¹·mg⁻¹ with 1% cholesterol.

DISCUSSION

Genetically determined cholesterol gallstone formation in mice can provide important insights into pathogenesis and genetic modifiers of cholesterol gallstone disease in humans. Because there is exceptionally close homology between mouse and human genomes (9), the inbred mouse is a most valuable research tool for investigating human gallstone genes. Cholesterol cholelithiasis does not occur spontaneously in healthy inbred mice, and the addition of 1% cholesterol and 0.5% cholic acid to an otherwise well-balanced rodent mouse diet is essential for inducing cholesterol gallstone formation (2, 21, 34, 39). Other small experimental animals such as prairie dogs form cholesterol gallstones after feeding the same amount of cholesterol without exogenous BS (24) because their principal BS is TC. However, mice have a more hydrophilic BS pool composition with 46–54% TC and 34–42% Tβ-MC (Ref. 39 and Fig. 2), compared with the 91–94% TC of prairie dogs (24).

Fig. 4. Cholesterol, lecithin, and BS outputs (µmol·h⁻¹·kg body wt) during first hour of interruption of enterohepatic circulation in mice (n = 5 each) after feeding different diets for 1yr. For mice fed 1% cholesterol alone, biliary lipid outputs are similar to those of mice fed chow, and both groups display significantly lower outputs (P < 0.05) compared with mice fed 1% cholesterol plus 0.5% cholic acid. See text for further description.

Fig. 5. Percent cholesterol absorption in mice (n = 5 each) determined by dual-isotope plasma ratio method (35, 46) as functions of chow, 1% cholesterol, 1% cholesterol plus 0.5% cholic acid, and 0.5% cholic acid feeding. Mice fed 1% cholesterol alone had similar %cholesterol absorption to mice fed chow, and both groups were significantly lower (P < 0.001) compared with mice fed both 1% cholesterol plus 0.5% cholic acid or 0.5% cholic acid. Although %cholesterol absorption in mice fed 0.5% cholic acid was slightly higher, they were not statistically different from mice fed 1% cholesterol plus 0.5% cholic acid. See text for further description.
ably contributing to the high cholesterol secretion rates, thereby further favoring cholesterol supersaturation, and 3) promotion of mucin gel formation, cholesterol crystals, and gallstones as well as the enlarged gallbladders only when both cholesterol and cholic acid were added to the diet, whereas addition of cholesterol alone did not reproduce these biliary phenotypes.

Cholic acid replaces T-β-MC with TC, which enhances intestinal cholesterol absorption. In the natural state, 40% or more of the BS pool of mice fed chow is composed of T-β-MC (Refs. 1, 39 and Fig. 2), and under these conditions cholesterol is only modestly absorbed from the intestine (Fig. 5). The reason apparently is that T-β-MC is a very poor cholesterol solubilizer with similar properties to the taurine conjugate of ursodeoxycholic acid (UDCA) (5, 25, 37). In fact, compared with TC, T-β-MC may be considered an "inhibitor" of intestinal cholesterol absorption. It is known that in cholesterol gallstone patients (16, 28) administration of UDCA reduces intestinal cholesterol absorption significantly, and this has been confirmed by us in gallstone-susceptible C57L/J mice (40). After feeding 1% cholesterol plus 0.5% cholic acid, we have observed (39) that BS compositions in bile were altered dramatically after 1 day of feeding, with TC increasing to 70–80% and T-β-MC decreasing to 3–10%. These profound changes in BS hydrophobicity induced by cholic acid in bile and the intestine should markedly increase micellar cholesterol solubility (6, 37). Because cholic acid increased cholesterol absorption from the intestine significantly via TC, this should elevate the cholesterol content of the liver (36), which in turn would increase bioavailability of cholesterol to enhance biliary cholesterol hypersecretion. Because insulin is apparently necessary for T-β-MC synthesis in mice (1), diabetes mellitus increases synthesis and secretion of endogenous TC in bile because of a spontaneous decrease in T-β-MC content. Therefore, alloxan-diabetic mice (1) fed 1% cholesterol without cholic acid form cholesterol gallstones reproducibly and spontaneously. We also observed that, in each dietary group (Fig. 5), the percent cholesterol absorption is slightly less than that in mice without dietary cholesterol, but the differences were not statistically significant. Most likely this difference is secondary to the effect of the radioisotope dilution on specific activity of cholesterol in the upper small intestine.

Recently, we (40) determined that genetic variations in cholesterol absorption efficiency are positively and significantly correlated with cholesterol gallstone prevalence in 11 strains of inbred mice. Furthermore, percent cholesterol absorption was shown to be significantly (P < 0.001) higher in the gallstone-susceptible C57L/J strain (37 ± 7%) and compared with the resistant AKR strain (24 ± 8%) (40) engendering a biliary cholesterol concentration that is significantly higher in C57L/J mice than in AKR mice (39). Our observations are not in agreement with the recent results of Sehayek et al. (33), whose findings were that biliary cholesterol concentration was inversely correlated with percent cholesterol absorption (33). In the future, it will be
imperative to investigate the mechanisms whereby dietary cholesterol influences biliary cholesterol secretion and how secretion rates of biliary lipids may regulate intestinal cholesterol absorption.

Cholic acid elicits a lithogenic phase change in bile with enlarged gallbladder and accumulation of mucin gel. Because the more hydrophobic BS (TC) replaced the hydrophilic BS (T-β-MC), all crystallization pathways are shifted to the right on the bile phase diagram, i.e., to higher lecithin contents (39). This phase change facilitates solid cholesterol crystallization (37, 38) during lithogenesis of cholesterol-supersaturated biles in mice fed cholesterol plus cholic acid. In contrast, when a hydrophilic BS is fed, all crystallization pathways would be shifted to lower lecithin contents and cholesteryl crystallization is delayed markedly (37, 38). In fact, the addition of 0.1–0.3% hyocholic acid, also a very hydrophilic BS (8), to a murine lithogenic diet prevents cholesterol monohydrate crystals from forming in mice (12) and similarly hyodeoxycholic acid, which is of approximate hydrophilicity to UDCA (8), has been shown to prevent cholelith gallstone formation in hamsters (41). Of interest is that, compared with hepatic biles (Fig. 1), the lipid compositions of gallbladder biles from mice fed chow or cholesterol alone became appreciably less saturated with cholesterol and also contained lower moles percent lecithin. In contrast, the relative lipid compositions of gallbladder and hepatic biles from mice fed cholesterol plus cholic acid remained indistinguishable (Fig. 1). This is consistent with the concept that the gallbladder absorbs cholesterol and indeed some lecithin from bile (20, 26), but this function is likely to be damaged in mice fed cholesterol plus cholic acid, as evidenced by both mucin production and absence of changes in relative lipid compositions (Fig. 1). Furthermore, we found that gallbladder size was significantly enlarged by ∼50% (see results) in mice fed the lithogenic diet for 1 yr compared with those fed for 8 wk (39), probably from cholesterol toxicity to smooth muscle cells (45). The enlarged gallbladder is most likely hypomotile and may facilitate cholesterol crystallization and gallstone formation (13). Also, feeding cholesterol plus cholic acid to mice is known to cause an acute inflammatory reaction in the gallbladder wall with mucosal hyperplasia and mucin hypersecretion (42). It is likely also that cholesterol-supersaturated bile, or TC, or both may stimulate mucin production and secretion (23). Thereafter, by forming a cholesterol-enriched gel layer (24, 39), mucin may provide an environment for continuous cholesterol crystal growth and trapping of crystals as well as precluding further mucosal absorption of cholesterol molecules (Fig. 1).

Response of hepatic cholesterol and BS synthesis to long-term feeding of 1% cholesterol plus 0.5% cholic acid. Dietary cholesterol suppresses hepatic cholesterol synthesis in the rat by >95%, whereas cholic acid does so to a lesser extent (18). Consistent with our previous studies (21), this effect was less pronounced in C57L/J mice fed cholesterol or cholesterol plus cholic acid and did not reach significance. Impaired downregulation of hepatic cholesterol synthesis in C57L/J mice fed 1% cholesterol plus 0.5% cholic acid (21) may contribute to biliary cholesterol hypersecretion and the formation of cholesterol-supersaturated biles. Although the lithogenic diet reduced cholesterol 7α-hydroxylase activities in mice (29), feeding cholic acid alone also suppressed hepatic BS synthesis in rats (18). The current results showed that feeding cholesterol alone did not influence activities of cholesterol 7α-hydroxylase. This suggests that a diminished conversion of cholesterol to BS induced by cholic acid and its deoxycholic acid metabolite (Fig. 2) may contribute to increased cholesterol bioavailability and augmented hepatic cholesterol secretion.

Addition of cholesterol or cholic acid alone to chow diet does not induce cholesterol gallstone formation. We observed that feeding cholesterol alone did not influence gallbladder size, hepatic secretion of biliary lipids, biliary CSI values, or mucin accumulation in the gallbladder, nor did it induce cholesterol gallstone formation. Also reported by Fujino and colleagues (14) is that feeding 0.5% cholic acid alone did not induce cholesterol gallstone formation in mice. Furthermore, they (14) observed that feeding 1.0% cholic acid induced high contents of free fatty acids (mainly palmitic acid) in hepatic biles possible due to hepatocyte toxicity, as might have been anticipated when feeding 1.5% cholic acid induced mouse mortality (14).

In summary, we have continued our pathophysiological investigation of the inbred mouse, which is now emerging as a model par excellence for the study of cholesterol gallstone disease because the genetic resources are so rich and quantitative trait loci for several lith genes have been discovered (21). In this study, we have focused critically on the essential role of 0.5% cholic acid for cholelithogenesis by a lithogenic diet, whereas feeding 1% cholesterol alone even for 1 yr is nonlithogenic. We show here that by suppressing T-β-MC and increasing TC levels in bile, cholic acid promotes cholesterol absorption from the intestine and inhibits one of the principal disposition pathways for cholesterol, that of de novo BS synthesis. Probably most important of all, cholic acid via TC alters the phase relations of biliary lipids in bile that solid cholesterol crystallization becomes possible in concentrated gallbladder biles. We note in closing that native murine strains such as deer mice (Peromyscus maniculatus) (32) can form cholesterol gallstones spontaneously in the wild, and it will be interesting to explore whether the deer mouse has spontaneous mechanisms for increasing intestinal absorption and phase separation of cholesterol by reducing hydrophilic BS synthesis.

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