Increases in biliary cholesterol-to-bile acid ratio in pregnant hamsters fed low and high levels of cholesterol

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Yao, Li Hang, Paul A. Dawson, and Laura A. Woollett. Increases in biliary cholesterol-to-bile acid ratio in pregnant hamsters fed low and high levels of cholesterol. Am J Physiol Gastrointest Liver Physiol 284: G263–G268, 2003; 10.1152/ajpgi.00332.2002.—Gallstones develop when the secretion of cholesterol is elevated compared with the secretion of bile acids into bile. One of the risk factors for the formation of gallstones is pregnancy. Because the pregnancy-induced increase in hepatic cholesterol synthesis rates could play a critical role in the development of cholesterol stones, the aim of the present study was to determine whether stone formation, as assessed by the ratio of cholesterol to bile acids in bile, could be ablated by blocking the pregnancy-induced increase in hepatic sterol synthesis rates. Golden Syrian hamsters were fed either ground chow or chow supplemented with 0.5% cholesterol for 3 wk and studied in the nonpregnant state or in late gestation. In chow-fed animals, a 1.6-fold increase in the ratio of cholesterol to bile acids occurred simultaneously with a sevenfold increase in hepatic steroid synthesis rates. In cholesterol-fed dams, an increase in the ratio of cholesterol to bile acids occurred even with the lack of induction of hepatic steroid synthesis rates during pregnancy. Thus it appears that the marked induction of hepatic sterol synthesis rates during gestation is not essential for the pregnancy-induced cholesterol saturation of bile when cholesterol is fed to animals.

Gallstones; gestation

GALLSTONE FORMATION OCCURS more readily under several different conditions, including pregnancy, usage of oral contraceptives, obesity, rapid weight loss, age, and hypertriglyceridemia (2, 14). Three primary changes in the biliary environment involved in cholesterol precipitation and subsequent cholesterol gallstone formation (reviewed in Refs. 2 and 6) include cholesterol supersaturation, cholesterol nucleation and crystal growth, and gallbladder hypomotility. Biliary cholesterol supersaturation occurs when the ratio of cholesterol to bile acid increases as the result of an increase in cholesterol and/or a decrease in bile acid secretion into the bile. Bile supersaturation with cholesterol is a necessary step for gallstone formation, but not all individuals with supersaturated bile form gallstones, implying that additional factors aid in the nucleation of cholesterol (14). Some of the factors implicated in cholesterol nucleation or crystallization include mucin, concanavalin A-binding glycoprotein, α1-acid glycoprotein, and calcium (reviewed in Ref. 14). Hypomotility of the gallbladder is also required for cholesterol gallstone formation, because crystals precipitate when cholesterol is exposed to the nucleating factors for extended periods of time (14).

There are two sources of biliary cholesterol: cholesterol that is newly synthesized within the liver and cholesterol that is taken up by the liver as lipoproteins (3, 21, 24–26, 28, 33). A change in the input of hepatic cholesterol can alter the quantity of cholesterol secreted into the bile and/or the ratio of cholesterol to bile acid in the bile. For example, an acute induction in hepatic sterol synthesis rate can lead to an acute increase in the amount of cholesterol secreted into the bile (3). As synthesis rates return to normal, biliary cholesterol secretion rates also normalize. Comparable results can occur when sterol synthesis rates are induced chronically with cholestyramine or suppressed chronically with dietary cholesterol in rats (33). Similarly, when the amount of exogenous lipoprotein-cholesterol taken up by the liver is altered, a simultaneous change in the ratio of biliary cholesterol to bile acid concentrations occurs (15, 28).

During gestation, cholesterol-saturated bile forms as the result of an increase in the secretion of newly synthesized cholesterol into the bile and a decrease in the transit time of lipids through the enterohepatic circulation (12). In hamsters, it has been demonstrated that the amount of newly synthesized hepatic sterol, one of the sources of biliary cholesterol, increases markedly late in gestation (19). Thus if the pregnancy-induced increase in the saturation of bile is due to an increase in hepatic sterol synthesis rates, then preventing the increase in sterol synthesis rates could reduce the saturation of bile with cholesterol during pregnancy. In the present study, diet-induced inhibition of hepatic sterol synthesis rates during gestation...
did not deter the increase in the cholesterol-to-bile acid ratio partly because of the constricted bile acid pool size (BAPS) in cholesterol-fed pregnancy dams and partly because the additional dietary cholesterol appears to somewhat compensate for the reduced hepatic sterol synthesis rates.

**MATERIALS AND METHODS**

**Animals and diets.** Nonpregnant female and male golden Syrian hamsters weighing 90–100 g (Charles River, Kingston, NJ) were maintained in a temperature- and humidity-controlled room with alternating light and darkness. Hamsters were fed one of two diets. Diets consisted of either plain ground chow (Teklad, Madison, WI) or ground chow supplemented with 0.5% cholesterol (wt/wt). After 3 wk of dietary treatment, the time it takes for the cholesterol-fed hamster to reach a new steady state (30), hamsters were either studied (0 days gestation) or mated and studied at 14 days (hamsters had a gestational period of 15.5 days). All protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

**Cholesterol concentrations in the liver and plasma.** Hamsters were anesthetized and exsanguinated from the abdominal aorta. Tissue samples were cut into standard (34). Plasma cholesterol concentration was determined with 0.5% cholesterol (wt/wt). After 3 wk of dietary gas liquid chromatography using stigmastanol as an internal standard (34). Plasma cholesterol concentration was determined enzymatically (Boehringer-Mannheim, Indianapolis, IN).

BAPS. The liver, including the gallbladder, was removed and placed in 100% ethanol. The small intestine was isolated and combined with the liver. Tissue samples were cut into 3 x 3-mm pieces and heated for 2 h at 65°C. Samples were centrifuged, and supernatants were filtered. The remaining pellet was homogenized, and the extraction process was repeated. A portion of the sample was dried under nitrogen with 5β-cholic acid-7α,12α-diol as an internal standard. The sample was redissolved in methanol, water, acetic acid, and sodium chloride (vol/vol/vol/vol/wt, 50:21.4:2.34:0.1, pH 4.45) and then sonicated and spun. The resulting supernatant was dried down under nitrogen and reconstituted in methanol plus mobile phase A (15% isopropanol, 15% acetonitrile, and 70% 20 mM acetic acid). Just before use, the sample was filtered through a 0.45-μm PVDF syringe filter ( Pall Gelman Laboratory, Ann Arbor, MI), and 50 μl of sample plus internal standard was injected onto a 5-μm ODS Hypersil (C18) 4.6 x 250-mm analytical column (Keystone Scientific, Bellefonte, PA). A linear gradient mobile phase system was used as described (43) using an evaporative light scattering detector (ELSD; Alltech Associates, Deerfield, IL) (22). Bile acids were identified on the basis of the retention time of known standards and quantified on the basis of the amount of internal standard added to the sample.

**Biliary lipid and bile acid composition.** Hamsters were anesthetized and exsanguinated from the abdominal aorta. Bile was collected directly from the gallbladder with a 30-gauge needle. Concentrations of cholesterol and phospholipids were determined enzymatically (Boehringer-Mannheim) and chemically (29), respectively. Concentration of bile acids was measured by HPLC. Briefly, gallbladder bile (5 μl) was added to ethanol containing an internal standard. The sample was heated at 65°C for 1 h, dried down under nitrogen, and redissolved in methanol plus mobile phase A (vol/vol, 1:3). The sample was filtered, and bile acids were separated and quantified by HPLC using an ELSD as described (43).

**In vivo de novo sterol synthesis rates.** Hamsters were anesthetized lightly with diethyl ether and given a bolus injection of [3H]H2O ip (50 mCi). After 1 h, the animals were anesthetized, samples of liver and blood were collected and saponified, and the amount of digitonin-precipitable sterols (DPS) was determined (5, 11). The hepatic sterol synthesis rates were expressed as nanomoles of [3H]H2O converted to sterol per gram per hour.

In a second set of studies, hamsters were injected with [3H]H2O as just described. After 1 h, animals were anesthetized and gallbladder bile was collected with a 30-gauge needle. The bile (50 μl) was saponified, and the amount of DPS was determined. The amount of newly synthesized cholesterol present in the bile was expressed as nanomoles of [3H]H2O converted to sterol per milliliter per hour.

**Immunoblots.** Small intestines were collected, and the lumens were cleared of its contents. The distal 25% of the intestines were collected to ensure pure ileum. Tissues were rapidly frozen in liquid nitrogen and stored at −70°C. Intestines were thawed on ice, and apical membranes were isolated as described (39). Intestinal apical (75 μg) membrane proteins were separated on denaturing polyacrylamide gels and visualized with enhanced chemiluminescence (ECL Plus; Amersham Pharmacia Biotech, Piscataway, NJ) using an anti-hamster apical sodium-dependent bile acid transporter (ASBT) antibody (39) as the primary antibody. The relative densities within each band of each gel were measured using National Institutes of Health Scion Image software. Each gel contained one sample from a control-fed nonpregnant dam, a control-fed pregnant dam, a cholesterol-fed nonpregnant dam, and a cholesterol-fed pregnant dam. Within each gel, the control-fed nonpregnant female membranes corresponded to a relative value of 1.

**Analyses of data.** Values are presented for all experimental data as means ± SE. Differences between the nonpregnant and pregnant hamsters on each diet were determined using Student’s t-test to detect significance (P < 0.05).

**RESULTS**

Because the purpose of the study was to test the ability of dietary cholesterol to block the pregnancy-induced increase in hepatic sterol synthesis, cholesterol synthesis rates initially were measured in nonpregnant and pregnant dams fed Chow and Chow supplemented with cholesterol. As seen in Fig. 1A, the nonpregnant control dam synthesized a significant amount of sterol in the liver (1,507 ± 243 nmol·h⁻¹·g⁻¹). By 14 days of gestation, liver sterol synthesis rates increased sevenfold (P < 0.01). In the presence of dietary cholesterol, cholesterol synthesis rates were suppressed to a minimal value before gestation (32 ± 4 nmol·h⁻¹·g⁻¹). Synthesis rates remained minimal at 14 days of gestation and did not increase during pregnancy in the control dams. A significant amount of DPS (118 ± 5 nmol·h⁻¹·ml⁻¹) was secreted into the bile in the nonpregnant hamsters, and the amount secreted increased ninefold during gestation (Fig. 1B) (P < 0.01). In dams with suppressed hepatic sterol synthesis rates, minimal amounts of newly synthesized cholesterol were present in the bile at 0 and 14 days of gestation (5 ± 2 nmol·h⁻¹·ml⁻¹). The amount of [3H]DPS present in the blood of the cholesterol-fed dams was nominal (6 ± 3 nmol·h⁻¹·ml⁻¹), indicating that the contribution of [3H]DPS synthesized within
the extrahepatic tissues to biliary [3H]DPS was minor within the 1-h study.

The cholesterol-to-bile acid ratio was then measured in nonpregnant and pregnant dams fed either the control diet or the cholesterol-containing diet. As expected, the cholesterol-to-bile acid molar ratio increased 1.8-fold during pregnancy (Fig. 2) (P < 0.05). Interestingly, even when hepatic sterol synthesis rates were suppressed, dams fed cholesterol had a 1.6-fold increase in the cholesterol-to-bile acid ratio during pregnancy (P < 0.02). The cholesterol-to-phospholipid molar ratio (×100) remained significantly unchanged during pregnancy and went from 35.3 ± 15.0 to 31.8 ± 5.1 in control-fed dams (P > 0.05) and from 44.7 ± 6.1 to 36.3 ± 5.3 in cholesterol-fed dams (P > 0.05).

Due to the pregnancy-induced increase in the cholesterol-to-bile acid ratio in the dams fed cholesterol as well as control-fed dams, the BAPS was then measured in these same animals. In nonpregnant females not fed any added cholesterol, the BAPS was 20.6 ± 0.8 μmol per animal (Fig. 3). During gestation, the pool size increased 1.7-fold (P = 0.03). In females fed cholesterol, the BAPS was similar in size to that measured in the control dams (27.4 ± 3.6 μmol per animal). In contrast to the control animals, BAPS in cholesterol-fed dams decreased during gestation (P = 0.04). The pool size was not the only parameter in bile acid metabolism that varied with gestation and diet; bile acid composition also varied with gestation and type of diet fed (Table 1). In nonpregnant females fed the control diet, 81.5 ± 2.2% of the bile acid pool was composed of cholic acid conjugates with a majority of the remaining bile acids consisting of primarily chenodeoxycholic acid conjugates and some deoxycholic acid. By late gestation, females fed the control diet had a significant enrichment of the amount of conjugates of cholic acid (P = 0.001) to >90% and a decrease in the relative
The present study demonstrates how sterol metabolism is markedly affected during times of a net loss of sterol by the pregnant dam [to the fetus (41)] and in the presence of elevated levels of steroidogenic hormones. They also demonstrate how the processes may be affected differently in the presence or absence of exogenous sterol.

First, this study shows that hepatic sterol synthesis rates increase markedly during pregnancy of chow-fed hamsters. Elevated sterol synthesis rates in the liver can be induced in numerous animal species by bringing about a negative sterol balance across the liver, such as that found with dietary cholestyramine or psyllium (9, 32). During pregnancy, a significant amount of circulating cholesterol is taken up by the extraembryonic fetal tissues (1, 40, 42) and removed irreversibly from the circulation. Uptake of cholesterol by these tissues constitutes a net loss of cholesterol from the body, because the cholesterol is used for hormone synthesis (38) or is transported to the fetal tissues (17, 41) and thus does not return to the maternal liver. Support for this net loss of lipid is that hypocholesterolemia develops in early-to-mid gestation (8). In response to the loss of sterol to the developing tissue, the liver increases sterol synthesis rates, thereby eventually inducing hypercholesterolemia in late gestation (18). The elevated synthesis rates can lead to an increase in the amount of cholesterol secreted into the bile, and in some cases to cholesterol-saturated bile or gallstones.

Second, the biliary cholesterol-to-bile acid molar ratio increases in pregnant hamsters. For there to be a change in the cholesterol-to-bile acid ratio, there must be an increase in the amount of cholesterol secreted into the bile or a decrease in the amount of bile acids secreted into the bile (reviewed in Refs. 2 and 6). Mechanisms responsible for the change in ratios are most likely different between the hamsters fed the low- and high-cholesterol diets. In the dams fed a low-cholesterol diet, there was markedly more newly synthesized cholesterol secreted into the bile during pregnancy. This increase in cholesterol could be at least part of the reason for the increase in the cholesterol-to-bile acid ratio during pregnancy. In contrast to data obtained from the dams fed the low-cholesterol diet, the BAPS was contracted in the pregnant dams fed the high-cholesterol diets. The decrease in size was most likely not due to a change in reabsorption of bile acids from the ileum, because protein levels of ASBT were similar during pregestation and gestation. An interesting note is that, even in the absence of any newly synthesized cholesterol, the cholesterol-to-bile acid ratio is similar or greater in cholesterol-fed compared with control-fed dams at either state of gestation. Thus

Table 1. Effect of pregnancy and diet on biliary bile acid composition

<table>
<thead>
<tr>
<th>Pregnancy Status, Diet</th>
<th>Cholic Acid</th>
<th>Chenodeoxycholic Acid</th>
<th>Deoxycholic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpregnant, control</td>
<td>81.5 ± 2.2</td>
<td>13.3 ± 1.8</td>
<td>3.4 ± 0.9</td>
</tr>
<tr>
<td>Nonpregnant, CH-fed</td>
<td>76.9 ± 3.0</td>
<td>18.6 ± 3.3</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td>Pregnant, control</td>
<td>92.2 ± 0.7</td>
<td>5.8 ± 0.6</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Pregnant, CH-fed</td>
<td>77.2 ± 3.3</td>
<td>17.6 ± 2.1</td>
<td>1.7 ± 0.6</td>
</tr>
</tbody>
</table>

Values are percent in means ± SE; n = 5–6 animals. Animals were fed with chow or chow plus cholesterol (0.5% wt/wt) for 3 wk, after which dams were studied (nonpregnant) or mated and studied 14 days later (pregnant). CH, cholesterol.
biliary cholesterol appears to be derived from exogenous, or diet-derived, as well as newly synthesized sources.

Third, this study shows that, during gestation, dams fed a low-cholesterol diet had a 1.6-fold increase in BAPS, whereas dams fed cholesterol had a reduction in BAPS. Any mechanism responsible for differences in BAPS could not be the result of just elevated steroidogenic hormone levels, because animals fed either diet would have marked elevations in concentrations during pregnancy. Also, mechanisms are most likely not due to either changes in the enterohepatic circulation, which is slower during pregnancy of animals and humans due to slow gallbladder emptying (12), transport of bile acids out of the liver during pregnancy, which is inhibited (31), or levels of ASBT that are normal during pregnancy. The increase in the pool size of the chow-fed animals is due to dramatically more cholesterol being synthesized in the liver, leading to more substrate for the enzymes of the classical pathway. The decrease in pool size of the cholesterol-fed animals could be that they do not have an increase in newly synthesized cholesterol and thus not an increase in substrate supply for 7α-hydroxylase (cytochrome P-450 7a1; CYP7A1).

Fourth, the ratio of cholic and deoxycholic acids to chenodeoxycholic acid changes from 6.4 to 16.1 during pregnancy in chow-fed dams and stays relatively constant during pregnancy in cholesterol-fed dams. This change in bile acid composition in chow-fed dams indicates that there is an alteration in enzymatic activity or in the pathway used to synthesize bile acids during pregnancy. Though not absolute, the alternative bile acid synthetic pathway preferentially synthesizes chenodeoxycholic acid, whereas the classical pathway preferentially synthesizes cholic acid (23, 36). Thus the marked increase in the ratio of cholic plus deoxycholic acids to chenodeoxycholic acid that occurs in pregnant chow-fed dams supports a shift or an increase in synthesis via the classical pathway in these animals.

Classical and alternative pathways responsible for the synthesis of bile acids are located in different subcellular locations and are minimally regulated at three different levels. The classical pathway occurs in microsomes with CYP7A1 as the initial enzymatic reaction, and the alternative pathway occurs in the mitochondria with 27α-hydroxylase as the initial enzyme (23, 36). One level of regulation occurs at the transcription rate of the various rate-limiting enzymes. The classical pathway is tightly regulated at the level of CYP7A1 transcription by negative feedback inhibition of bile acids via the farnesyl X receptor and short heterodimeric partner or by the pregnane X receptor (reviewed in Ref. 7). The transcription rates of 27α-hydroxylase and oxysterol CYP7B1 of the alternative pathway also are regulated by bile acids in most (reviewed in Ref. 36), though possibly not all (27), systems. A common point of regulation in both pathways occurs at the levels of 12α-hydroxylase (CYP8B1) (35). A second level of regulation of the various pathways can occur as the result of the amount of accessible substrate. In the present study, there was a significant increase in hepatic sterol synthesis rates of the control-fed dams. If newly synthesized sterol were the preferred substrate for CYP7A1, the proportion of cholic acid and its derivatives to chenodeoxycholic acid would increase and BAPS would enlarge, similar to what occurs in the present study. A third level of regulation is the activities of transport proteins responsible for the movement of the sterol substrates across the mitochondrial membrane. Thus changes in transport proteins, such as the steroidogenic acute regulatory protein, may play a role in substrate supply for the enzymes of the various pathways. It should also be noted that steroidogenic hormones can have a marked influence on sterol metabolism. For example, progesterone is a ligand for pregnane X receptor (13), estrogen suppresses steroidogenic acute regulatory protein levels in developing ovaries (10), and estrogen upregulates hepatic LDL receptor levels (37). Dissecting the interacting role of steroidogenic sex hormones and sterol supply on bile acid synthesis rates and substrate supply will need to be studied further.

To conclude, the dam's first requirement during pregnancy appears to be the presentation of enough cholesterol to the developing fetal tissues. When enough cholesterol is taken up by the extraembryonic tissues to place the dams in a negative sterol balance, hepatic sterol synthesis rates increase, leading to cholesterol-saturated bile and/or an increase in the cholesterol-to-bile acid ratio. Although some of the extra hepatic cholesterol may be converted to bile acids, the increase in secretion of cholesterol to the bile is greater. When the female is fed enough cholesterol to compensate for the net loss of cholesterol to the fetal tissues, the cholesterol-to-bile acid ratio still increases during pregnancy, possibly due to a reduction in the amount of bile acids synthesized and the use of diet-derived cholesterol for biliary cholesterol. Thus consumption of a cholesterol-containing diet will not prevent the development of cholesterol-saturated bile, as indicated by the cholesterol-to-bile acid ratio, and thus possibly gallstone disease during pregnancy.

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