

Supporting Materials and Methods

Microscopic study of gallbladder bile and gallstones. At 8 weeks on the lithogenic diet, mice (n=10 per group) were fasted overnight with free access to water. After anesthetization with pentobarbital, a cholecystectomy was carefully performed. To observe the effect of GPR30 or ER α on G-1-induced gallstone phenotypes, gallbladder bile was examined by polarizing light microscopy without a cover slip and then with a cover slip using phase contrast optics for the presence of mucin gels, liquid crystals, solid cholesterol monohydrate crystals, sandy stones, and gallstones according to previously established criteria (1). The images of plate-like cholesterol monohydrate crystals and gallstones were analyzed by a Carl Zeiss Imaging System with an AxioVision Rel 4.6 software (Carl Zeiss Microimaging GmbH, Göttingen, Germany). After microscopic analysis, pooled gallbladder bile was frozen and stored at -20°C for further lipid analysis.

Biliary lipid secretion study. After anesthetization with pentobarbital, the cystic duct was doubly ligated and cholecystectomy was performed. Subsequently, 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 (2). The first hour sample of hepatic bile was harvested by gravity in OVX mice (n=5 per group). During surgery and hepatic bile collection, mouse body temperature was maintained at $37\pm 0.5^{\circ}\text{C}$ with a heating lamp and monitored with a thermometer. After the volumes of hepatic bile samples were determined, hepatic bile was examined by polarizing light microscopy. All bile samples were frozen and stored at -20°C for further lipid analyses.

Biliary lipid analysis. Total and individual bile salt, as well as cholesterol and phospholipid concentrations in the gallbladder and hepatic bile were determined as

described previously (3, 4). Cholesterol saturation indexes (CSI) of the gallbladder and hepatic bile samples were calculated from the critical tables (5). Individual bile salt species were measured by high-performance liquid chromatography (HPLC) (6) and hydrophobicity indexes (HI) of individual hepatic bile samples were calculated according to published methods (7). The relative lipid composition of pooled gallbladder bile was plotted on condensed phase diagrams appropriate to their mean total lipid concentrations at ~10 g/dL. For graphic analysis, the phase boundaries and crystallization pathways were extrapolated from model bile systems developed for taurocholate at 37°C and a total lipid concentration of 10 g/dL (8).

Dynamic measurement of gallbladder emptying function. To study the lithogenic role of GPR30 in impairing gallbladder contractility, a dynamic measurement of gallbladder emptying rate was performed in OVX mice (n=4 per group) in response to exogenously administered sulfated cholecystokinin octapeptide (CCK-8) at 17 nmol/kg body weight dissolved in 100 µL of phosphate buffered saline (PBS) solution, or 100 µL of PBS solution (as a control) after 2 weeks of G-1 treatment at 0 or 1 µg/day and feeding the lithogenic diet. Gallbladder emptying function was analyzed by a difference in gallbladder volumes before and after the intravenous injection of CCK-8 or PBS (9). Gallbladder volumes were calculated using the following formula, assuming an ellipsoid shape of the organ:

$$\text{Gallbladder volume } (\mu\text{L}) = \text{length (mm)} \times \text{width (mm)} \times \text{depth (mm)} \times \pi / 6$$

Quantitative real-time PCR assay. Using RNeasy Midi (Qiagen, Valencia, CA), total RNA was extracted from fresh liver tissues in two groups (n=4 per group) of OVX mice: ones were fed the chow diet and treated with G-1 at 0 or 1 g/day for 2 weeks, and

others were fed the lithogenic diet and treated with G-1 at 0 or 1 g/day for 8 weeks. Reverse-transcription reaction was performed using the SuperScript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with 5 µg of total RNA and random hexamers to generate cDNA. Primer Express Software (Applied Biosystems, Foster City, CA) was used to design the primers based on sequence data available from GenBank. Quantitative real-time PCR assays of the hepatic *Era*, *Erβ*, and *Gpr30* genes and the genes involved in the regulation of hepatic lipid metabolism, including ATP-binding cassette (ABC) transporter G5 (*Abcg5*), *Abcg8*, *Abcb4*, *Abcb11*, cholesterol 7 α -hydroxylase (*Cyp7a1*), and sterol 27-hydroxylase (*Cyp27a1*), acyl-coenzyme A:cholesterol acyltransferase 2 (*Acat2*), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*), sterol regulatory element-binding protein 2 (*Srebp2*), low-density lipoprotein receptor (*Ldlr*), scavenger receptor class B type I (*Sr-b1*), and farnesyl diphosphate synthase (*Fdps*) were performed in triplicate according to previously established methods (10). The sequences of the primers for these genes are listed in **Supplementary Table S1**. Relative mRNA levels were calculated using the threshold cycle of an unknown sample against a standard curve with known copy numbers. To obtain a normalized target value, the target amount was divided by the endogenous reference amount of mouse *β-Actin* as an internal control.

Statistical method. All data are expressed as mean \pm SD. Statistically significant differences among groups of mice were assessed by Student's *t*-test, Mann-Whitney U-tests, or Chi-square tests. If the F-value was significant, comparisons among groups of mice were further analyzed by a multiple comparison test. Analyses were performed with

SuperANOVA software (Abacus Concepts, Berkeley, CA). Statistical significance was defined as a two-tailed probability of less than 0.05.

Supporting Results

Supplementary Table S1 lists the primer sequences of the genes analyzed in the paper.

Supplementary Figure S1. Percent bile salt species in individual hepatic bile from OVX wild-type, GPR30 (-/-), and ER α (-/-) mice fed the lithogenic diet and treated with G-1 at 0 or 1 $\mu\text{g}/\text{day}$ for 8 weeks. Because the lithogenic diet contains 0.5% cholic acid, taurocholate (TC) becomes the major bile salts replacing most tauro- β -muricholate (T- β -MC) in these mice. Abbreviations: TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; T- ω -MC; tauro- ω -muricholate; TUDC, taurocholate.

Supplementary Figure S2. Effects of G-1 on expression of the genes involved in the regulation of hepatic lipid metabolism in chow-fed OVX mice. The relative mRNA levels of the genes in OVX wild-type mice receiving no G-1 and fed chow are set at 1. The data exhibit expression of the genes encoding hepatic lipid transporters and enzymes for the regulation of bile salt and cholesterol synthesis in OVX wild-type, GPR30 (-/-), and ER α (-/-) mice fed chow and treated with G-1 at 0 or 1 $\mu\text{g}/\text{day}$ for 2 weeks. * $P < 0.01$ and # $P < 0.05$, compared with OVX wild-type mice receiving no G-1 and fed chow. See text for further description and for abbreviations.

Supporting References:

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7. Wang DQ, Tazuma S, Cohen DE, Carey MC. Feeding natural hydrophilic bile acids inhibits intestinal cholesterol absorption: studies in the gallstone-susceptible mouse. *Am J Physiol Gastrointest Liver Physiol* 2003;285:G494-502.

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Supplementary Table S1. Primer and probe sequences in mRNA quantification by real-time PCR

Gene	Accession Number	Forward	Reverse	Probe
<i>Era</i>	NM_007956	5'-CCAGCAGTAACGAGAAAGGAAAC-3'	5'-TCATTGCACACGGCACAGT-3'	5'-TCATGGAGTCTGCCAAGGAGACTCGC-3'
<i>Erβ</i>	NM_010157	5'-AAGCTGGCTGACAAGGAACTG-3'	5'-CCACAAAGCCAGGGATTTTC-3'	5'-TGCACATGATTGGCTGGGCCA-3'
<i>Gpr30</i>	NM_029771	5'-ACAGCTTCCTGGGAGAGACCTT-3'	5'-CTTGAATCTGGATGGCAGCAT-3'	5'-CCTGCCGGCTCTGAACCGCTT-3'
<i>Abcg5</i>	AF312713	5'-CCTGCAGAGCGACGTTTTTC-3'	5'-GCATCGCTGTGTATCGCAAC-3'	5'-AGCAGCCTCACTGTGCGCGAGA-3'
<i>Abcg8</i>	AF324495	5'-TGGATAGTGCCTGCATGGATC-3'	5'-AATTGAATCTGCATCAGCCCC-3'	5'-CAAGCTGTCGTTCCCTCCGGTGGTG-3'
<i>Abcb4</i>	NM_008830	5'-GGAAATCATTGGTGTGGTAAGTCA-3'	5'-CACGGCCATAGCGGATATTTT-3'	5'-AGCCCGTGCTGTTCTCTACTACGATCGC-3'
<i>Abcb11</i>	NM_021022	5'-TCTGGTACGGCTCCAGACTTG-3'	5'-GCTGCTATTATGACACAGAGGAAAAT-3'	5'-AAGCGAGTACACACCAGGGACACTGATC-3'
<i>Cyp7a1</i>	NM_007824	5'-TCGTGATCCTCTGGGCATCT-3'	5'-CCAAGTGCATTAAGTGTGGGTAAA-3'	5'-AGGAGGCTCTGCGGCTCTCC-3'
<i>Cyp27a1</i>	NM_024264	5'-TGACATGGGCCCTGTACCA-3'	5'-TCCCAGGGTTATCAGCCTCTT-3'	5'-CAGAGATCCAGGAGGCCTTGAC-3'
<i>Acat2</i>	AF078751	5'-TTTGCTCTATGCCTGCTTCATC-3'	5'-GGTTCCCGGCTCATGTTG-3'	5'-TGGGCCGCCTCTGTGTCCCT-3'
<i>Hmgcr</i>	M62766	5'-ATTCTGGCAGTCAGTGGGAACT-3'	5'-CCTCGTCCTTCGATCCAATTT-3'	5'-CACCGACAAGAAGCCTGCTGCCA-3'
<i>Srebp2</i>	AF374267	5'-TGAAGCTGGCCAATCAGAAAA-3'	5'-CCACATCACTGTCCACCAGACT-3'	5'-CAAGCTCCTGAAGGGCATCGACCTG-3'
<i>Ldlr</i>	NM_010700	5'-GCTCCATAGGCTATCTGCTCTTCA-3'	5'-TGCGGTCCAGGGTCATCT-3'	5'-CAACCGCCACGAGGTCCGG-3'
<i>Sr-b1</i>	U37799	5'-TTCACGGGCGTCCAGAA-3'	5'-GATCTTGCTGAGTCCGTTCCA-3'	5'-TTCAGCAGGATCCATCTGGTGGACAA-3'
<i>Fdps</i>	BC048497	5'-CGGGCAGACTCTAGACCTCATG-3'	5'-CGATTTGTACCTCTTTTCAGTGTATCTAC-3'	5'-AGCACCCAGGGCCATGTGGA-3'
<i>β-Actin</i>	NM_007393	5'-GCTCTGGCTCCTAGCACCAT-3'	5'-GCCACCATCCACACAGAGT-3'	5'-AAGATCATTGCTCCTCCTGAGCGCAA-3'

See text for abbreviations.



