Defects in the Leptin Axis Reduce Abundance of the ABCG5-ABCG8 Sterol Transporter in Liver*§

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ABGG5 (G5) and ABCG8 (G8) are ABC half-transporters that dimerize within the endoplasmic reticulum, traffic to the cell surface, and mediate cholesterol excretion into bile. Mice harboring defects in the leptin axis (db/db and ob/ob) have reduced biliary cholesterol concentrations. Rapid weight loss brought about by administration of leptin or dietary restriction increases biliary cholesterol excretion. We hypothesized that the reduction in biliary cholesterol in mice harboring defects in the leptin axis is associated with a reduction in G5G8 transporters and that levels of the transporter would increase with leptin administration and dietary restriction. We examined mRNA and protein levels for G5 and G8 in db/db and ob/ob mice. In both models G5 and G8 protein levels were reduced. In ob/ob mice, both leptin administration and dietary restriction increased G5 and G8 protein and biliary cholesterol concentrations. Finally, we examined the effects of tauroursodeoxycholate, which has been shown to increase biliary cholesterol excretion and function as a molecular chaperone. Tauroursodeoxycholate increased G5 and G8 protein and biliary cholesterol concentrations in both wild-type and db/db mice. Our results indicate that the mechanism for reduced biliary cholesterol excretion in db/db and ob/ob mice involves reductions in G5 and G8 protein levels and that this may occur at the level of G5G8 heterodimer assembly within the endoplasmic reticulum.

ABC65 (G5) and ABC68 (G8) play a major role in the elimination of dietary and endogenously synthesized sterols in humans and mice (1–5). G5 and G8 are ATP binding cassette (ABC)2 half-transporters that dimerize in the endoplasmic reticulum (ER) before trafficking of the functional G5G8 sterol transporter to the apical surface where it promotes the excretion of sterols from hepatocytes and enterocytes (3, 6–8). In the liver, the G5G8 sterol transporter appears to be the major route for cholesterol excretion into bile. Mice deficient in G5, G8, or both transporters have 80–90% reductions in biliary cholesterol concentrations (3–5). Conversely, expression of a human transgene in mice results in a 6–8-fold increase in biliary cholesterol concentration and supersaturation of bile (7).

In the absence of dietary or pharmacological perturbations, biliary cholesterol concentrations correlate with Abcg5/Abcg8 genocopy (9). Activation of the liver X receptor (LXRα, NR1H3) by cholesterol feeding or administration of an agonist increases G5 and G8 mRNA as well as biliary cholesterol concentrations (10). In addition, these effects are absent in mice lacking LXRαβ, suggesting that LXR is the principal regulator of G5 and G8 expression in response to dietary cholesterol (11). Although biliary cholesterol excretion generally correlates with expression levels of G5 and G8 mRNAs, exceptions include increases in biliary cholesterol excretion after treatment with diosgenin or tauroursodeoxycholate (TUDCA) and in liver transplant patients after surgery (12–14). The uncoupling of biliary cholesterol excretion from G5 and G8 expression levels suggests that other pathways may contribute to biliary cholesterol excretion. However, it should be noted that the effects of both diosgenin and TUDCA were dependent on the presence of G5 and G8 (14, 15), indicating that post-transcriptional regulation of G5G8 may influence G5G8 sterol transporter abundance and activity or that the pathways which supply cholesterol to the G5G8 sterol transporter can be regulated.

Given the role of the G5G8 sterol transporter in biliary cholesterol excretion, perhaps it is not surprising that quantitative trait locus mapping studies have identified Abcg5/Abcg8 as a lithogenic locus (16–18). However, the Abcg5/Abcg8 locus is only one of more than 20 quantitative trait loci associated with increased or decreased cholesterol gallstone susceptibility, indicating that many genetic factors are involved in lithogenesis (19). Furthermore, G5G8 transgenic mice do not develop cholesterol gallstones despite supersaturation of bile (7). In humans, obesity is considered a major risk factor for cholesterol gallstone formation (20–23). Surprisingly, two mouse models of obesity, Leprd (db/db) and Lepob (ob/ob), are protected from cholesterol gallstone formation when fed lithogenic diets that contain cholesterol and cholic acid (24). Indeed, db/db and ob/ob mice have lower levels of biliary cholesterol than their wild-type counterparts (24, 25). Replacement of leptin results in rapid weight loss, reductions in apolipoprotein B (apoB)-containing lipoproteins, increases in biliary cholesterol concentrations, and restoration of cholesterol gallstone susceptibility (26, 27). Although the effects on weight loss, biliary cholesterol, and plasma cholesterol are also observed in mice that...

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2. The abbreviations used are: ABC, ATP binding cassette; G5, ABCG5; G8, ABCG8; TUDCA, tauroursodeoxycholate; STZ, streptozotocin; FPLC, fast protein liquid chromatography; ER, endoplasmic reticulum; LXR, liver X receptor; PBS, phosphate-buffered saline; RT, reverse transcription; SREBP-1, sterol regulatory element-binding protein 1.
are pair-fed to match caloric intake, the control mice are still resistant to cholesterol gallstone formation, indicating that the 2–3-fold increases in biliary cholesterol concentrations are insufficient to promote gallstone formation. The precise mechanisms responsible for leptin-dependent cholesterol gallstone formation are not clear but appear to include alterations in phospholipid excretion, gallbladder motility, and bile acid hydrophobicity among others (26–29).

The metabolic consequences of leptin deficiency that include dyslipidemia, insulin resistance, steatosis, inflammation, and others compromise liver function and may be responsible for reduced biliary cholesterol excretion. Within the liver evidence of ER stress has been reported in both diet-induced obesity and in ob/ob mice (30). Furthermore, alleviation of ER stress by administration of chemical chaperones restores insulin sensitivity in ob/ob mice (31). The assembly of the G5G8 sterol transporter occurs in the ER and is an inefficient process whereby most of the G5 and G8 monomers fail to find their respective partners and are rapidly degraded (6). Interestingly, the overwhelming majority of disease-causing mutations in either G5 or G8 that result in sitosterolemia are due to failures in the assembly and trafficking of the G5G8 sterol transporter (32). The assembly of the complex is dependent upon N-linked glycans that interact with the ER chaperone calnexin (32). Under conditions of ER stress, it is plausible that the assembly of the G5G8 sterol transporter is compromised. We hypothesized that the reduction in biliary cholesterol in mice harboring defects in the leptin axis is associated with a reduction in G5G8 transporters. We examined mRNA and protein levels for each half-transporter in db/db and ob/ob mice. We also examined the effects of leptin administration, dietary restriction, and administration of the chemical chaperone TUDCA. Our findings indicate that G5 and G8 protein levels are reduced in db/db and ob/db mice compared with controls in the absence of changes in mRNA levels. In addition, both calorie restriction and TUDCA restore G5G8 protein levels and biliary cholesterol concentrations. Our results indicate that the mechanism for reduced biliary cholesterol excretion in db/db and ob/ob mice involves destabilization of the G5G8 transporter and suggests this effect may occur at the level of G5G8 heterodimer assembly within the ER.

**EXPERIMENTAL PROCEDURES**

**Reagents and Buffers**—General chemical reagents were obtained from Sigma. Recombinant murine leptin was obtained from Biomyx Technology (San Diego, CA). Protein sample buffer was 30 mM Tris base, 10 mM EDTA, pH 6.8, 3% SDS, 20% glycerol, 0.00625% bromophenol blue. Membrane buffer was 250 mM sucrose, 2 mM MgCl₂, 20 mM, pH 7.5. Buffer A was 20 mM Tris, pH 7.6, 137 mM NaCl, 0.2% Tween 20, 5% milk. Buffer B was 20 mM Tris, pH 7.6, 137 mM NaCl, 0.2% Tween 20. Calnexin and GRP78 were purchased from Nventa (San Diego, CA). Secondary antibodies and enhanced chemiluminescence reagents were purchased from Pierce. Rabbit anti-ABCG5 and mouse anti-ABCG8 antibodies have been previously reported (7, 8).

**Animals and Treatments**—In the first experiment male and female db/db mice on the C57BL/6J background were used between 8 and 12 weeks of age. All mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in a temperature-controlled room with 12:12 light:dark cycle (6:00 a.m. to 6:00 p.m.). Upon arrival mice were allowed to adapt to the environment for 1 week. All mice were maintained on standard rodent chow (Harlan Teklad 2014S). Analysis of the data from the first experiment indicated no differential effects due to sex. In all subsequent experiments male mice were used. Tissues from type 1 diabetic mice were generously provided by Dr. Ming Gong (University of Kentucky). STZ treatment was conducted as previously described (33). ob/ob mice were injected once daily with equal volumes of saline or leptin (10 µg/g body weight, intraperitoneally) for 7 days as previously described (27). To achieve isocaloric intake, mice treated with saline were pair-fed with mice treated with leptin (27). Mice treated with TUDCA received twice-daily injections (intraperitoneally, 7:00 a.m. and 7:00 p.m.) of 250 mg/kg (500 mg/kg/day) as previously described (31).

Blood glucose levels were measured using a standard glucometer from a drop of blood obtained by tail-vein prick. Control and streptozotocin (STZ) mice were killed by exsanguination under ketamine xylazine anesthesia. All others were euthanized with CO₂ after a 4-h fast beginning shortly after “lights on.” Blood was collected from the right ventricle with a 1-cc syringe fitted with a 20-gauge hypodermic needle. Gall bladder bile was collected with an insulin syringe fitted with a 26-gauge needle. Bile samples were stored at −20 °C until analysis. Serum was separated by centrifugation and stored at 4 °C for fast protein liquid chromatography (FPLC) fractionation. Livers and other tissues were excised, rinsed with PBS to remove blood, and snap-frozen in liquid nitrogen. Tissue samples were stored at −80 °C until analyses.

**Lipid Analyses**—Serum was fractionated by FPLC using Superox 6 HR10/30 column to separate lipoprotein fractions. Serum cholesterol concentrations and cholesterol content in FPLC fractions were determined by colorimetric-enzymatic assays (Wako Chemicals, Richmond, VA). The concentration of cholesterol in gallbladder bile was measured as previously described by gas chromatography-mass spectroscopy (34).

**Preparation of Membrane Proteins**—A total of 100–200 mg of liver or intestine (duodenum, jejunum, and ileum) was homogenized by a Polytron homogenizer in 1.2 ml of membrane buffer containing protease inhibitors (Roche Diagnostics). The crude preparation was centrifuged at 2000 × g for 10 min at 4 °C. The supernatant was collected and centrifuged at 100,000 × g for 45 min at 4 °C. The membrane pellet was suspended in protein sample buffer. Protein concentrations were determined by BCA assay (Pierce).

**SDS-PAGE and Immunoblot Analysis**—Protein sample buffer was added to samples to achieve uniform concentrations, β-mercaptoethanol was added to a final concentration of 1.2% (v/v), and samples were heated to 95 °C for 5 min. Proteins were size-fractionated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated in buffer A for 30 min at 22 °C. Primary antibodies were diluted in buffer A and incubated with membranes for 60 min at 22 °C. Membranes were washed 3 times for 5 min in buffer B.
Horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Pierce) were diluted (1:20,000) and incubated with membranes for 30 min at 22 °C. Membranes were washed 3 times for 5 min in buffer B and visualized by enhanced chemiluminescence (Pierce). Protein loading was assessed by visual inspection of Ponceau S-stained membranes and by re-blotting with GRP78 and calnexin. Expression levels were semi-quantified by densitometry. Radiography films were scanned as .tiff images, and sum intensities were quantified using Eastman Kodak Co. Molecular Imaging software (Version 4.0). Film background was subtracted from all bands of interest. Expression levels for G5 and G8 were normalized to signals for GRP-78 obtained by re-blotting of stripped membranes.

Quantitative Real-time PCR—Total RNA was extracted from tissues using RNeasy mini kit (Qiagen, Valencia, CA). Samples of total RNA (1.0 μg) from mouse livers were transcribed using SuperScript™ First Strand Synthesis system (Invitrogen) using random hexamers in a final total reaction volume 20 μl. Primers (Table 1) have been previously described (35) but were validated to ensure amplification of single transcripts and covariation of amplification efficiencies with the invariant control (cyclophilin) according to the manufacturer’s instructions (Applied Biosystems, Guide and User Bulletin 2). Quantitative RT-PCR was performed on an Applied Biosystems 7700 sequence detection system. Standard reaction volume was 30 μl containing 1× SYBR Green PCR master mix (Applied Biosystems), 1 μl of cDNA template, and 150 nm concentrations of each oligonucleotide. Initial steps of RT-PCR were 10 min at 95 °C. Cycles (n = 40) consisted of a 15-s melt at 95 °C followed by a 1-min annealing/extension at 60 °C. All reactions were performed in triplicate. Means of the differences in threshold cycle (Ct) values from cyclophilin and their S.D. were calculated for each treatment group (ΔCt). The relative abundance of each transcript within treatment groups was determined by subtracting the control group mean difference from the ΔCt means. Two bands are routinely observed for both G5 and G8. For the purpose of presentation, equal amounts of membrane protein from each animal were pooled against G5 and G8. For the purpose of presentation, equal amounts of membrane protein from each animal were pooled against G5 and G8. For the purpose of presentation, equal amounts of membrane protein from each animal were pooled against G5 and G8.

RESULTS

Steady-state levels of immunoreactive G5 and G8 were evaluated in livers of male and female db/db mice and their heterozygous controls (Fig. 1). db/db mice were significantly heavier (48 ± 5 versus 28 ± 3 g) and had elevated fasting glucose levels (185 ± 18 versus >475 mg/dl; four had values in excess of 500 mg/dl, the limit of the glucometer). Membrane proteins were prepared from livers of obese and control male and female mice (n = 10 per genotype, 5 per sex) and subjected to SDS-PAGE and immunoblotting using antibodies directed against G5 and G8. For the purpose of presentation, equal amounts of membrane protein from each animal were pooled (Fig. 1A). Two bands are routinely observed for both G5 and G8. The higher molecular weight, diffuse bands correspond to the post-Golgi forms of each protein, whereas the lower molecular weight bands correspond to the reticular forms that have yet to heterodimerize and traffic to the cell surface. It should be noted that for G5, the lower molecular weight band present in the G5 immunoblot contains both specific and nonspecific signals as discussed under “Results.” Membrane preparations were also blotted for Bip and G2 as controls for equal protein loading and general effects on protein processing. Con, control. B, each sample was analyzed individually, and the relative abundance of proteins was determined by densitometry. The signal intensities for G5 and G8 were divided by the values obtained for Bip. Error bars represent the S.D. for ratios of G5 or G8 to Bip.

Error bars represent the S.D. of the differences among means for control and groups of db/db mice. C, the relative abundance of G5 and G8 mRNAs were quantified by RT-PCR. Error bars represent the S.D. of the differences among means for control and groups of db/db mice. D, cholesteryl concentrations in gall-bladder bile. Error bars represent the S.D.
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were observed for calreticulin, there appeared to be a modest reduction in the abundance of calnexin.

We also evaluated the expression of two additional ABC transporters, ABCG2 (G2) and ABCA1 (A1). ABCG2 is the most closely related member of the G-subfamily to G5 and G8. Like G5 and G8, it is a glycoprotein that forms a dimer within the ER before the transport of the functional complex to the apical surface where it mediates excretion of dietary compounds into the biliary space (36, 37). However, it is not involved in hepatobiliary transport of cholesterol and does not interact with either G5 or G8 (8, 38). The expression of G2 appears to be greater in male control mice than in the remaining three groups, but there were no differences between female control and db/db mice, indicating that unlike reductions in G5G8, differences in G2 could not be independently attributed to the presence of the db mutation. Like G5 and G8, ABCA1 (A1) expression is regulated by LXR. We evaluated the expression of A1 by immunoblotting (S1). In both male and female mice, A1 levels were reduced when compared with lean controls.

Densitometric analysis of signal intensities for G5 and G8 was conducted for each mouse as described under “Experimental Procedures.” The data are expressed as the ratio of signal intensities for G5 and G8 to Bip (Fig. 1B). The effect of genotype was significant for both G5 and G8 (p < 0.01). However, there was no effect of sex or a genotype by sex interaction. In both male and female mice, levels of G5 and G8 were reduced between 50 and 60% in db/db mice compared with controls. G5 and G8 protein levels were also determined in three segments of the small intestine. Although there was a tendency for reduced protein levels in each segment, none reached statistical significance (not shown).

To determine whether the reduction in G5 and G8 protein was associated with a reduction in their respective mRNAs, total RNA was isolated and analyzed by quantitative RT-PCR (Fig. 1C). The analysis indicates that there was no change in G5 or G8 message levels. By way of control, we also evaluated mRNA levels for genes known to be elevated in mice harboring defects in the leptin axis. Both peroxisome proliferator-activated receptor-γ and SREBP-1c mRNA levels were increased by ~3-fold in the livers of db/db mice compared with controls (not shown). There was no difference in Cyp7A1 mRNA, but it should be noted that variability within groups was high for this transcript. The data show that the reduction in G5 and G8 protein occurred in the absence of changes in mRNA for each half-transporter, indicating that differences in G5 and G8 protein levels were not associated with a decrease in transcription.

To determine whether the reduction in G5 and G8 protein correlated with reduced biliary cholesterol concentrations, gall bladder bile was analyzed by gas chromatography-mass spectroscopy (Fig. 1D). Bile was collected from control and db/db mice after a 4-h fast beginning shortly after lights on. Cholesterol concentrations were reduced by 45% in db/db mice compared with controls (2.2 ± 0.53 versus 0.9 ± 0.56 mM). Phospholipid concentrations were measured in pooled bile of C57 and db/db mice. Phospholipid concentrations were slightly elevated in db/db mice compared with controls (14.0 versus 17.7 mM) as has been reported previously (24).

Like db/db mice, ob/ob mice harbor a deficiency in the leptin axis and are hyperphagic and obese. However, the defect in the leptin axis can be overcome by daily injections of recombinant leptin. For these experiments we used a model of leptin replacement that has been shown to induce weight loss and restore plasma lipoprotein profiles and biliary cholesterol concentrations to wild-type levels (26, 27). Four groups of mice (n = 3 per group) were compared. Wild-type control and ob/ob control mice were not treated. A second group of ob/ob mice received daily injections of recombinant leptin (10 μg/g/day) for each of 7 days. Because of profound effect of leptin on food intake in ob/ob mice, a third group of ob/ob mice was treated with daily injections of the carrier (saline) for 7 days. Mice assigned to the saline group were pair-fed to mice receiving daily injections of leptin to achieve isocaloric intake and similar degrees of weight loss. Body weight was monitored daily in the ob/ob mice assigned to the leptin-treated and their pair-fed controls (Fig. 2A). Body weight declined to a similar extent over the 7-day period in these two groups of ob/ob mice, indicating that caloric intake was similar in the two groups.

Following a 4-h fast after the last injection on day 7, mice were killed, and tissues were harvested for analysis. Serum cholesterol concentrations were greater in untreated ob/ob than in wild-type mice (Fig. 2B, p < 0.05). Leptin treatment reduced serum cholesterol concentrations in ob/ob mice (p < 0.05) to levels similar to those observed in wild-type mice. There was also a tendency for reduced serum cholesterol in pair-fed mice, but it failed to reach statistical significance. FPLC analysis of pooled plasma indicates that the reduction in serum cholesterol concentrations in leptin-treated and pair-fed controls was confined to the low density lipoprotein-containing fractions (13–18) and was slightly offset by modest increases in the high density lipoprotein-containing fractions (18–22, Fig. 2C).

Immunoblot analysis of liver membrane preparations from wild-type and ob/ob mice confirmed results in db/db mice that indicate a reduction in G5 and G8 protein in mice harboring defects in the leptin axis (Fig. 3A, densitometry not shown). Administration of leptin increased G5 and G8 protein in ob/ob mice to levels greater than those observed in wild-type mice. Immunoreactive G5 and G8 levels were restored in ob/ob mice that were pair-fed to leptin-treated mice. This result indicates that dietary restriction alone can restore G5 and G8 protein levels in ob/ob mice and that the reductions in immunoreactive G5 and G8 in ob/ob mice are not a direct effect of leptin signaling but, rather, a secondary effect of leptin deficiency. Immunoreactive levels of G2 were constant across all groups, indicating that global changes in protein synthesis and processing are insufficient to explain the reduction of G5 and G8 in ob/ob mice and their stabilization in leptin-treated and pair-fed mice. In addition, no differences were appreciated among treatment groups for calnexin, calreticulin, or A1 (S2). Although it is plausible that this difference could be due to differences between the two genotypes, it should be noted that the db/db mice were substantially older and had higher body weights and fasting glucose levels than the ob/ob mice.

A one-way analysis of variance indicated differences among treatment groups for biliary cholesterol concentrations. The reduction in immunoreactive G5 and G8 in untreated ob/ob
mice relative to wild-type mice correlated with a reduction in biliary cholesterol concentration (Fig. 3B). Conversely, the restoration of G5 and G8 either by leptin-treatment or calorie restriction in their pair-fed controls increased biliary cholesterol concentrations. However, the additional increase in G5 and G8 protein levels after leptin treatment was not associated with a further increase in biliary cholesterol concentration. As with db/db mice, ob/ob mice had slightly elevated biliary phospholipid concentrations (15.5 versus 16.7 mM). Although leptin treatment decreased biliary phospholipid concentrations, they remained constant in pair-fed controls (13.3 versus 16.6 mM).

Finally, we evaluated the levels of G5 and G8 mRNA (Fig. 3C). As in db/db mice, there was no reduction in mRNA levels for either G5 or G8 in ob/ob mice compared with wild-type controls, indicating that the reduction in G5G8 occurred post-transcriptionally. If anything, there is a modest increase, although these differences were not significant. We also evaluated the expression of LXRα, SREBP-1C, and Cyp7A1. The data suggest that there is a modest, but consistent increase in LXRα,
SREBP-1c, and G5 and G8 mRNAs in ob/ob mice when compared with wild-type controls regardless of treatment but no change in Cyp7A1. When compared with untreated ob/ob mice, neither leptin treatment nor calorie restriction resulted in a significant increase in message levels for either G5 or G8. In conclusion, G5 and G8 protein levels are reduced in ob/ob mice and can be restored with either leptin replacement or dietary restriction, both of which increase biliary cholesterol content. However, changes in G5 and G8 protein levels and biliary cholesterol concentrations do not correlate with changes in mRNA levels for either transcript.

Both db/db and ob/ob mice are obese, insulin-resistant, and routinely studied as a model of type 2 diabetes. Leptin treatment and dietary restriction are associated with increased insulin sensitivity. To determine whether a lack of insulin signaling in the absence of obesity could explain the post-transcriptional reductions in G5G8, we evaluated levels of G5 and G8 in a mouse model of type I diabetes. Livers from control and STZ-treated mice were generously provided by Dr. Ming Gong (University of Kentucky). Membranes were prepared from livers of male control and STZ-treated male mice (n = 3/group) were analyzed by SDS-PAGE (50 μg/lane) and immunoblotting. Shown are body weights (B) and blood glucose levels (C) at the termination of the experiment. The asterisk denotes nonspecific band observed using the antibody directed against mouse G5. Error bars represent the S.D. The dagger (†) denotes significant differences at p < 0.05.

G5G8 heterodimer is formed in the ER in a glycan-dependent manner that involves calnexin, we hypothesized that TUDCA might increase biliary cholesterol excretion by stabilizing the G5G8 heterodimer in mice with defects in the leptin axis. To test this hypothesis we treated wild-type and db/db mice with either PBS (control) or TUDCA.

Body weight and plasma glucose concentrations were analyzed using two-way repeated measures analysis of variance. For body weight, there were no differences between weights on day 1 and day 10 within any of the treatment groups (Fig. 5A). For blood glucose, the treatment by time interaction tended to be significant (p = 0.07). A post hoc analysis indicated that plasma glucose levels were lower on day 10 than on day 1 in db/db mice treated with TUDCA but not in db/db control mice (Fig. 5B). However, it should be noted that recorded plasma glucose levels declined slightly in PBS-treated mice, elevating the p value for the treatment by time interaction. Plasma cholesterol data were analyzed using a two way analysis of variance comparing genotype, treatment with TUDCA, and their interaction (Fig. 5C). Although genotype was associated with a modest, but significant increase in plasma cholesterol levels (p < 0.05), significant differences were not detected for treatment or the treatment by genotype interaction. FPLC fractionation of pooled serum confirmed that the elevations in serum cholesterol concentrations were largely due to increases in low density lipoprotein-containing fractions, although high density lipoprotein-containing fractions also had increased cholesterol content (Fig. 5D).

Immunoblot analysis indicates that TUDCA increased G5 and G8 protein levels in both wild-type and db/db mice (Fig. 6A). Consistent with previous observations, biliary cholesterol concentrations were lower in control, db/db mice compared with wild-type controls (Fig. 6B). Interestingly, the expression of G2 appears to be modestly reduced in db/db mice when compared with controls, an observation that is consistent with...
that observed in Fig. 1. However, levels of G2 were not affected in either wild-type or db/db mice by treatment with TUDCA, indicating that the effects of TUDCA show some degree of specificity. Calnexin, calreticulin, and A1 were neither affected in either wild-type or db/db mice by treatment with TUDCA, as expected, db/db mice have elevated mRNAs encoding SREBP-1c, and Cyp7A1 as determined by RT-PCR. A recent report indicates that expression of calreticulin can facilitate the trafficking of G5G8 transporters to the cell surface, suggesting that chaperone activity can be both limiting and overcome experimentally (46). We evaluated the expression of calnexin and calreticulin in our experiments. In the first experiment, calnexin levels were modestly reduced. However, in subsequent experiments this finding was not reproducible. There are significant differences between the db/db mice used in the experiment presented in Fig. 1 and the remainder of the studies that may account for the differences in calnexin expression. First, the mice are on the KS strain of C57Bl/6 mice. Second, they were considerably older and had greater fasting glucose levels. Regardless of these differences, reductions in G5G8 abundance and biliary cholesterol concentrations are consistent across all experiments, indicating that reduced calnexin

that observed in Fig. 1. However, levels of G2 were not affected in either wild-type or db/db mice by treatment with TUDCA, indicating that the effects of TUDCA show some degree of specificity. Calnexin, calreticulin, and A1 were neither affected by genotype nor treatment with TUDCA (S3).

TUDCA treatment was associated with increased biliary cholesterol concentrations regardless of genotype \((p < 0.05)\); however, the genotype by treatment interaction was not significant, indicating that TUDCA increased biliary cholesterol concentrations irrespective of genotype. Conversely, TUDCA administration only slightly increased biliary phospholipids in db/db mice \((17.2 \text{ versus } 18.0)\) but had no effect in wild-type mice in this twice-daily injection protocol.

Last, we evaluated mRNAs for G5, G8, LXRα, SREBP-1C, and Cyp7A1. Consistent with earlier studies, there were no changes in G5 and G8 mRNAs between wild-type and db/db mice nor were there differences in LXRα or Cyp7A1. As expected, db/db mice have elevated mRNAs encoding SREBP-1c; treatment with TUDCA suppressed Cyp7A1 mRNA in both genotypes. In conclusion, TUDCA increased G5 and G8 protein levels and biliary cholesterol concentrations in both wild-type and db/db mice independently of changes in mRNA levels for either protein.

**DISCUSSION**

The major findings of this study are that defects in the leptin axis are associated with reductions in the levels of immunoreactive G5G8 sterol transporter. These reductions are not associated with decreased levels of mRNAs encoding either subunit. The reduction in G5G8 sterol transporter levels can be restored with caloric restriction and further enhanced by leptin replacement. Similarly, administration of TUDCA can restore immunoreactive G5 and G8 in db/db mice but also increases G5G8 in wild-type mice. For caloric restriction, leptin replacement, and TUDCA treatment, the increases in G5G8 abundance are associated with increased biliary cholesterol concentrations. To the best of our knowledge this is the first report that establishes a role for post-transcriptional regulation of the G5G8 sterol transporter.

Beyond the absence of a functional leptin axis, the molecular mechanism for the post-transcriptional reduction in G5G8 transporter levels in db/db and ob/ob mice is not known. A number of possibilities exist and include a decrease in the rate of translation, assembly of the complex within the ER, and stability of the post-Golgi complex. Studies in cell culture indicate that the bulk of G5 and G8 monomers fail to find their respective partners and are rapidly degraded (6). This may also be true in vivo, since the reticular forms of both G5 and G8 have half-lives of approximately 2 h and are generally visible in immunoblots of hepatic and intestinal membrane preparations. This property would not be unique to G5G8 since as much as 80% of all of the cystic fibrosis membrane conductance regulator (ABCC7) fails to reach the cell surface and is degraded (40, 41). Indeed, much like cystic fibrosis, sitosterolemia is a disease that is generally due to failures in protein trafficking (32, 42). The use of chemical chaperones to rescue misfolded cystic fibrosis conductance regulator mutants that otherwise retain activity is currently under investigation for the treatment of cystic fibrosis (43–45). This possibility has not been explored for sitosterolemia. A recent report indicates that expression of calreticulin can facilitate the trafficking of G5G8 transporters to the cell surface, suggesting that chaperone activity can be both limiting and overcome experimentally (46). We evaluated the expression of calnexin and calreticulin in our experiments. In the first experiment, calnexin levels were modestly reduced. However, in subsequent experiments this finding was not reproducible. There are significant differences between the db/db mice used in the experiment presented in Fig. 1 and the remainder of the studies that may account for the differences in calnexin expression. First, the mice are on the KS strain of C57Bl/6 mice. Second, they were considerably older and had greater fasting glucose levels. Regardless of these differences, reductions in G5G8 abundance and biliary cholesterol concentrations are consistent across all experiments, indicating that reduced calnexin

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FIGURE 6. Expression of G5 and G8 and biliary cholesterol concentrations in control and TUDCA-treated wild-type and db/db mice. A, membrane preparations from livers of male untreated (PBS) and TUDCA-treated wild-type and db/db mice were analyzed by SDS-PAGE (50 μg/lane) and immunoblotting. B, cholesterol concentrations in gall-bladder bile. Error bars represent the S.D. C, the relative expression of mRNAs encoding G5, G8, LXRα, SREBP-1C, and Cyp7A1 as determined by RT-PCR. Error bars represent the S.D. of the differences among means for wild-type (wt) control and remaining groups of mice. The asterisk denotes nonspecific band observed using the antibody directed against mouse G5.
expression in older db/db mice is insufficient to explain the reduction in G5G8 abundance and activity.

Although diabetes in the absence of leptin deficiency failed to reduce G5G8 transporter levels, calorie restriction in ob/ob mice largely restored levels of the complex. This effect is presumably metabolic in nature and may be due to a reduction in hepatic ER stress. A recent study indicates that alleviation of ER stress is sufficient to correct hyperglycemia in ob/ob mice (31). In the present study we treated db/db mice with TUDCA using the same treatment paradigm previously reported. We did see a reduction in plasma glucose levels in db/db mice when comparing day 1 to day 10 of treatment in post hoc analysis. In addition, we saw an increase in G5G8 transporters compared with control, db/db mice, indicating that TUDCA treatment was sufficient to correct the effect of leptin deficiency on G5G8 levels. Interestingly, the effect of TUDCA was also observed in wild-type mice. This observation suggests that chaperone activity may be limiting in wild-type mice and supports in vitro data suggesting that a significant number of G5 and G8 monomers fail to reach their final destination. However, the interpretation of the effect of TUDCA on G5G8 is limited by the fact that the mechanism by which TUDCA acts as a molecular chaperone is not known, and its ability to suppress Cyp7A1, presumably via farnesoid X-activated receptor, indicates that the molecule elicits responses beyond its effects as a molecular chaperone. Furthermore, TUDCA has been shown to acutely increase biliary cholesterol secretion when infused into mice (47). It is unlikely that this effect involved alterations in the assembly of G5G8 transporters via chaperone activity, although these experiments cannot preclude this possibility. Furthermore, feeding cholate to mice has been shown to increase both G5G8 abundance and mRNA, an effect lost in farnesoid X-activated receptor-deficient mice (9). A careful study of the effects of individual bile acids on complex synthesis, assembly, and transport will be required to elucidate the mechanisms by which they influence activity of the G5G8 transporter.

Beyond the ER, the regulation of the G5G8 sterol transporter is essentially unstudied. Recent progress has been made using purified G5G8 complexes and demonstrates that sterols are indeed substrates for the G5G8 transporter (48, 49). However, this approach does not address the possibility of post-translational regulation of G5G8 transporter mass observed in the present study. There is precedent for this type of regulation of ABC transporters. ABCA1 contains a PEST domain that mediates the degradation of the protein unless stabilized by binding to apoA-I (50). Conversely, polyunsaturated fatty acids destabilize ABCA1 through a distinct mechanism (51). The primary sequence of G8 contains a PEST domain, but there has been no report of regulated degradation of the G5G8 transporter by this or any other mechanism.

The effect of leptin on G5G8 is not entirely explained by caloric restriction and weight loss. The dose of leptin used in the present study increased G5G8 to levels greater than those observed in wild-type mice. However, this increase was not associated with a further increase in biliary cholesterol concentrations. This observation is largely consistent with those of Cohen and co-workers (26, 27), which show that whereas leptin infusion increases biliary cholesterol concentrations relative to untreated ob/ob mice, pair-feeding mice to match caloric intake with those receiving leptin also increases biliary cholesterol secretion. In these studies, biliary cholesterol concentrations in pair-fed mice were greater than those observed in leptin-treated mice evaluated after 14 days of leptin replacement on control or lithogenic diets. The uncoupling of G5G8 protein levels with biliary cholesterol concentrations suggests that G5G8 transporter activity is regulated beyond abundance of mRNA and protein. These may include pathways that supply cholesterol to the transporter, alter the subcellular localization of G5G8, or directly affect transport activity within the canalicular membrane. Although such mechanisms have yet to be reported, the present study cannot preclude their involvement. Therefore, our conclusions are limited to correlating the abundance of G5G8 with the activity of the transporter in vivo. Whether or not such mechanisms regulate G5G8 awaits investigation.

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