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GRP78 rescues the ABCG5 ABCG8 sterol transporter in db/db mice

Yuhuan Wang[a], **Kai Su**[a], **Nadezhda S. Sabeva**[d], **Ailing Ji**[c], **Deneys R. van der Westhuyzen**[b,c], **Fabienne Foufelle**[e], **Xia Gao**[f], and **Gregory A. Graf**[a,b]

[a]Department of Pharmaceutical Sciences, University of Kentucky, Lexington, KY, USA

[b]Graduate Center for Nutritional Sciences, Saha Cardiovascular Research Center and Barnstable Brown Kentucky Diabetes and Obesity Center, Lexington, KY, USA

[c]Departments of Internal Medicine and Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY, USA

[d]Department of Neuroscience, Universidad Central del Caribe, Bayamón, PR, USA

[e]INSERM, UMR-S 872, Centre de recherches des Cordeliers, 15 rue de L'école de Médecine, Paris, France

[f]Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada

Abstract

Objective—Mice lacking leptin (ob/ob) or its receptor (db/db) are obese, insulin resistant, and have reduced levels of biliary cholesterol due, in part, to reduced levels of hepatic G5G8. Chronic leptin replacement restores G5G8 abundance and increases biliary cholesterol concentrations, but the molecular mechanisms responsible for G5G8 regulation remain unclear. In the current study, we used a series of mouse models to address potential mechanisms for leptin-mediated regulation of G5G8.

Methods and Results—We acutely replaced leptin in ob/ob mice and deleted hepatic leptin receptors in lean mice. Neither manipulation altered G5G8 abundance or biliary cholesterol. Similarly, hepatic vagotomy had no effect on G5G8. Alternatively, G5G8 may be decreased in

Address Correspondence To: Gregory A. Graf, Pharmaceutical Sciences, University of Kentucky, Room 345 Biopharmaceutical Complex, 789 South Limestone Street, Lexington, KY, 40536, Tel. 859-257-4749, Fax 859-257-7564, Gregory.Graf@uky.edu.

Author contributions

YW designed experiments, performed experiments, interpreted data, and wrote the manuscript. KS and NS designed experiments, performed experiments, and interpreted data. AJ and XG performed some experiments. FF and DvdW provided some experimental materials and conceived the idea of the study. GG conceived the idea of the study, designed experiments, supervised its completion, wrote and edited the final manuscript. All authors contributed in writing and critical revision of the manuscript have approved its final version.

Conflict of Interest

The authors have no conflicts of interest to disclose.

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ob/ob and db/db mice due to ER dysfunction, the site of G5G8 complex assembly. Overexpression of the ER chaperone GRP78 using an adenoviral vector restores ER function and reduces steatosis in ob/ob mice. Therefore, we determined if AdGRP78 could rescue G5G8 in db/db mice. As in ob/ob mice, AdGRP78 reduced expression of lipogenic genes and plasma triglycerides in the db/db strain. Both G5 and G8 protein levels increased as did total biliary cholesterol, but in the absence of changes in G5 or G8 mRNAs. The increase in G5G8 was associated with increases in a number of proteins, including the ER lectin chaperone, calnexin, a key regulator of G5G8 complex assembly.

Conclusions—Leptin signaling does not directly regulate G5G8 abundance. The loss of G5G8 in mice harboring defects in the leptin axis is likely associated with compromised ER function.

Keywords

Chaperones; cholesterol; leptin; lipids and lipoprotein metabolism; obesity

1. Introduction

The *ABCG5 ABCG8* locus encodes a pair of ABC half transporters that form a G5G8 complex that promotes the secretion of cholesterol into bile and opposes the absorption of dietary sterols in the small intestine [1]. Mutations in either *ABCG5* or *ABCG8* cause Sitosterolemia (OMIM, #210250), a recessive monogenic disorder characterized by elevated plasma cholesterol and plant sterols, tendon and tuberous xanthomas, and accelerated atherosclerosis [1]. G5G8 deficiency also results in reduced cholesterol elimination, exacerbated hepatic insulin resistance, and the development of nonalcoholic fatty liver disease (NAFLD) in a mouse model of diet-induced obesity [2]. Conversely, accelerated biliary cholesterol secretion through G5G8 overexpression improves glycemic control and hepatic insulin signaling in db/db mice [3]. In LDL receptor deficient mice, expression of a G5G8 transgene reduces atherosclerosis, suggesting that therapeutics that accelerate G5G8 activity may be beneficial in the prevention and treatment of cardiovascular disease [4, 5].

G5G8 heterodimers are formed in the endoplasmic reticulum (ER) in an N-linked glycan dependent manner facilitated by the lectin chaperones calnexin and calreticulin [6, 7]. Overexpression of calreticulin increases the abundance of the G5G8 complex at the cell surface, indicating that protein folding and G5G8 complex formation is a limiting factor that determines G5G8 abundance and activity [6]. G5G8 complex formation and trafficking to the cell surface requires simultaneous expression of both G5 and G8 [8, 9], which is accomplished by a common promoter containing response elements for a number of transcription factors including liver X receptors (LXR) α and β, hepatocyte nuclear receptor 4α (HNF4α), GATA transcription factors, orphan nuclear receptor liver receptor homolog-1 (LRH-1), thyroid hormone receptor, and forkhead box O1A (FOXO1) [10–14]. The upregulation of G5G8 by FOXO1 is clinically significant because it mechanistically ties an increased risk for cholesterol gallstones to a hepatic insulin resistance [15]. Additionally, quantitative trait locus mapping has identified *Abcg5Abcg8* as a lithogenic locus in mice, and polymorphisms in both *ABCG5* and *ABCG8* have been associated with increased risk of cholesterol gallstones in humans [16].

Mice lacking leptin (ob/ob) or its receptor (db/db) are obese and insulin resistant, but are paradoxically resistant to the formation of cholesterol gallstones when challenged with a lithogenic diet [17]. Multiple mechanisms appear to contribute to this phenotype, including a downregulation of hepatic G5G8 and a reduction in biliary cholesterol [18–24]. Leptin replacement in ob/ob mice increases hepatic G5G8 and cholesterol concentrations in gallbladder bile, suggesting that leptin may directly regulate G5G8 abundance and activity [22, 24]. However, in the present study, leptin administration in ob/ob mice failed to acutely increase hepatic G5G8. In addition, hepatic branch vagotomy failed to alter G5G8 in obese mice, indicating that centrally acting leptin was not a direct regulator of G5G8 abundance. Likewise, deletion of hepatic leptin receptors had no effect on G5G8.

Caloric restriction can partially rescue G5G8 and biliary cholesterol concentrations in db/db mice, suggesting that other mechanisms secondary to obesity may destabilize the G5G8 complex in mice that lack a functional leptin axis [24, 25]. Markers of ER stress are elevated in the liver of ob/ob mice and are associated with the development of hepatic insulin resistance and fatty liver disease [26, 27]. Alleviation of ER stress through the chemical chaperones 4-phenyl butyrate and tauroursodeoxycholic acid (TUDCA) restores insulin signaling and glycemic control [26]. We previously reported that TUDCA increases G5G8 in db/db mice; however, it has a virtually identical effect in lean C57Bl mice in the absence of ER stress [24]. Furthermore, TUDCA stimulates bile flow and increase biliary cholesterol secretion in lean mice presenting no ER stress, suggesting its effects on G5G8 may be independent of chaperone function [28, 29]. Indeed, TUDCA has a number of effects beyond chaperone functions, including opposing mitochondrial depolarization, caspase activation, and apoptosis [30–32]. Therefore, whether the reduction of hepatic G5G8 in ob/ob and db/db mice is a consequence of ER dysfunction remains unclear.

GRP78 is an ER chaperone and component of unfolded protein response (UPR). In the face of ER stress, induction of GRP78 plays an essential role in promoting protein folding and assembly, targeting aberrant protein for degradation, and increasing the folding capacity of ER. Hepatic ER stress also contributes to increased lipogenesis and steatosis by promoting the processing of sterol regulatory element binding proteins (SREBPs) [33]. SREBPs are among a family of ER membrane proteins that traffic to the Golgi in response to metabolic signals and are proteolytically processed to release their respective transcription factor domains [34]. Exogenous expression of GRP78 by adenoviral administration reduces lipogenesis and steatosis by preventing the unregulated trafficking and activation of SREBP1-c [33]. Since ER folding capacity is a limiting factor in G5G8 abundance, we tested the hypothesis that adenovirus encoding GRP78 (AdGRP78) would restore G5G8 in db/db mice. As predicted, G5G8 abundance and biliary cholesterol increased following expression of AdGRP78. These results reveal a role for ER stress as a mechanism for reduced G5G8 in mice lacking a functional leptin axis.

2. Materials and Methods

2.1. Ethics statement

All animal procedures conform to Public Health Service policies for humane care and use of laboratory animals and were approved by the institutional animal care and use committee at the University of Kentucky (Animal protocol 2007-0172).

2.2. Chemicals, reagents and antibodies

General chemicals were purchased from Sigma, immunoblotting reagents from Pierce, real-time PCR reagents from Applied Biosystems, mouse recombinant leptin from Biomyx Technology (San Diego, CA), and mouse leptin ELISA from EMD Millipore. Calnexin and GRP78 antibodies were purchased from Nventa (San Diego, CA). The α-tubulin and β-actin antibodies were purchased from Cell Signaling and Sigma, respectively. Anti-SR-BI was purchased from Novus. Anti-ABCA1 antibody was a generous gift from Manson Freeman (Harvard Medical School, Boston, MA). Anti-calreticulin antibody was purchased at Stressgen Bioreagents Corp. Total and phospho-protein kinase-like endoplasmic reticulum kinase (PERK) antibodies were purchased from Cell Signaling Technology. Experiments presented in Figure 1 were conducted with previously described antibodies directed against G5 and G8 [8, 9]. Stocks of the rabbit polyclonal antibody directed against G5 have become limited and suitable commercially available sources have not been identified. Therefore, we contracted ProSci Inc. (Poway, CA; NIH/OLAW assurance #A4550-01) to synthesize a peptide from the N-terminus of the rat ortholog of G5 (MGELPFLSPEGARGPHINRGSLSSLEC) for antibody development in chickens. Antibodies were affinity purified and tested for suitability in western blotting and immunofluorescence microscopy applications in mouse and rat livers. The chicken anti-G5 polyclonal antibody was used for experiments presented in Figures 2, 3, 5, and S2.

2.3. Animal husbandry

Male mice lacking functional leptin (ob/ob, stock #000632), and the leptin receptor (db/db, stock #000697) on the C57BL/6J background and their lean littermate controls were purchased from The Jackson Laboratory (Bar Harbor, ME). Upon arrival mice were allowed to acclimatize for a period of 7 days prior to initiation of studies. Mice harboring two copies of the floxed leptin receptor allele ($ObR^{f/f}$) were provided by Dr. Jeffrey Friedman (The Rockefeller University) and maintained in our colony [35]. Animals were housed in individually ventilated cages in a temperature-controlled room with a 14:10 light: dark cycle and provided with enrichment in the form of acrylic huts and nesting material. All mice were maintained on standard rodent chow (Harlan Teklad 2014S).

2.4. Leptin treatment

Mice lacking leptin (ob/ob, n=3) were injected (i.p.) with either saline or 10 mg/kg mouse recombinant leptin within 30 min of “lights-on” at 06:00. All mice were placed in clean cages with full access to water, but without food. Blood and tissues were collected 4, 8, and 16 h following leptin administration. For the 16 h time-point, a second injection of leptin

was administered at 8 h. Blood leptin levels were determined at the time of tissue collection by ELISA.

2.5. Adenoviral mediated hepatic gene expression

AdGRP78 and a control virus (AdEmpty) were previously reported [33]. Adenovirus encoding Cre-recombinase (AdCre) was purchased from Microbix Biosystems, Inc. (Mississauga, Ontario, Canada). AdCre, AdGRP78, and AdEmpty were amplified in HEK293Q cells and purified on cesium chloride gradients as previously described [8]. Purified adenovirus was diluted in sterile saline and mice (8–12 weeks) were injected through the tail vein with 4×10^{12} particles/kg body weight. Analysis of AdCre injected $ObR^{f/f}$ and wild-type (ObR^{WT}) mice were conducted two weeks following viral delivery. Analysis of control and GRP78 expressing mice was conducted five days following infection.

2.6. Immunoblot and Quantitative Real-time PCR

Total membrane preparations from liver samples were prepared and proteins analyzed by SDS-PAGE (50μg/lane) and immunoblotting as previously described [24]. The signals were quantified by densitometry using ImageJ software. Total RNAs were extracted from liver tissue using RNA STAT-60 (Tel-Test, Inc) and subjected to cDNA synthesis with iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA). To determine relative abundance of transcripts, RT-PCR was conducted using SYBRGreen as detector on Applied Biosystem 7900HT fast-Real Time PCR System (Carlsbad, CA) [2]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for the normalization of data in all RT-PCR experiments.

2.7. Plasma and biliary lipid analysis

Plasma TG concentrations were determined enzymatically using the L-type TG M kit. Biliary cholesterol concentrations were determined using the cholesterol E kit (Wako Chemicals, Richmond, VA).

2.8. Statistical Analysis

All statistical analyses were conducted using GraphPad Prism. Data are expressed as mean ± SEM. Data were analyzed by two-tailed t-test, one-way ANOVA, or two-way ANOVA as indicated in figure legends. Post-hoc comparisons were conducted by using Dunnett's tests for one-way ANOVA and Bonferroni post-tests for two-way ANOVA, respectively. Differences were considered significant at $P < 0.05$.

3. Results

To determine if leptin could acutely increase hepatic G5G8 abundance, we chose a 10 mg/kg dose that we previously reported to restore hepatic G5G8 in ob/ob mice following seven days of treatment [24]. To ensure that this dose was biologically active over the course of our study, we monitored food intake for three consecutive days prior to, and following, a single dose of leptin (Fig 1A). Leptin administration immediately suppressed food intake to levels generally observed in WT mice over the first 24 h, indicating that leptin was centrally

active over the course of our experiment. Interestingly, food intake remained significantly suppressed for at least seven days following this single dose.

Ob/ob mice were injected with saline or leptin and tissues collected 4, 8, and 16 h after administration. For the 16 h time-point, a second injection of leptin was administered at 8 h. Blood leptin levels were determined by ELISA at the termination of the experiment (Fig 1B). Lean control mice had leptin levels of 1.5 ng/ml, while levels in saline treated ob/ob mice were at the lower limit of detection in our assay. Serum leptin was 30 ng/ml at 4 h and declined to 8 ng/ml by 8 h, but increased to 20 ng/ml at 16 h following the second injection. While these levels of leptin were substantially higher than lean controls, they are typical of obese mice maintained on high fat diets [36]. Hepatic levels of G5 and G8 were analyzed by immunoblotting (Fig S1). Densitometric analysis of immunoblots of hepatic G5 and G8 confirmed that G5 and G8 protein levels were low in ob/ob mice compared to lean littermates (Fig 1C, D). Administration of leptin failed to increase G5 or G8 at 4, 8, or 16 h following the initial injection, indicating that centrally acting leptin had no immediate impact on hepatic G5G8 protein.

As an alternative approach to hormone replacement, hepatic G5G8 was examined in obese mice that had undergone hepatic vagotomy to determine if vagal mediated effects of chronic, centrally-acting leptin could regulate G5G8 abundance. Hepatic vagotomy was performed in anesthetized 8-week-old male mice on the C57Bl/6 background as previously described [37]. Mice were allowed to recover, maintained on standard chow diet for one week after vagotomy, and then switched to high fat diet (Bio-Serv, #F3282) feeding for 10 weeks. At termination, liver tissues were harvested and hepatic levels of G5 and G8 analyzed by immunoblotting (Fig 2). Neither G5 nor G8 protein levels were affected by hepatic branch vagotomy, indicating that centrally acting leptin failed to regulate hepatic G5G8 via vagal innervation in obese mice.

Although expression of the long, signaling form of the leptin receptor is low in peripheral tissues, a number of studies indicate functions for peripheral leptin receptor isoforms [38, 39]. Hepatic leptin receptors from mice harboring floxed leptin receptor alleles ($ObR^{f/f}$) were selectively depleted with AdCre. WT and $ObR^{f/f}$ mice were injected with AdCre and analyzed 2 weeks following treatment (Fig 3). Commercially available antibodies for leptin receptor proved unsatisfactory for hepatic protein level determination. An RT-PCR assay detecting all ObR isoforms demonstrated a 70% reduction in hepatic mRNA levels (Fig 3A). Food intake was not affected over this period and there were no differences in body weight (data not shown). Neither G5 nor G8 mRNA or protein levels were affected by depletion of hepatic leptin receptors (Fig 3A, B). Similarly, no changes were observed in biliary cholesterol concentrations (Fig 3C). These results failed to support direct effects of centrally or peripherally acting leptin on G5G8 abundance.

Expression of the ER chaperone protein GRP78 has been shown to alleviate markers of ER stress and reverse key features of the fatty liver phenotype in ob/ob mice [33]. The formation and trafficking of the G5G8 heterodimer is critically dependent upon the ER calnexin/calreticulin chaperone system. Therefore, we tested the hypothesis that elevated expression of GRP78 could restore G5G8 in db/db mice. A small cohort of lean mice (n = 3)

was also administered AdEmpty and AdGRP78 as an additional control. Tissues were harvested 5 days following viral administration. AdGRP78 increased hepatic GRP78 compared to mice infected with the control virus (Fig 4A). Immunoblot analysis demonstrated a reduction in phospho-PERK following GRP78 expression, indicating a reduction in UPR signaling (Fig 4A, B). As previously published in ob/ob mice, expression of AdGRP78 reduced expression of SREBP1-c, as well as its target genes acetylCoA carboxylase (ACC1) and fatty acid synthase (FAS), in the lipogenic pathway (Fig 4C). Similarly, plasma TGs and liver to body weight ratios were elevated in obese, db/db mice and reduced by elevated GRP78 expression to levels observed in lean controls (Fig 4D, E). These data confirmed the primary observations previously made in ob/ob mice and extended them to the db/db model.

As previously reported in both ob/ob and db/db mice, hepatic G5 protein expression was reduced in db/db mice compared with their lean controls (Fig S2). No difference was observed in the expression of GRP78, calnexin, or calreticulin. In db/db mice, AdGRP78 increased G5 and G8 by 2.2 and 2.4 fold, respectively. The increase in G5 and G8 protein occurred in the absence of an increase in mRNA encoding either protein, consistent with a mechanism of increased efficiency of G5G8 complex formation within the ER (Fig 5A, B). An increase in protein expression of the ER chaperone calnexin was observed in the absence of an increase at its mRNA level (Fig 5A, B). Total cholesterol content in gallbladder bile was elevated most likely due to increased G5G8 (Fig 5C). We also observed significant, albeit modest, increases in both mRNA and protein expression of SR-BI, which is another contributor to biliary cholesterol secretion (Fig 5A, B) [29, 40, 41]. However, there was no direct relationship between SR-BI and G5G8 abundance as shown in SR-BI deficient mice (Fig S3). Protein expression of ABCA1 also increased in db/db mice following AdGRP78 treatment (Fig 5A).

4. Discussion

The key finding of the present study is that expression of the ER chaperone, GRP78, restores hepatic G5G8 in db/db mice, whereas there is no apparent direct central or peripheral effect of leptin signaling on the complex. In the context of previously published studies that established a role for biliary cholesterol secretion in opposing ER stress, the present study suggests a reciprocal relationship between ER function and G5G8-mediated biliary cholesterol secretion.

Leptin signaling

Leptin replacement reduces food intake and induces weight loss in ob/ob mice. Therefore, it is difficult to establish which effects are truly dependent upon central or peripheral leptin signaling or are secondary to changes in energy balance. We previously reported the restoration of G5G8 in ob/ob mice following long-term leptin replacement [24]. While an increase in immunoreactive G5G8 was observed in pair-fed controls, leptin replacement resulted in a far greater increase, suggesting a direct role for leptin signaling as a regulator of hepatic G5G8 abundance. In the present study, leptin administration failed to acutely increase G5G8 after 4, 8, or 16 h when plasma levels of leptin were more than sufficient to mediate the central effects of this hormone. The absence of an effect over this period fails to

support a role for centrally acting leptin on hepatic G5G8 abundance. While it is possible that centrally acting leptin may take a greater period of time to restore hepatic G5G8, the abundance of G5 and G8 mRNAs increase after a 24 h fast in a PPARα-dependent fashion [25]. Consequently, observations at later time-points would be confounded by induction of the PPARα-mediated fasting response.

Long-term stimulation of the hepatic branch of the vagus nerve reduces body weight and fat mass in rats [42]. Although effects of leptin in liver have been shown to be mediated by vagal stimulation or by hepatic leptin receptors, neither pathway appears to regulate hepatic G5G8 abundance [43, 44]. In our studies, mice that had undergone hepatic branch vagotomy failed to show any difference in hepatic levels of G5G8 compared with their sham-operated controls. Similarly, depletion of hepatic leptin receptors also failed to reduce G5G8. While these results support a hepatic leptin receptor-independent mechanism for reduced G5G8 in db/db and ob/ob mice, this experiment was limited by incomplete ObR depletion at the mRNA level and that hepatic leptin receptor depletion could not be confirmed at the protein level.

Restoration of G5G8 by AdGRP78 in db/db mice

The increase in G5G8 following AdGRP78 treatment was consistent with a mechanism that includes the improvement of ER folding capacity and greater efficiency of G5G8 complex formation within the ER. When expressed individually in cells, G5 and G8 have short half-lives and are rapidly degraded [9]. Co-expression allows for the formation of G5G8 heterodimers through a process that is dependent on the presence of their N-linked glycans and interactions with calnexin [7]. In cultured cells, co-expression of either calnexin or calreticulin with G5 and G8 increases the appearance of G5G8 at the cell surface [6]. It is important to note that GRP78 does not bind either G5 or G8 [7]. Therefore, it is unlikely to directly facilitate G5G8 folding. An increase in calnexin was also observed and is more likely to account for G5G8 rescue in db/db mice (Fig 5A). However, whether the increase in calnexin is required for the effect of GRP78 on G5G8 abundance is unknown. Apart from increased G5G8 complex formation in the ER, other mechanisms that may account for increased G5G8 levels include an increase in the rates of translation for each monomer or increased stability of the mature, post-Golgi complex. While these alternate explanations have not been formally explored, the parallel increase in the immature and mature forms of G5 and G8 suggest that post-Golgi complex stability was not altered as this would result in selective accumulation of the mature form of each protein.

ER function and G5G8

ER stress is thought to play a causative role in the development of liver dysfunction in mice lacking leptin or its receptor and may directly contribute to the reduction in G5G8 in db/db mice. However, markers of ER stress are also induced in mice following high fat feeding and G5G8 levels are unaffected or even increased depending on the lipid composition of the diet [45–47]. While these observations were suggestive of a direct effect of leptin signaling on G5G8 abundance, direct effects of leptin could not be demonstrated. The reduction in G5G8 associated with ER stress in ob/ob and db/db mice may simply be a matter of degree. The extent of obesity, steatosis, insulin resistance, and ER dysfunction is generally greater in

these mice compared those with an intact leptin axis and challenged with various high fat diets. Alternatively, the lack of reduction in G5G8 protein in other models may reflect a compensatory increases in G5G8 transcription that overcomes reduced efficiency of complex formation within the ER. In addition, we previously reported that other glycoproteins, including the closely related family member ABCG2, were not affected in mice harboring defects in leptin or its receptor [24]. Rescue of the complex by AdGRP78 implicates ER dysfunction as a mechanism contributing to reduced G5G8, but what accounts for the selective depletion of G5G8 in db/db and ob/ob mice remains unknown. Perhaps G5G8 assembly is particularly sensitive to ER dysfunction. Alternatively, there may be some synergistic effect of the absence of leptin signaling and fatty liver phenotype on G5G8 abundance.

We previously reported that the loss of G5G8 increased markers of ER stress in high fat-fed mice, establishing that biliary cholesterol secretion is essential to maintain hepatocyte function [2]. Excess unesterified cellular cholesterol is known to induce ER stress in a number of cell types, including hepatocytes [48–50]. Conversely, ER stress reduces ABCA1 and SR-BI and impairs cholesterol efflux in cultured hepatocytes [51]. Similar to G5G8, AdGRP78 increased ABCA1 and SR-BI in db/db mice, suggesting that ER dysfunction perturbs multiple facets of hepatic sterol metabolism in db/db mice. Collectively, the data support a reciprocal relationship between ER function and cholesterol metabolism in which disturbances in cholesterol homeostasis contribute to ER stress and ER stress contributes to disruptions in cellular cholesterol metabolism.

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Abbreviations

G5G8	ABCG5 ABCG8
ACC1	acetyl-CoA carboxylase
AdGRP78	adenoviral vector encoding GRP78
AdCre	adenoviral vector encoding Cre-recombinase
FOXO1	forkhead box O1A
ER	endoplasmic reticulum
eiF2α	eukaryotic translation initiation factor 2α
AdEmpty	empty adenoviral vector
FAS	fatty acid synthase
GRP78	glucose regulated protein 78-kDa

GWAS	Genome-wide association study
HF	high fat
HNF4α	hepatic nuclear receptor 4 α
HV	hepatic vagotomy
KO	knockout
ob/ob	leptin deficient mice
ObR	leptin receptor
db/db	leptin receptor deficient mice
LXR	liver X receptor isoforms
NAFLD	nonalcoholic fatty liver disease
LRH-1	orphan nuclear receptor liver receptor homolog-1
PERK	protein kinase-like endoplasmic reticulum kinase
PPARα	peroxisome proliferator activated receptor α
SR-BI	scavenger receptor BI
SREBP1	sterol regulatory element binding protein-1
TUDCA	tauroursodeoxycholic acid
TGs	triglycerides
UPR	unfolded protein response
WT	wild-type

References

1. Kidambi S, Patel SB. Sitosterolaemia: Pathophysiology, clinical presentation and laboratory diagnosis. J Clin Pathol. 2008; 61(5):588–94. [PubMed: 18441155]
2. Su K, Sabeva NS, Liu J, et al. The abcg5 abcg8 sterol transporter opposes the development of fatty liver disease and loss of glycemic control independently of phytosterol accumulation. J Biol Chem. 2012; 287(34):28564–75. [PubMed: 22715101]
3. Su K, Sabeva NS, Wang Y, et al. Acceleration of biliary cholesterol secretion restores glycemic control and alleviates hypertriglyceridemia in obese, db/db mice. Arterioscler Thromb Vasc Biol. 2013; 34(1):26–33. [PubMed: 24202306]
4. Basso F, Freeman LA, Ko C, et al. Hepatic abcg5/g8 overexpression reduces apob-lipoproteins and atherosclerosis when cholesterol absorption is inhibited. J Lipid Res. 2007; 48(1):114–26. [PubMed: 17060690]
5. Wilund KR, Yu L, Xu F, et al. High-level expression of abcg5 and abcg8 attenuates diet-induced hypercholesterolemia and atherosclerosis in ldlr−/− mice. J Lipid Res. 2004; 45(8):1429–36. [PubMed: 15175362]
6. Okiyoneda T, Kono T, Niibori A, et al. Calreticulin facilitates the cell surface expression of abcg5/g8. Biochem Biophys Res Commun. 2006; 347(1):67–75. [PubMed: 16806071]
7. Graf GA, Cohen JC, Hobbs HH. Missense mutations in abcg5 and abcg8 disrupt heterodimerization and trafficking. J Biol Chem. 2004; 279(23):24881–8. [PubMed: 15054092]

8. Graf GA, Yu L, Li WP, et al. Abcg5 and abcg8 are obligate heterodimers for protein trafficking and biliary cholesterol excretion. J Biol Chem. 2003; 278(48):48275–82. [PubMed: 14504269]
9. Graf GA, Li WP, Gerard RD, et al. Coexpression of atp-binding cassette proteins abcg5 and abcg8 permits their transport to the apical surface. J Clin Invest. 2002; 110(5):659–69. [PubMed: 12208867]
10. Sumi K, Tanaka T, Uchida A, et al. Cooperative interaction between hepatocyte nuclear factor 4{alpha} and gata transcription factors regulates atp-binding cassette sterol transporters abcg5 and abcg8. Mol Cell Biol. 2007; 27(12):4248–60. [PubMed: 17403900]
11. Freeman LA, Kennedy A, Wu J, et al. The orphan nuclear receptor lrh-1 activates the abcg5/abcg8 intergenic promoter. J Lipid Res. 2004; 45(7):1197–206. [PubMed: 15121760]
12. Bonde Y, Plosch T, Kuipers F, et al. Stimulation of murine biliary cholesterol secretion by thyroid hormone is dependent on a functional abcg5/g8 complex. Hepatology. 2012; 56(5):1828–37. [PubMed: 22829162]
13. Sabeva NS, Liu J, Graf GA. The abcg5 abcg8 sterol transporter and phytosterols: Implications for cardiometabolic disease. Curr Opin Endocrinol Diabetes Obes. 2009; 16(2):172–7. [PubMed: 19306529]
14. Back SS, Kim J, Choi D, et al. Cooperative transcriptional activation of atp-binding cassette sterol transporters abcg5 and abcg8 genes by nuclear receptors including liver-x-receptor. BMB Rep. 2013; 46(6):322–7. [PubMed: 23790976]
15. Biddinger SB, Haas JT, Yu BB, et al. Hepatic insulin resistance directly promotes formation of cholesterol gallstones. Nat Med. 2008; 14(7):778–82. [PubMed: 18587407]
16. Rudkowska I, Jones PJ. Polymorphisms in abcg5/g8 transporters linked to hypercholesterolemia and gallstone disease. Nutr Rev. 2008; 66(6):343–8. [PubMed: 18522623]
17. Bouchard G, Johnson D, Carver T, et al. Cholesterol gallstone formation in overweight mice establishes that obesity per se is not linked directly to cholelithiasis risk. J Lipid Res. 2002; 43(7): 1105–13. [PubMed: 12091495]
18. Graewin SJ, Kiely JM, Lu D, et al. Leptin regulates gallbladder genes related to gallstone pathogenesis in leptin-deficient mice. Journal of the American College of Surgeons. 2008; 206(3): 503–10. [PubMed: 18308222]
19. Graewin SJ, Lee KH, Tran KQ, et al. Leptin-resistant obese mice do not form biliary crystals on a high cholesterol diet. J Surg Res. 2004; 122(2):145–9. [PubMed: 15555610]
20. Tran KQ, Graewin SJ, Swartz-Basile DA, et al. Leptin-resistant obese mice have paradoxically low biliary cholesterol saturation. Surgery. 2003; 134(2):372–7. [PubMed: 12947343]
21. Hyogo H, Roy S, Paigen B, et al. Leptin promotes biliary cholesterol elimination during weight loss in ob/ob mice by regulating the enterohepatic circulation of bile salts. J Biol Chem. 2002; 277(37):34117–24. [PubMed: 12114517]
22. Hyogo H, Roy S, Cohen DE. Restoration of gallstone susceptibility by leptin in c57bl/6j ob/ob mice. J Lipid Res. 2003; 44(6):1232–40. [PubMed: 12671032]
23. Goldblatt MI, Swartz-Basile DA, Svatek CL, et al. Decreased gallbladder response in leptin-deficient obese mice. J Gastrointest Surg. 2002; 6(3):438–42. discussion 43–4. [PubMed: 12022998]
24. Sabeva NS, Rouse EJ, Graf GA. Defects in the leptin axis reduce abundance of the abcg5-abcg8 sterol transporter in liver. J Biol Chem. 2007; 282(31):22397–405. [PubMed: 17561514]
25. Kok T, Wolters H, Bloks VW, et al. Induction of hepatic abc transporter expression is part of the pparalpha-mediated fasting response in the mouse. Gastroenterology. 2003; 124(1):160–71. [PubMed: 12512040]
26. Ozcan U, Yilmaz E, Ozcan L, et al. Chemical chaperones reduce er stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science. 2006; 313(5790):1137–40. [PubMed: 16931765]
27. Ozcan U, Cao Q, Yilmaz E, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science. 2004; 306(5695):457–61. [PubMed: 15486293]
28. Oude Elferink RP, Ottenhoff R, van Wijland M, et al. Uncoupling of biliary phospholipid and cholesterol secretion in mice with reduced expression of mdr2 p-glycoprotein. J Lipid Res. 1996; 37(5):1065–75. [PubMed: 8725158]

29. Dikkers A, Freak de Boer J, Annema W, et al. Scavenger receptor bi and abcg5/g8 differentially impact biliary sterol secretion and reverse cholesterol transport in mice. Hepatology. 2013; 58(1): 293–303. [PubMed: 23401258]
30. Xie Q, Khaoustov VI, Chung CC, et al. Effect of tauroursodeoxycholic acid on endoplasmic reticulum stress-induced caspase-12 activation. Hepatology. 2002; 36(3):592–601. [PubMed: 12198651]
31. Benz C, Angermuller S, Tox U, et al. Effect of tauroursodeoxycholic acid on bile-acid-induced apoptosis and cytolysis in rat hepatocytes. J Hepatol. 1998; 28(1):99–106. [PubMed: 9537871]
32. Benz C, Angermuller S, Otto G, et al. Effect of tauroursodeoxycholic acid on bile acid-induced apoptosis in primary human hepatocytes. Eur J Clin Invest. 2000; 30(3):203–9. [PubMed: 10691996]
33. Kammoun HL, Chabanon H, Hainault I, et al. Grp78 expression inhibits insulin and er stress-induced srebp-1c activation and reduces hepatic steatosis in mice. J Clin Invest. 2009; 119(5): 1201–15. [PubMed: 19363290]
34. Horton JD, Goldstein JL, Brown MS. Srebps: Activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest. 2002; 109(9):1125–31. [PubMed: 11994399]
35. Cohen P, Zhao C, Cai X, et al. Selective deletion of leptin receptor in neurons leads to obesity. J Clin Invest. 2001; 108(8):1113–21. [PubMed: 11602618]
36. Takanabe R, Ono K, Abe Y, et al. Up-regulated expression of microrna-143 in association with obesity in adipose tissue of mice fed high-fat diet. Biochem Biophys Res Commun. 2008; 376(4): 728–32. [PubMed: 18809385]
37. Gao X, van der Veen JN, Zhu L, et al. Vagus nerve contributes to the development of steatohepatitis and obesity in phosphatidylethanolamine n-methyltransferase deficient mice. J Hepatol. 2014
38. Lautenbach A, Breitmeier D, Kuhlmann S, et al. Human obesity reduces the number of hepatic leptin receptor (ob-r) expressing nk cells. Endocr Res. 2011; 36(4):158–66. [PubMed: 21973235]
39. Koch L, Wunderlich FT, Seibler J, et al. Central insulin action regulates peripheral glucose and fat metabolism in mice. J Clin Invest. 2008; 118(6):2132–47. [PubMed: 18451994]
40. Kozarsky KF, Donahee MH, Rigotti A, et al. Overexpression of the hdl receptor sr-bi alters plasma hdl and bile cholesterol levels. Nature. 1997; 387(6631):414–7. [PubMed: 9163428]
41. Wiersma H, Gatti A, Nijstad N, et al. Hepatic sr-bi, not endothelial lipase, expression determines biliary cholesterol secretion in mice. J Lipid Res. 2009; 50(8):1571–80. [PubMed: 19252221]
42. Banni S, Carta G, Murru E, et al. Vagus nerve stimulation reduces body weight and fat mass in rats. PLoS One. 2012; 7(9):e44813. [PubMed: 23028630]
43. German J, Kim F, Schwartz GJ, et al. Hypothalamic leptin signaling regulates hepatic insulin sensitivity via a neurocircuit involving the vagus nerve. Endocrinology. 2009; 150(10):4502–11. [PubMed: 19574396]
44. Li X, Wu X, Camacho R, et al. Intracerebroventricular leptin infusion improves glucose homeostasis in lean type 2 diabetic mkr mice via hepatic vagal and non-vagal mechanisms. PLoS One. 2011; 6(2):e17058. [PubMed: 21379576]
45. Li Y, Xu S, Giles A, et al. Hepatic overexpression of sirt1 in mice attenuates endoplasmic reticulum stress and insulin resistance in the liver. The FASEB Journal. 2011; 25(5):1664–79. [PubMed: 21321189]
46. Escolà-Gil JC, Llaverias G, Julve J, et al. The cholesterol content of western diets plays a major role in the paradoxical increase in high-density lipoprotein cholesterol and upregulates the macrophage reverse cholesterol transport pathway. Arteriosclerosis, Thrombosis, and Vascular Biology. 2011; 31(11):2493–9.
47. Gustavsson C, Parini P, Ostojic J, et al. Cocoa butter and safflower oil elicit different effects on hepatic gene expression and lipid metabolism in rats. Lipids. 2009; 44(11):1011–27. [PubMed: 19806378]
48. Hager L, Li L, Pun H, et al. Lecithin:Cholesterol acyltransferase deficiency protects against cholesterol-induced hepatic endoplasmic reticulum stress in mice. J Biol Chem. 2012; 287(24): 20755–68. [PubMed: 22500017]

49. Van Rooyen DM, Larter CZ, Haigh WG, et al. Hepatic free cholesterol accumulates in obese, diabetic mice and causes nonalcoholic steatohepatitis. Gastroenterology. 2011; 141(4):1393–403. 403 e1–5. [PubMed: 21703998]

50. Feng B, Yao PM, Li Y, et al. The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. Nat Cell Biol. 2003; 5(9):781–92. [PubMed: 12907943]

51. Rohrl C, Eigner K, Winter K, et al. Endoplasmic reticulum stress impairs cholesterol efflux and synthesis in hepatic cells. J Lipid Res. 2014; 55(1):94–103. [PubMed: 24179149]

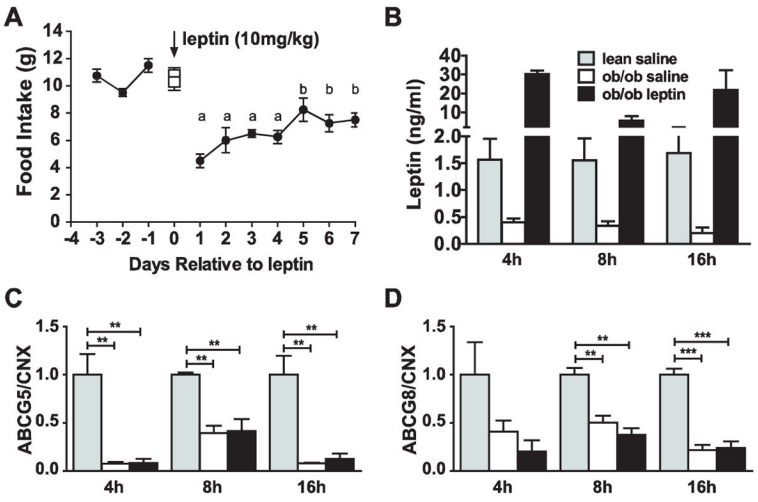

Figure 1. Leptin acutely suppresses food intake, but fails to restore hepatic G5G8 in ob/ob mice

(A) Food intake was monitored for three consecutive days in ob/ob mice (n=4). A single dose of leptin (10 mg/kg, ip) was administered (Day 0) and food intake monitored for seven additional days. Intake following leptin treatment was compared to the three-day pre-treatment mean by one-way ANOVA. A Dunnett's test was used to determine differences from the three-day pre-treatment mean (a, $p<0.01$; b, $p<0.05$). (B–D) In a second cohort of lean and ob/ob mice (n=3), tissues were harvested at 4, 8, and 16 h following the initial injection of either saline or leptin. (B) Serum leptin levels at the time of tissue collection were determined by ELISA. (C–D) Relative protein abundance was determined by densitometry using ImageJ software after normalization to calnexin (CNX). Data are mean ± SEM and were analyzed by one-way ANOVA. Post-hoc comparisons were conducted using Dunnett's tests ** $p<0.01$, ***$p<0.001$.

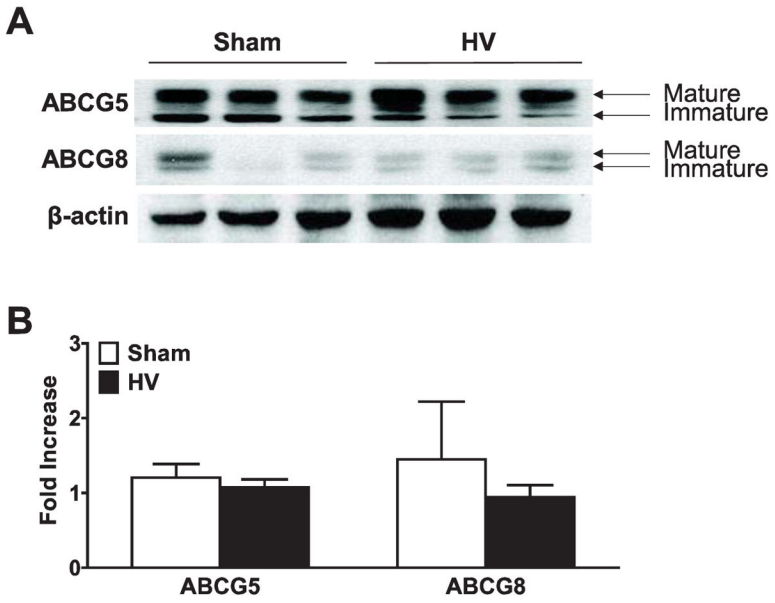

Figure 2. Hepatic branch vagotomy fails to alter the abundance of G5G8 in the setting of obesity

Liver tissues of obese mice that had undergone hepatic vagotomy and their sham-operated controls (n = 3) were harvested after being maintained on high fat feeding for 10 weeks. (A) Hepatic levels of G5 and G8 were analyzed by immunoblotting. (B) Relative protein abundance was determined by densitometry using ImageJ software after normalization to β-actin. Data are mean ± SEM. Differences were determined by two-tailed t-test.

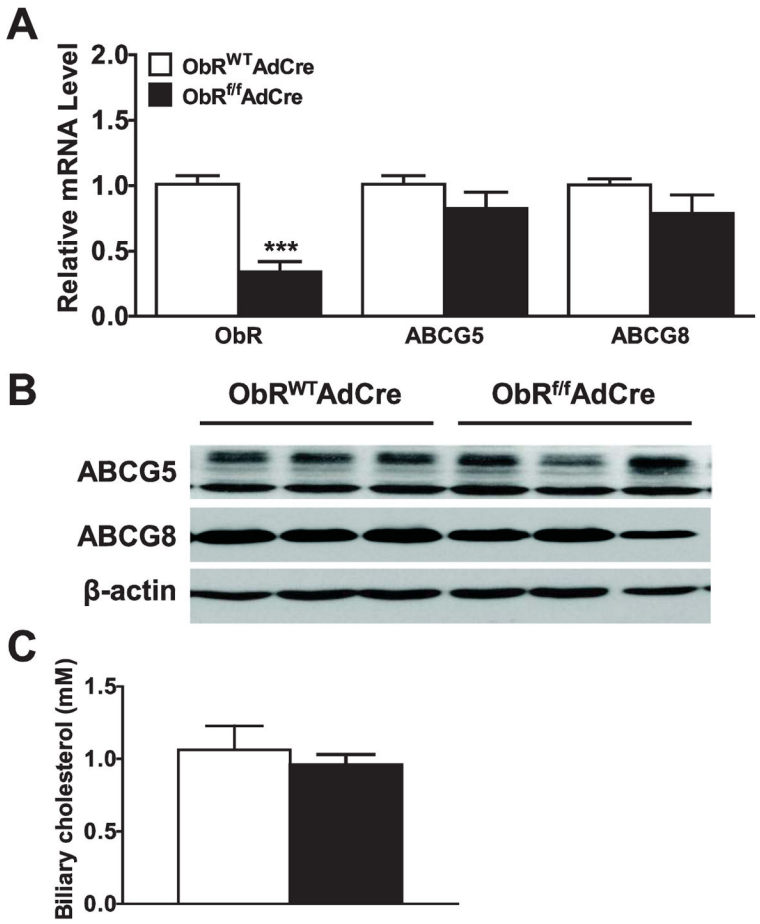

Figure 3. Depletion of hepatic leptin receptors does not reduce G5G8

An adenoviral vector encoding Cre recombinase (AdCre) was administered to WT (n = 3) and mice harboring two floxed alleles for leptin receptor ($ObR^{f/f}$, n = 3). (A) Hepatic levels of leptin receptor (ObR), G5 and G8 mRNA were quantified by RT-PCR. (B) The abundance of hepatic ABCG5 and ABCG8 protein was determined by immunoblotting. (C) Biliary cholesterol concentrations were determined by colorimetric-enzymatic assay. Data are mean ± SEM. Differences were determined by two-tailed t-test ***p<0.001.

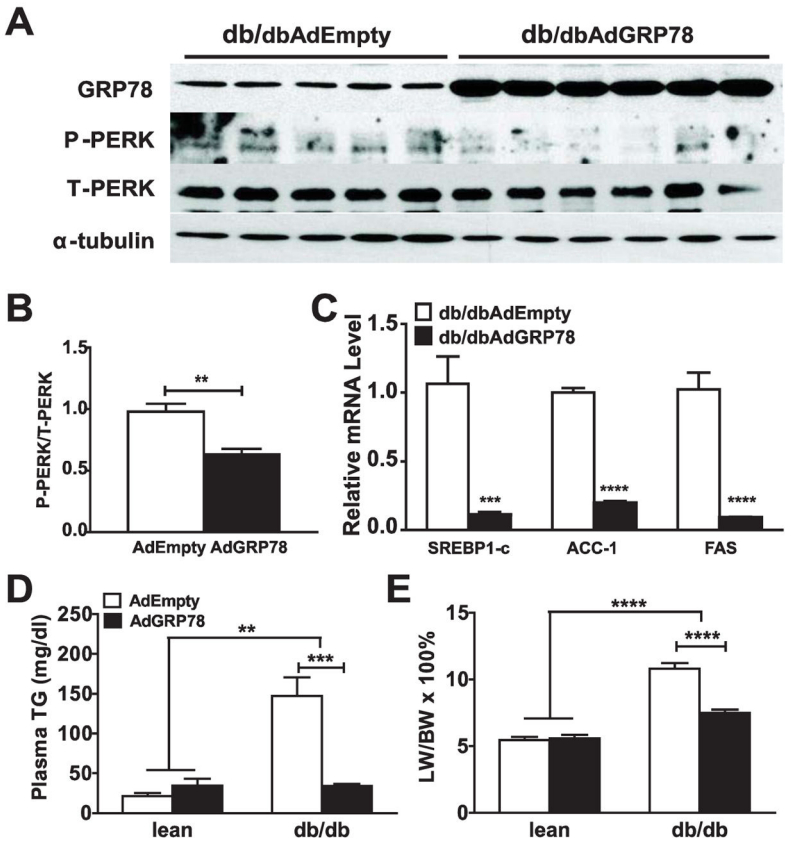

Figure 4. AdGRP78 alleviates ER stress, reduces lipogenic gene expression, and normalizes plasma TGs and liver weight in db/db mice

Control (AdEmpty) and GRP78 (AdGRP78) adenoviral vectors were administered to lean (n = 3) and db/db (n = 5 WT and 6 KO) mice. Tissues were harvested for analysis 5 days following viral expression. (A) GRP78, total and phosphorylated (P-) protein kinase-like endoplasmic reticulum kinase (PERK) in db/db mice were assessed in hepatic lysates by SDS-PAGE and immunoblotting. α-tubulin was used as a loading control. (B) The ratio of phosphorylated to total PERK was determined by desitometry using ImageJ. (C) The mRNA for lipogenic genes SREBP1-c, acetylCoA carboxylase (ACC1), and fatty acid synthase (FAS) was determined by RT-PCR. (D) Plasma TGs and (E) liver weight to body weight ratio (LW/BW) were determined in both lean and db/db mice. Data are mean ± SEM and were analyzed by two-tailed t-test (B–C) and two-way ANOVA followed by Bonferroni post-tests (D-E) ** p<0.01, ***p<0.001, ****p<0.0001.

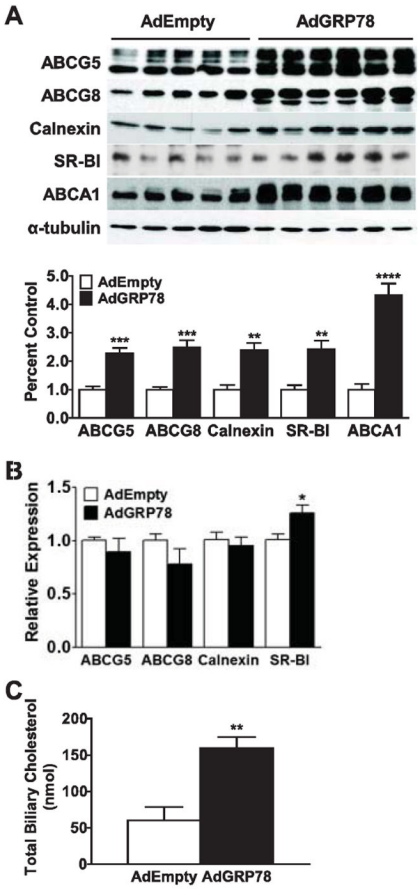

Figure 5. AdGRP78 increases G5G8 at the protein level and elevates biliary cholesterol in db/db mice

(A) Immunoblot analysis of hepatic G5, G8, calnexin, SR-BI, and ABCA1. Data were analyzed by densitometry and normalized to α-tubulin. (B) Levels of G5, G8, calnexin, and SR-BI mRNA were determined by RT-PCR. (C) Total biliary cholesterol was determined by colorimetric-enzymatic assays. Data are mean ± SEM (n=5 WT and 6 KO). Differences were determined by two-tailed t-test ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$.