Acceleration of Biliary Cholesterol Secretion Restores Glycemic Control and Alleviates Hypertriglyceridemia in Obese db/db Mice

Kai Su,* Nadezhda S. Sabeva,* Yuhuan Wang, Xiaoxi Liu, Joshua D. Lester, Jingjing Liu, Shuang Liang, Gregory A. Graf

Objective—Recent studies support a role for cholesterol in the development of obesity and nonalcoholic fatty liver disease. Mice lacking the ABCG5 ABCG8 (G5G8) sterol transporter have reduced biliary cholesterol secretion and are more susceptible to steatosis, hepatic insulin resistance, and loss of glycemic control when challenged with a high-fat diet. We hypothesized that accelerating G5G8-mediated biliary cholesterol secretion would correct these phenotypes in obese mice.

Approach and Results—Obese (db/db) male and their lean littermates were administered a cocktail of control adenovirus or adeno-viral vectors encoding ABCG5 and ABCG8 (AdG5G8). Three days after viral administration, measures of lipid and glucose homeostasis were determined, and tissues were collected for biochemical analyses. AdG5G8 increased biliary cholesterol and fecal sterol elimination. Fasting glucose and triglycerides declined, and glucose tolerance improved in obese mice expressing G5G8 compared with mice receiving control adenovirus. These changes were associated with a reduction in phosphorylated eukaryotic initiation factor 2α and c-Jun N-terminal kinase in liver, suggesting alleviation of endoplasmic reticulum stress. Phosphorylated insulin receptor and protein kinase B were increased, indicating restored hepatic insulin signaling. However, there was no reduction in hepatic triglycerides after the 3-day treatment period.

Conclusions—Accelerating biliary cholesterol secretion restores glycemic control and reduces plasma triglycerides in obese db/db mice. (Arterioscler Thromb Vasc Biol. 2014;34:26-33.)

Key Words: bile • cholesterol • insulin resistance • liver steatosis • obesity

The causal role of cholesterol in the development of cardiovascular disease is well-established, but an emerging body of literature indicates that cholesterol may also contribute to the development of obesity and the severity of nonalcoholic fatty liver disease (NAFLD). Dietary cholesterol and fat act synergistically to promote obesity and the development of NAFLD in C57BL6/J mice. Similarly, the addition of cholesterol to a high-fat, high-sucrose diet also exacerbates the development of insulin resistance and steatosis in low-density lipoprotein receptor (LDLR)–deficient mice. In a mouse model of Alström syndrome, hepatic cholesterol accumulation is associated with disruptions in both cholesterol uptake and elimination pathways and correlates with the severity of liver phenotypes. Conversely, the inhibition of cholesterol absorption with ezetimibe (EZ) opposes the development of obesity, insulin resistance, and steatosis in both rats and mice. Mice lacking the target of EZ, Niemann-Pick C1-like 1 protein, are protected from NAFLD and the development of obesity and insulin resistance. However, the addition of cholesterol to the diet reverses this phenotype, suggesting that the presence of cholesterol in the intestine modulates metabolism independently of its absorption. In the LDLR model challenged with a high-cholesterol diabetogenic diet, EZ reduced hepatic steatosis but not other obesity phenotypes such as adipose tissue inflammation. EZ was shown to produce a modest, but significant, reduction in hepatic fat and liver enzymes in a Japanese population of patients with NAFLD. In addition, a recent clinical trial suggests that EZ may improve measures of insulin resistance in diabetic insulin-resistant subjects.

The ABCG5 ABCG8 (G5G8) heterodimer functions as a xenobiotic transporter that opposes the absorption of phytosterols and their accumulation in plasma and tissues in both mice and humans. G5G8 also promotes cholesterol secretion...
contributing to either ER stress or hepatic phenotypes of either
and biliary cholesterol, but whether reduced G5G8 deficiency
Both strains are also characterized by reduced G5G8 protein
dition.19–21 In mice lacking functional G5G8, the prevention
can contribute to biliary cholesterol secretion under some con-
tion.16,18 However, it should be noted that alternative pathways
G5G8 accounts for 70% to 90% of biliary cholesterol secre-
tribution to intestinal cholesterol elimination remains unclear,
into bile and the intestinal lumen.16,17 Although its relative con-
tribution to intestinal cholesterol elimination remains unclear,
and G5G8 accounts for 70% to 90% of biliary cholesterol secre-
junctions.19–21 In mice lacking functional G5G8, the prevention
of phytosterol accumulation by treatment with EZ, deletion
of Niemann-Pick C1–like 1 protein, or feeding a plant sterol–
free diet corrects metabolic phenotypes associated with G5G8
deficiency, indicating that compensatory mechanisms over-
come the loss of G5G8-dependent biliary and intestinal secre-
tion.22–24 When challenged with a plant sterol–free, high-fat
diet, the absence of G5G8 accelerates the development of obe-
sity and insulin resistance and increases hepatic cholesterol,
steatosis, and inflammation, thereby revealing an essential
role for G5G8 in metabolism, independent of its opposition to
phytosterol absorption.22

The phenotype of obese G5G8-deficient mice was associ-
ated with increased eukaryotic initiation factor 2α (eIF2α)
phosphorylation and upregulation of activating transcription
factor (ATF) 4 and tribbles 3 (Trb3), suggesting that endo-
plasmic reticulum (ER) stress contributes to the accelerated
loss of glycemic control and amplified steatosis after high-
fat feeding. ER stress has been linked to the development of insulin resistance and the upregulation of lipogenesis in genetically obese leptin-deficient (ob/ob) mice.25,26 Mice lacking either leptin (ob/ob) or its receptor (db/db) are hyperpha-
gic, insulin-resistant, and develop severe hepatic steatosis.27
Both strains are also characterized by reduced G5G8 protein
and biliary cholesterol, but whether reduced G5G8 deficiency
contributes to either ER stress or hepatic phenotypes of either
strain is not known.28-30

We hypothesized that increased G5G8-mediated biliary
cholesterol secretion would alleviate metabolic phenotypes
in genetically obese mice in which G5G8 activity is com-
promised. Adenoviral vectors encoding G5G8 (AdG5G8)
were used to accelerate biliary cholesterol secretion in db/
db mice. After AdG5G8 delivery, plasma glucose and tri-
glycerides (TGs) were reduced and glucose tolerance was
improved. These changes were associated with decreased
expression of lipogenic genes, increased Akt phosphoryla-
tion, and reduced eIF2α signaling, suggesting that G5G8-
mediated biliary cholesterol secretion alleviates ER stress
and restores insulin signaling. However, steatosis was not
reduced in this short-term experiment. Additional stud-
ies in which stable expression of G5G8 is achieved will be
required to determine whether accelerated biliary chole-
sterol secretion can improve hepatic steatosis in models of
obesity and insulin resistance.

Materials and Methods

Biliary Cholesterol Elimination

We have previously used adenoviral vectors to transiently
increase G5G8 and biliary cholesterol.31 Before initiat-
ing studies in db/db mice, we conducted a pilot study to
confirm that AdG5G8 could increase fecal sterol output
during the planned treatment period. Fecal neutral sterols
increase within 2 days and remain elevated for ≤6 days
after AdG5G8 delivery (Figure I in the online-only Data
Supplement). Obese (db/db) mice and their lean littermates
were injected with a cocktail of adenoviral particles encod-
ing ABCG8 and an empty vector (control) or both ABCG5
and ABCG8 (AdG5G8), and tissues were dissected 3 days
after viral delivery. G5 and G8 are obligate heterodimers
that require coexpression for complex formation, trafficking
to the cell surface, and biliary cholesterol secretion. Both
G5 and G8 contain N-linked glycans and heterodimer for-
mation, and trafficking to the cell surface can be monitored
by the appearance of the mature, post-Golgi forms of both
proteins by SDS-PAGE and immunoblot analysis.32 The
immature form of recombinant G8 is observed in all mice,
but the mature form is only observed in mice receiving the
G5 adenovirus (Figure 1A). Similarly, the mature form of
recombinant G5 is only observed in AdG5G8-injected mice
that express functional G5G8. AdG5G8 did not increase
intestinal levels of the G5G8 transporter, nor did viral cock-
tails differentially affect liver enzymes or the expression of
inflammatory genes in liver (Figure II in the online-only
Data Supplement).

We next measured biliary cholesterol and fecal neutral
sterols as indirect measures of G5G8 function. AdG5G8
increased biliary cholesterol concentrations in both lean and
obese mice by 5.2-fold and 5.7-fold, respectively (Figure 1B).
We also blotted for the class B, type 1 scavenger receptor
because this protein can mediate G5G8-independent biliary
 cholesterol secretion.30 Class B, type 1 scavenger receptor
is reduced in db/db mice compared with lean controls but is
not elevated in either lean or db/db mice after AdG5G8 treat-
ment (Figure 1A). The overall ANOVA indicated a significant
increase in fecal neutral sterols in AdG5G8-injected mice,
regardless of the genotype (P<0.01). Post hoc analysis indi-
cated 4.4-fold and 2.9-fold increases in lean and obese mice,
respectively. Although levels of G5G8 protein are lower in db/
db mice compared with lean controls, biliary cholesterol and
fecal neutral sterol levels increase to a similar extent.
The loss of glycemic control and hepatic phenotypes in obese G5G8-deficient mice were associated with increased activation of some components of the unfolded protein response (UPR), in particular phosphorylation of eIF2α. Therefore, we determined whether AdG5G8 reduced phosphorylated eIF2α and suppressed other components of the UPR in livers of db/db mice. Immunoblot analysis demonstrated a reduction in phospho-eIF2α but not in total eIF2α in AdG5G8-treated obese mice compared with control virus (Figure 3A and 3B). The reduction in phospho-eIF2α was associated with less ATF4 mRNA expression and its downstream target, Trb3 (Figure 3C). Trb3 is a negative regulator of insulin-mediated Akt phosphorylation in liver. The decrease in Trb3 was associated with an increase in phospho-Akt but not total Akt, suggesting that alleviation of ATF4-Trb3 signaling plays a role in the restoration of insulin signaling in the livers of AdG5G8-treated db/db mice.

To further investigate UPR signaling, we blotted for total and phosphorylated c-Jun N-terminal kinase and pancreatic eIF2α kinase (PERK). Consistent with reduced ER stress, phospho-c-Jun N-terminal kinase declined after AdG5G8 administration. However, we did not observe elevations in phospho-PERK. In addition, other components of the UPR are largely unaffected in db/db mice treated with AdG5G8 compared with control virus. This includes expression of the ATF6 target genes glucose-regulated protein (GRP) 78, GRP94, CCAAT-enhancer-binding proteins (C/EBP)-homologous protein, and X box–binding protein 1. However, we did detect a modest but significant reduction in spliced X box–binding protein 1, consistent with reduced inositol-requiring enzyme 1 activation. Collectively, these data support a role for accelerated biliary cholesterol secretion in the reduction of eIF2α signaling, but the kinase and initiating events remain to be resolved.

### Glycemic Control, Hepatic ER Stress, and Insulin Signaling

We previously reported that the absence of G5G8 accelerates the loss of glycemic control in high-fat–fed mice. To determine whether increased G5G8 and accelerated biliary cholesterol secretion could restore glycemic control in db/db mice, we measured fasting glucose and conducted a glucose tolerance test. Overexpression of G5G8 had no effect on fasting glucose in lean mice, nor did it alter glucose disposal in glucose tolerance tests. AdG5G8 decreased plasma fasting glucose in db/db mice to levels that were similar to lean controls (Figure 2A). AdG5G8 decreased plasma glucose at 30 and 60 minutes after glucose administration (Figure 2B). There was also a significant reduction in the mean area under the curve for blood glucose in db/db mice treated with AdG5G8 compared with control virus (Figure 2B, inset).

We next evaluated hepatic insulin signaling. Livers from db/db mice were collected 15 minutes after administration of insulin and subjected to SDS-PAGE and immunoblot analysis. There was an increase in tyrosine-phosphorylated insulin receptor and a decrease in serine-phosphorylated insulin receptor substrate 1 (Figure 2C and 2D), indicating improvements in hepatic insulin sensitivity. An insulin tolerance test revealed a decrease in the area under the curve for blood glucose, but fasting insulin was only modestly lower in AdG5G8-treated mice and failed to reach statistical significance (Figure 2E and 2F).

### Plasma and Hepatic Lipids

We broadly assessed changes in genes related to insulin signaling in db/db mice using pooled RNA from animals infected with either control virus or AdG5G8 and analyzed by an insulin signaling pathway polymerase chain reaction array (Figure III in the online-only Data Supplement). The majority of genes assayed were unaltered, but key genes in both the lipogenic and gluconeogenic pathways were suppressed, including the lipogenic transcription factor, sterol regulatory–binding protein (SREBP). We confirmed suppression of mRNAs for SREBP1 and its target genes acetyl coenzyme A carboxylase and fatty acid synthase by reverse-transcriptase polymerase chain reaction in individual mice (Figure 4A). Consistent with the reduction in mRNA, full-length SREBP1 protein was lower after AdG5G8 delivery (Figure 4B and 4C). There was also a tendency toward lower levels of cleaved SREBP in nuclear extracts. However, there was no difference in hepatic TG levels 72 hours after AdG5G8 treatment in either lean or db/db mice (Figure 4D). We next measured plasma TGs and TG secretion rates (Figure 4E and 4F). TGs were normalized in db/db mice, and the TG secretion rate was reduced (542±31 versus 428±23 mg/dL per hour; P<0.01) after AdG5G8 administration in db/db mice. However, levels of apolipoprotein B (ApoB) and microsomal TG transfer protein mRNAs were unchanged (not shown).
Despite elevations in biliary and fecal sterols, plasma cholesterol was paradoxically increased after AdG5G8 administration in both lean and obese mice (Figure 5A). Fast protein liquid chromatography fractionation of pooled serum revealed that the increase in cholesterol was caused by the accumulation of large particles. High-density lipoprotein levels, which are characteristically elevated in db/db mice, declined in both genotypes. A significant fraction of biliary cholesterol is reabsorbed in the small intestine. Therefore, we tested the hypothesis that the unexpected increase in plasma cholesterol after AdG5G8 administration could be averted by coadministration of the cholesterol absorption inhibitor, EZ. In a separate cohort of lean strain-matched mice, we pretreated mice with EZ before administration of control or AdG5G8 virus. As in lean and db/db littersmates, AdG5G8 increased plasma cholesterol and promoted the accumulation of large cholesterol ester–enriched particles but not in mice in which intestinal absorption was inhibited by pretreatment with EZ (Figure 5D). Immunoblot analysis of peak fractions revealed that these enriched particles but not in mice in which intestinal absorption was inhibited by pretreatment with EZ (Figure 5D).

**Discussion**

The major findings of the present study are that increased G5G8-mediated biliary cholesterol secretion restores glycemic control, improves hepatic insulin signaling, and reduces plasma triglycerides in obese and insulin-resistant db/db mice. These phenotypic differences were associated with reduced markers of ER stress and increased measures of insulin signaling in liver. Protein levels of 3-hydroxy-3-methylglutaryl-coenzyme A reductase and LDLR were determined in an independent cohort of db/db mice. Data are mean±SEM (n=3–6). Horizontal lines terminating in vertical bars denote significant difference from control virus within genotype. *P<0.05, ***P<0.001, ****P<0.0001.
of G5G8 and accelerated biliary cholesterol secretion during a longer period would promote clearance of hepatic TGs will require alternative approaches to transient expression by adenoviral gene delivery.

**G5G8 and the UPR**

This study is the converse of our previous work showing that the absence of G5G8 exacerbates the development of insulin resistance, steatosis, and inflammation in diet-induced obesity. In the previous study, the development of hepatic insulin resistance was associated with an increase in eIF2α signaling and reduced phospho-Akt. Consistent with this pathway playing a causative role in hepatic insulin resistance in G5G8-deficient mice, the overexpression of G5G8 in db/db mice reduced eIF2α signaling and increased phospho-Akt. An interesting feature of UPR activation in G5G8 deficiency and its alleviation by AdG5G8 is that it is largely limited to the PERK arm of the UPR. The prevailing model for activation of the UPR is the release of inositol-requiring enzyme 1 (IRE1), ATF6, and PERK from GRP78 by the accumulation of misfolded and unfolded proteins. Although there is some evidence for selective activation of UPR signaling, it is important to note that multiple stress-activated kinases can phosphorylate eIF2α. In addition, the specificity may have nothing to do with selective activation of the UPR but is rather an alternate mechanism for activating eIF2α. In addition, a recent report indicates that PERK and inositol-requiring enzyme 1 are responsive to changes in membrane lipid composition, independently of changes in protein folding in the ER. Whether membrane cholesterol content similarly affects UPR signaling is not known. The question of how G5G8 influences UPR signaling remains unresolved in the present study because we have yet to mechanistically link the absence of G5G8 to activation of a specific eIF2α kinase or other element of the UPR.

ER stress and the UPR are also thought to play a direct role in the upregulation of lipogenesis in leptin-deficient mice. In these studies, suppression of the UPR through adenoviral expression of GRP78 represses hepatic lipogenesis and lowers hepatic TGs. Although AdG5G8 reduced plasma TGs and lipogenic gene expression, this was not sufficient to reduce hepatic lipids. These differences may be a matter of degree in which GRP78 more strongly represses lipogenesis or could be because of the disruptions in cholesterol homeostasis associated with AdG5G8. We did not observe changes in GRP78 after AdG5G8 administration in obese mice; the effect of GRP78 and the alleviation of ER stress by this mechanism on G5G8 may be independent of leptin signaling and that ER stress may directly affect G5G8.

**Leptin and G5G8**

In both ob/ob and db/db mice, G5G8 protein levels are posttranscriptionally reduced and can be increased by caloric restriction or the administration of molecular chaperones. This suggests that reductions in G5G8 are independent of leptin signaling and that ER stress may directly affect G5G8.
Figure 4. Adenoviral vectors encoding G5G8 (AdG5G8) reduces lipogenic gene expression and lowers plasma triglycerides (TGs). Obese (db/db) male mice and their lean littermates were administered control or AdG5G8 virus. A, Total RNA was extracted from liver 72 hours after viral administration and was analyzed by reverse-transcriptase polymerase chain reaction for expression of lipogenic genes. B, Full-length and processed sterol regulatory element–binding protein (SREBP) 1 were analyzed by immunoblotting in whole cell lysates and nuclear extracts, respectively. C, Densitometry of SREBP immunoblots. Signal intensities are normalized to calnexin (CNX) and expressed as percent of the control mean. D and E, Plasma and hepatic TGs were determined by enzymatic colorimetric assay. F, TG secretion rates were determined after injection of Triton WR 1339. Data are mean±SEM (n=3–4). *P<0.05, **P<0.01, ****P<0.0001.

abundance.\(^{30}\) Perhaps this is not surprising given the dependence of G5G8 formation on lectin chaperones within the ER.\(^{37}\) Conversely, neither diabetes mellitus nor obesity alone is sufficient to reduce hepatic G5G8.\(^{30}\) We recently evaluated another model of obesity and insulin resistance for abundance of G5G5 at the protein level: LDLR-deficient mice maintained on a high-fat, high-cholesterol, Western-type diet for 16 weeks. In this model, G5 protein levels are increased because another model of obesity and insulin resistance for abundance.\(^{30}\) Perhaps this is not surprising given the dependence of G5G8 formation on lectin chaperones within the ER. Conversely, neither diabetes mellitus nor obesity alone is sufficient to reduce hepatic G5G8.\(^{30}\) We recently evaluated another model of obesity and insulin resistance for abundance of G5G5 at the protein level: LDLR-deficient mice maintained on a high-fat, high-cholesterol, Western-type diet for 16 weeks. In this model, G5 protein levels are increased because of the activation of liver X receptor by high-cholesterol diet (Figure V in the online-only Data Supplement). Thus, destabilization of hepatic G5G8 seems to be a feature of obesity limited to genetic models that lack a functional leptin axis. In addition, the upregulation of G5G8 and increased biliary cholesterol secretion may play a protective role in the insulin-resistant liver. Consistent with this idea, ablation of hepatic insulin signaling through liver-specific deletion of insulin receptor increases G5G8 mRNA in a forkhead box O1A–dependent manner.\(^{38}\)

G5G8 and Cholesterol Homeostasis

The increase in plasma cholesterol levels after AdG5G8 administration in db/db mice was unexpected. The ability of EZ to block this effect of AdG5G8 indicates that this is most likely because of intestinal reabsorption of cholesterol from enriched bile. An enterohepatic pool of cholesterol has been described and is thought to expand during the development of obesity in mice.\(^{39}\) The apparent expansion of this pool of cholesterol resulted in perturbations of plasma and hepatic cholesterol homeostasis that are difficult to unravel and are beyond the scope of the present study. However, this observation highlights the fact that accelerating biliary cholesterol secretion is ineffective in reducing plasma cholesterol unless there is a concomitant increase in intestinal G5G8 or other secretory mechanisms to oppose reabsorption. Similar observations were made in transgenic mice. A liver-specific G5G8 transgene was ineffective in reducing plasma cholesterol or atherosclerosis in the absence of EZ, whereas a human G5G8 transgene under the control of the endogenous promoter that drives expression in both liver and intestine can reduce cholesterol homeostasis that are difficult to unravel and are beyond the scope of the present study. However, this observation highlights the fact that accelerating biliary cholesterol secretion is ineffective in reducing plasma cholesterol unless there is a concomitant increase in intestinal G5G8 or other secretory mechanisms to oppose reabsorption. Similar observations were made in transgenic mice. A liver-specific G5G8 transgene was ineffective in reducing plasma cholesterol or atherosclerosis in the absence of EZ, whereas a human G5G8 transgene under the control of the endogenous promoter that drives expression in both liver and intestine can reduce cholesterol and atherosclerosis.\(^{30,41}\)

The primary implication of these findings is that the intestine mitigates the effectiveness of cholesterol elimination by the liver. The role of the intestine in cholesterol elimination has regained attention, with recent studies characterizing transintestinal cholesterol elimination.\(^{42}\) Although G5G8 is thought to be responsible for increasing transintestinal cholesterol elimination in response to liver X receptor agonists, there is a G5G8-independent component that is yet to be elucidated.\(^{43}\) Although this pathway can compensate for disruptions in biliary cholesterol elimination, it is not clear...
how hepatic and intestinal pathways cooperate to maintain cholesterol homeostasis. In this and previous studies in which hepatic cholesterol secretion is significantly elevated, transintestinal cholesterol elimination is insufficient to prevent the reabsorption and accumulation of cholesterol in plasma.

**Limitations**

The primary limitation of the present study is the use of adenoviral vectors to test the effect of increased GS58-mediated biliary cholesterol elimination. These vectors dramatically increase protein expression that peaks between 48 and 72 hours after administration and then declines at a rate that is largely dependent on the half-life of the recombinant protein. In the present study, we cannot account for the simultaneous increase in fecal, biliary, hepatic, and plasma cholesterol in AdGS58-administered db/db mice. Several possibilities exist that include mobilization from extrahepatic tissues, such as adipose, or extrahepatic synthesis. Alternatively, there may be a robust, transient increase in hepatic synthesis followed by repression as cholesterol accumulates in liver. Given the dynamic nature of adenoviral-mediated gene expression, mice are unlikely to achieve steady state. Consequently, studies to evaluate the efficacy of accelerated biliary cholesterol elimination on hepatic steatosis will require approaches that result in sustained increases in GS58 expression. Such approaches will also facilitate examination of other features of NAFLD such as inflammation and apoptosis.

**Acknowledgments**

We thank Dr Victoria King (University of Kentucky) for liver samples from low-density lipoprotein receptor–deficient mice.

**Sources of Funding**

This work was funded by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (to G.A. Graf: R01DK080874) and the National Institute of General Medical Sciences (8 P20 GM103527-05) of the National Institutes of Health.

**Disclosures**

None.

**References**


fatty liver disease.

role for cholesterol in the development of obesity-related liver phenotypes. It also suggests that therapeutic approaches to actively reduce

characterized by elevated levels of endoplasmic reticulum stress. These findings add to a growing body of literature that supports a causal

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2008;23:1635–1648.


2012;23:85–90.

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Arterioscler Thromb Vasc Biol. 2014;34:26-33; originally published online November 7, 2013;
doi: 10.1161/ATVBAHA.113.302355
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplemental Figure I

Fig SI. Nine C57 mice were group housed in clean cages (3/cage) and feces collected for 48 hr prior to viral delivery (Day 0). AdG5G8 was administered on Day 0 and feces collected on day 2, 4, 6, 10 and 14. Feces from were dried, lipids extracted and analyzed by GC/MS. Data are mean ± SEM (n=3 cages) and were analyzed by 1-Way repeated measures ANOVA. Dunnett’s multiple comparison tests were used to determine differences from pretreatment fecal neutral sterols (Day 0, **p<0.01, ***p<0.001).
Supplemental Figure II

A

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B

![Graph showing ALT levels (U/L) for control and AdG5G8 groups.](image)

C

![Graph showing relative mRNA expression for control and AdG5G8 groups.](image)

Fig SII. Effects of AdG5G8 on intestinal G5 and G5 protein levels (A), liver enzymes (B), and hepatic expression of inflammatory cytokines in obese, db/db mice. Values are mean ± SEM (n=4).
Figure SIII. AdG5G8 suppresses insulin signaling genes in obese mice. Total RNA was pooled from three db/db mice administered control virus or AdG5G8 and analyzed by PCR array. A) PCR Array layout with gene symbol, fold change and flags indicated. B) Heat Map of gene expression data. C) Genes with greater than a two-fold difference in abundance in AdG5G8 compared to controls are plotted by fold-change. # above bars indicates confirmation of significant difference by rtPCR in individual mice. Acaca1 upregulation could not be confirmed by rtPCR.
Figure SIV. Effect of AdG5G8 on ApoE. A) Immunoblot and densitometry analysis of serum ApoE in control and AdG5G8 treated mice. B, C) Total RNA was extracted from liver and small intestine 72 hr following viral administration and analyzed by rtPCR. Data are mean ± SEM (n=4).
Supplemental Figure V

Figure SV. Hepatic membrane proteins (50 µg) from LDL receptor deficient mice maintained on a chow or western diet for 16 weeks were isolated and subjected to SDS-PAGE and immunoblot analysis for G5 and G8. β-actin was used as a loading control.
Supplemental Methods

Reagents:

General chemicals were purchased from Sigma. Horseradish peroxidase conjugated secondary antibodies and SuperSignal West Pico Chemiluminescent reagents were purchased from Thermo/Pierce. Real-time PCR reagents were purchased from Applied Biosystems. Calnexin, glucose-regulated protein 78-kD (GRP78), and glucose-regulated protein 94-kD (GRP94) antibodies were purchased from Nventa (San Diego, CA). Total and phospho-eIF2α, total and phospho-Akt, and β-actin antibodies were purchased from Cell Signaling. SREBP, insulin receptor and phospho-insulin receptor antibodies were purchased from Santa Cruz. Total and phospho-protein kinase-like endoplasmic reticulum kinase (PERK) and total and phospo-insulin receptor substrate 1 (IRS1) antibodies were purchased from Cell Signaling. The antibody to 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) was purchased from Upstate. The SR-BI antibody was a kind gift from Deneys R. van der Westhuyzen (University of Kentucky). Production of ABCG5 and ABCG8 antibodies has been previously reported. Phospholipid, cholesterol and triglyceride (TG) assays were purchased from Wako Chemical (Richmond, VA).

Animals and Treatments

Male, db/db (stock #000697) mice and their lean littermates were obtained from Jackson Laboratory (Bar Harbor, ME). A second cohort of strain matched lean mice were also purchased from the same vendor (C57BL6/J, stock #000664). Mice were housed in individually ventilated cages and provided enrichment in the form of nesting material and acrylic huts. The animal room was set to a 14:10 hour dark/light cycle and a temperature of 22 ± 3°C. Upon arrival mice were allowed to adapt to the environment for a period of 7 days. All mice were maintained on standard rodent chow diet (Harlan Teklad 2014S). Where indicated, the chow diet was formulated with ezetimibe (0.005% w/w) by Research Diets Inc. (New Brunswick, NJ). All animal procedures conform to PHS 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.

Recombinant adenoviral vectors containing no insert (AdEmpty) and cDNAs for ABCG5 and ABCG8 have been described previously.2,3 Adenoviral vectors were amplified in HEK293Q cells, purified on cesium chloride gradients, and dialyzed against physiological saline. On Day 0 of the experiment mice were placed in clean cages and injected with a total of 5×10^11 particles/kg via the tail vein. After 72 hours (Day 3), mice were placed in clean cages and a fasting glucose was determined following a 4 hour fast beginning shortly after “lights-on” using blood obtained from a tail-vein prick and a standard glucometer. The animals were anesthetized with ketamine:xylazine anesthesia, blood, gall bladder bile, tissues and feces were collected. Serum was harvested by centrifugation. Tissues were snap frozen in liquid nitrogen and stored at -80°C until analyzed.

A glucose tolerance test (GTT) was conducted in an independent cohort of db/db mice as previously described.4 Mice were fasted for four hours beginning at “lights-on”. Mice were injected with 20% glucose solution (10 μl/ gram of body weight i.p.). In db/db mice, an insulin sensitivity test was also conducted. Fasted mice were administered human recombinant insulin (1 IU/kg i.p.). In both assays, blood glucose levels were measured prior to and 30, 60, 90 and 120 minutes following glucose and insulin injections. Areas under the curve (AUC) were calculated for blood glucose levels for each individual mouse. Triglyceride (TG) secretion rates were determined in a separate cohort of db/db mice (n=4). Mice were administered TritonWR-1339 (650 mg/kg body weight i.v). Blood was collected using
heparinized capillary tubes before and 0.5, 1, 2 and 3 h following injections.

**Analytical Procedures**

The preparation of membrane proteins, SDS-PAGE and immunoblotting were conducted as previously described. Real-time PCR and XBP-1 splicing assays and were conducted as described previously. The Insulin Signaling Pathway PCR Array was purchased from QiAgen and conducted according to the manufacturer’s instructions.

Serum triglycerides and cholesterol in total and FPLC fractionated serum were measured with enzymatic colorimetric assays. Hepatic lipids were also determined using colorimetric assays following Folch extraction. Briefly, 100 mg of liver was ground using a Teflon pestle-glass homogenizer. Lipids were extracted in 2:1 chloroform:methanol at room temperature. The organic phase was dried under nitrogen gas, solubilized in 1 volume of 2% (v/v) Triton X-100 in chloroform, dried under streaming nitrogen, and solubilized in 2 volumes of water. Hepatic triglycerides, phospholipids and total cholesterol were normalized to wet tissue weight.

Fecal neutral sterols were analyzed as previously described. Total feces from the 72 hour period was collected, dried at 37°C, weighed and ground to powder. An aliquot of 0.125 g feces was placed into a glass tube with 1.25 ml ethanol and 0.25 ml 10N NaOH. Lipids were saponified at 72°C in a water bath for 2 hours and extracted (water, ethanol and petroleum ether, 1:1:1 v:v:v). 5α-cholestanene was used as internal standard. Following extraction, the organic phase was dried under a steam of nitrogen gas, and solubilized in hexane. The amount of neutral sterols (cholesterol, coprostanol and cholestanol) was quantified by GC/MS. Similarly, biliary cholesterol concentrations were determined by GC-MS from 5 ul of gallbladder bile following addition of the internal standard and Folch extraction.

**Statistical analysis**

Data are expressed as the mean ± SEM. Data were analyzed by two-way ANOVAs with genotype (lean vs. db/db) and treatment (control vs. AdG5G8) as factors. Bonferroni posttests were applied where indicated to determine differences due to genotype within control or AdG5G8 treated animals. Where only obese mice were considered, comparisons between control and AdG5G8 were conducted using a two-tailed t-test. Rates of TG secretion were determined by linear regression. Differences were considered significant at p < 0.05.

**Supplemental References**
