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Liver Specific Inactivation of Carboxylesterase 3/Triacylglycerol Hydrolase Decreases Blood Lipids Without Causing Severe Steatosis in Mice

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Carboxylesterase 3/triacylglycerol hydrolase (Ces3/TGH) participates in hepatic very low-density lipoprotein (VLDL) assembly and in adipose tissue basal lipolysis. Global ablation of *Ces3/Tgh* expression decreases serum triacylglycerol (TG) and nonesterified fatty acid levels and improves insulin sensitivity. To understand the tissue-specific role of Ces3/TGH in lipid and glucose homeostasis, we generated mice with a liver-specific deletion of *Ces3/Tgh* expression (L-TGH knockout [KO]). Elimination of hepatic *Ces3/Tgh* expression dramatically decreased plasma VLDL TG and VLDL cholesterol concentrations but only moderately increased liver TG levels in mice fed a standard chow diet. Significantly reduced plasma TG and cholesterol without hepatic steatosis were also observed in L-TGH KO mice challenged with a high-fat, high-cholesterol diet. L-TGH KO mice presented with increased plasma ketone bodies and hepatic fatty acid oxidation. Intrahepatic TG in L-TGH KO mice was stored in significantly smaller lipid droplets. Augmented hepatic TG levels in chow-fed L-TGH KO mice did not affect glucose tolerance or glucose production from hepatocytes, but impaired insulin tolerance was observed in female mice. *Conclusion*: Our data suggest that ablation of hepatic *Ces3/Tgh* expression decreases plasma lipid levels without causing severe hepatic steatosis. (HEPATOLOGY 2012;56:2154-2162)

Increased plasma triacylglycerol (TG) concentration represents an independent risk factor for cardiovascular disease.[1,2] Elevated levels of circulating TG-rich, apolipoprotein B (apoB)-containing lipoproteins (very low-density lipoprotein [VLDL] and chylomicrons) accompany insulin resistance and visceral obesity.[1,3-5] Secretion of VLDL depends on the availability of lipids for apoB lipidation, and therefore, intracellular VLDL assembly presents a potential pharmacological target for the treatment of hypertriglyceridemia and associated cardiovascular and metabolic diseases.[6,7]

Accumulating evidence suggests that the formation of apoB-containing lipoproteins is accomplished by a two-step process, where a primordial lipoprotein with low amounts of TG is initially synthesized, followed by the addition of TG from preexisting lipid storage pools.[8-10] Preformed intrahepatic TG was shown to be the source of 60%-80% of VLDL-TG; however, this TG is not transferred to the primordial lipoprotein *en bloc* but is delivered via a process involving lipolysis and re-esterification.[11-15] Triacylglycerol hydrolase (TGH), also termed carboxylesterase 3 (Ces3) in mice and carboxylesterase 1 in humans, has been suggested to play an important role in the provision of TG for the assembly of apoB-containing lipoproteins.[16-19]

Abbreviations: apoB, apolipoprotein B; CE, cholesteryl ester; Ces3, carboxylesterase 3; Es-X, esterase-x; FA, fatty acids; FC, free cholesterol; G6p, *glucose-6-phosphatase; HSL, hormone-sensitive lipase; KO, knockout; LD, lipid droplet; MTP, microsomal triglyceride transfer protein;* Pepck, *phosphoenolpyruvate carboxykinase; p-HSL, phosphorylated hormone-sensitive lipase; PL, glycero-phospholipids; TG, triacylglycerol; TGH, triacylglycerol hydrolase; VLDL, very low-density lipoprotein; WAT, white adipose tissue; WTD, Western-type diet.*

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One major concern with targeting VLDL assembly as drug therapy for hyperlipidemia is that inhibition of hepatic VLDL secretion may lead to nonalcoholic fatty liver disease and increased susceptibility to toxin-induced injury. Such condition has been observed upon chemical inhibition or genetic disruption of microsomal triglyceride transfer protein (MTP).[7,9,20,21] Nonalcoholic fatty liver disease is strongly associated with obesity and insulin resistance and is now considered to be the hepatic representation of the metabolic syndrome.[22-24] Peripheral insulin resistance may lead to fatty liver through increased circulating fatty acids (FA), glucose and insulin, which stimulate hepatic lipid synthesis and impair FA oxidation.[22,23,25]

Ces3/TGH is highly expressed in the liver and adipose tissue.[26] Although hepatic VLDL-apoB100 secretion was inhibited and plasma lipid levels were decreased in chow-fed Ces3/TGH-deficient mice, these mice did not develop fatty liver, which was attributed to both increased hepatic FA oxidation and decreased delivery of FA derived from lipolysis in white adipose tissue (WAT).[27] Ces3/TGH-deficient mice also exhibited improved systemic glucose clearance and insulin sensitivity, decreased pancreatic islet size, and expression of hepatic gluconeogenesis-related genes, suggesting that the improved insulin sensitivity and lack of hepatic steatosis may be the consequence of ablation of *Ces3/Tgh* expression in all tissues (liver, adipose tissue, and islets).[27]

In this study, we generated liver-specific Ces3/TGH-deficient mice (L-TGH knockout [KO]) using a conditional knockout strategy and investigated the role of hepatic Ces3/TGH in VLDL secretion and lipid and glucose metabolism.

Materials and Methods

Generation of Liver-Specific Ces3/TGH-Deficient (L-TGH KO) Mice. The steps to obtain L-TGH KO mice are outlined in Fig. 1A, and details are provided in the Supporting Information. All animal procedures were approved by the University of Alberta's Animal Care and Use Committee and were performed in accordance with the guidelines of the Canadian Council on Animal Care. Mice, housed three to five per cage, were exposed to a 12-hour light/dark cycle beginning with light at 8:00 AM. Adult male and female mice were fed *ad libitum* a chow diet (LabDiet, PICO laboratory Rodent Diet 20) or a high-fat, high-cholesterol diet [referred to as a Western-type diet (WTD)] (TD 88137, Harlan Teklad), containing 21% fat, 0.2% cholesterol by weight for 2 weeks. Twelve- to 16-week-old mice were used in the experiments.

Plasma Parameters. Plasma insulin levels were measured using the Rat/Mouse Insulin ELISA kit (Millipore Corporation, Billerica, MA). Plasma nonesterified FA levels were determined using the NEFA-C kit (Wako Chemicals). Plasma β-hydroxybutyrate levels were measured via the cyclic enzymatic method using Autokit Total Ketone Bodies (Wako Chemicals, Code No. 415-73301).

Determination of Liver and Plasma Lipids. The amounts of indicated lipids were determined in liver homogenates (0.5 mg of protein) or 50 μL of plasma by gas chromatography as described.[19]

Fast Protein Liquid Chromatography Analysis of Plasma Lipoproteins. Size-exclusion chromatography was used to determine lipid levels in VLDL and high-density lipoprotein fractions.[19,27]

RNA Isolation and PCR Analysis. Total liver RNA was isolated using Trizol reagent (Life Technologies, Inc.). Quantitative real-time PCR was employed. All primers were synthesized at Integrated DNA Technologies. Primers for the various genes are listed in Supporting Table 2.

In Vivo *Insulin Signaling.* Mice were fasted for 16 hours before 0.5 unit/kg body weight of bovine insulin or PBS was injected via the portal vein. Livers were harvested 5 minutes after injection. Cell lysates were immunoblotted for anti-AKT and anti-p-AKT (Cell signaling Technology). The ratio between p-AKT and AKT was employed to evaluate hepatic insulin sensitivity.[28-30]

Pyruvate Tolerance Test. Two g/kg body weight of sodium pyruvate (Sigma) was intraperitoneally injected into 4-hour fasted mice and blood was drawn at the indicated times after injection.[31] Blood glucose levels were monitored using blood glucose strips and the Accu-Check glucometer (Roche Diagnostics, Vienna, Austria).

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Additional Supporting Information may be found in the online version of this article.

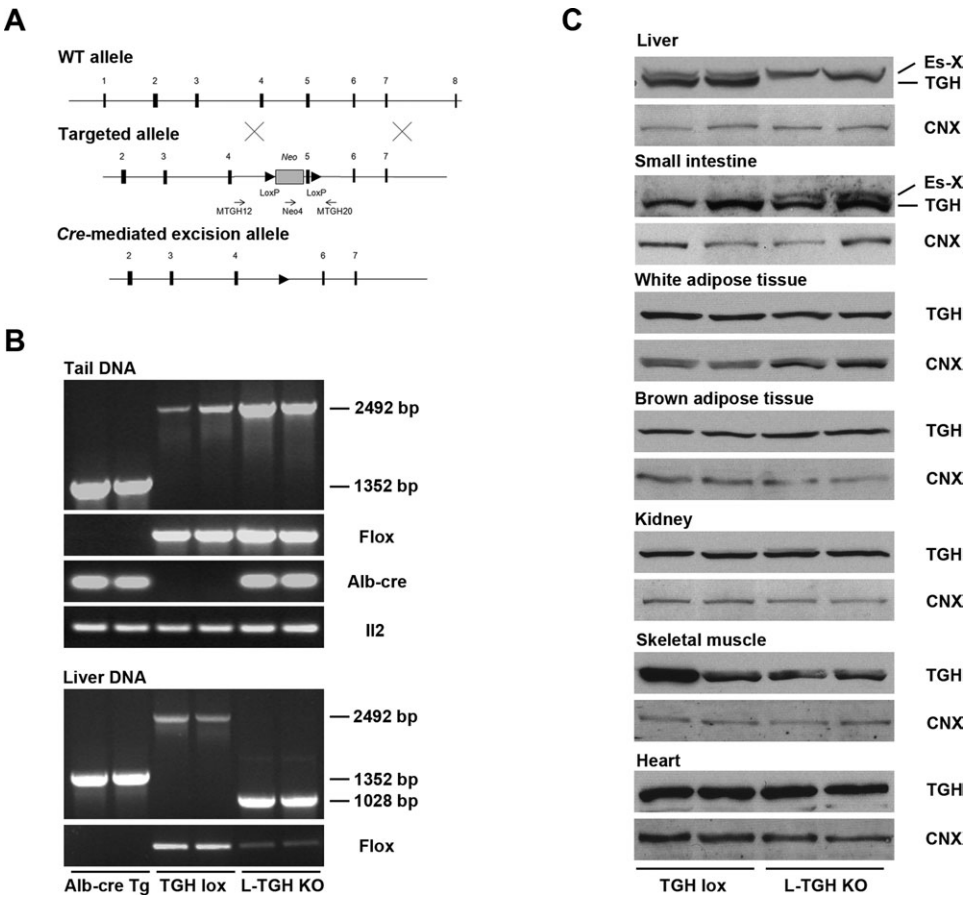

Fig. 1. Generation and characterization of L-TGH KO mice. (A) Exons 1-5 of the mouse *Tgh* gene are indicated by black boxes (top line). The middle line shows the wild-type allele that was replaced by recombination with the insertion cassette (arrowhead), LoxP site. The bottom line indicates the allele after *cre*-mediated excision. MTGH12 and MTGH20 are primers used to amplify genomic DNA specific in wild-type allele, Neo4 and MTGH20 are primers used to amplify the flox-specific product. (B) Genotyping of tail DNA and liver DNA. PCR analysis detected the presence of the LoxP sites (Flox), Alb-Cre transgene, wild-type and knockout allele, Il2 was amplified as an internal control. (C) Immunoblot analysis shows the liver-specific deletion of TGH. Es-X, esterase-X (Ces1/Es-x); TGH, Ces3/TGH; CNX, calnexin (loading control).

Oral Glucose Tolerance Test and Insulin Tolerance Test. Oral glucose tolerance test and insulin tolerance test were performed as described.[27]

Statistical Analysis. Data are presented as the mean ± SEM. Statistical significance was evaluated via unpaired two-tailed Student *t* test. Pyruvate tolerance test, oral glucose tolerance test, and insulin tolerance test results were analyzed via two-way analysis of variance followed by a Bonferoni posttest using GraphPad PRISM 4 software. Five to nine animals were used in each experimental group. $P < 0.05$ was considered a significant difference.

Results

Generation of Liver-Specific Ces3/TGH-Deficient (L-TGH KO) Mice. Mice with a floxed allele of *Ces3/Tgh* were generated (Fig. 1A) and crossed with albumin-*Cre* mice to produce liver-specific Ces3/TGH-deficient mice (L-TGH KO) (Fig. 1B). Ces3/TGH lox mice ($Tgh^{lox/lox}$/*Alb-Cre*$^{0/0}$) were used as controls. The presence of cell types in the liver other than hepatocytes accounted for some of the undeleted allele (Flox band) detected in the *Ces3/Tgh*-deficient liver samples (Fig. 1B). Ces3/TGH western blot analysis confirmed the lack of Ces3/TGH in the liver, but not in other tissues (Fig. 1C). Hepatic levels of mouse carboxylesterase 1 (Es-X), a related carboxylesterase family member found in the carboxylesterase gene cluster on chromosome 8, remained unaltered in the L-TGH KO mice (Fig. 1C).

L-TGH KO mice appeared outwardly unremarkable with normal activity and breeding behavior. They exhibited no differences in body weight, fat mass, or plasma FA levels when compared with TGH lox mice at 14-16 weeks of age (Supporting Table 1). No significant differences in tissue weight (liver, WAT, brown adipose tissue (BAT))/body weight ratios were observed between the genotypes (TGH lox and L-TGH KO).

Decreased Blood Lipid Levels in L-TGH KO Mice. Because the loss of Ces3/TGH in adipose tissues contributes to reduced FA release from adipose, which may consequently result in decreased liver TG stores and VLDL secretion, we investigated whether liver-specific Ces3/TGH deficiency is sufficient to inhibit VLDL secretion *in vivo*. L-TGH KO mice presented with decreased plasma concentration of free (unesterified) cholesterol (FC), cholesteryl esters (CE), glycero-

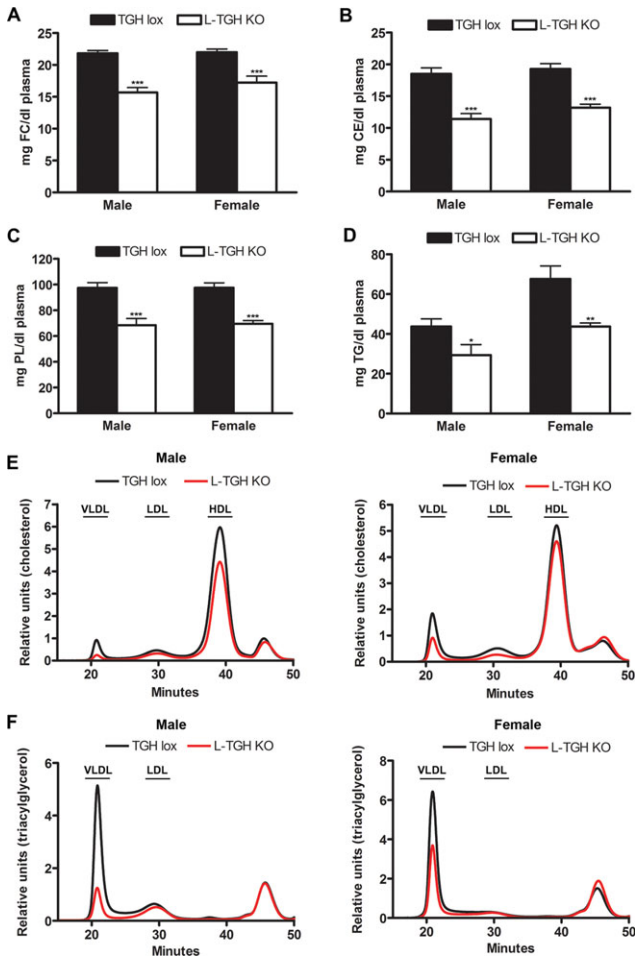

Fig. 2. Decreased blood lipids in L-TGH KO mice. Mice were fasted for 16 hours before blood collection. Plasma lipids were extracted and analyzed via gas chromatography. Plasma FC (A), CE (B), PL (C), and TG (D). Pooled plasma aliquots from fasted mice were analyzed for cholesterol (E) and TG (F) content in lipoprotein fractions by fast-protein liquid chromatography. Data are presented as the mean ± SEM (n = 5-9 per group). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus Ces3/TGH lox control group.

phospholipids (PL), and TG by 28.2%, 38.4%, 29.8%, and 32.8%, respectively, in male mice, and by 21.7%, 31.6%, 28.7%, and 35.4%, respectively, in female mice (Fig. 2A-D). L-TGH KO mice had significantly decreased levels of VLDL cholesterol (Fig. 2E) and VLDL TG (Fig. 2F). Total plasma cholesterol concentrations were significantly reduced in male and female L-TGH KO mice by 33% and 26%, respectively.

Increased Hepatic TG Accumulation in L-TGH KO Mice. While the concentrations of FC and PL in the livers of L-TGH KO mice did not differ from control mice (Fig. 3A,C), mice lacking hepatic Ces3/TGH had increased liver TG mass by 123.2% and 73.9% in males and females, respectively (Fig. 3D). Hepatic Ces3/TGH deficiency resulted in increased liver CE mass by 109.1% in male mice but only showed an increased trend in female mice (Fig. 3B). The results are consistent with diminished TG and CE hydrolysis, as TG and CE have been demonstrated to be physiological substrates of the enzyme.[18,32,33]

Augmented hepatic neutral lipid storage in L-TGH KO mice was accompanied by increased levels of lipid droplet-associated protein ADRP (perilipin 2), while the levels of another lipid droplet-associated protein TIP47 (perilipin 3) remained unchanged (Fig. 3E). MTP, a key protein involved in hepatic VLDL assembly, was not affected by the ablation of *Ces3/Tgh* expression in the liver (Fig. 3E). Increased hepatic lipid storage is closely associated with endoplasmic reticulum stress, which in turn has been shown to impair hepatic insulin signaling and apoB secretion.[34-36] Proteins CHOP and GRP78, two players involved in the unfolded protein response to endoplasmic reticulum stress,[34,37] were not altered in L-TGH KO (Fig. 3E).

L-TGH KO Hepatocytes Have Altered Lipid Droplet Morphology and Number. Hepatocytes isolated from L-TGH KO mice contained a higher number of smaller lipid droplets (LDs) (Fig. 4A-D). The average area of an individual LD was decreased in L-TGH KO hepatocytes by approximately 20%, and the

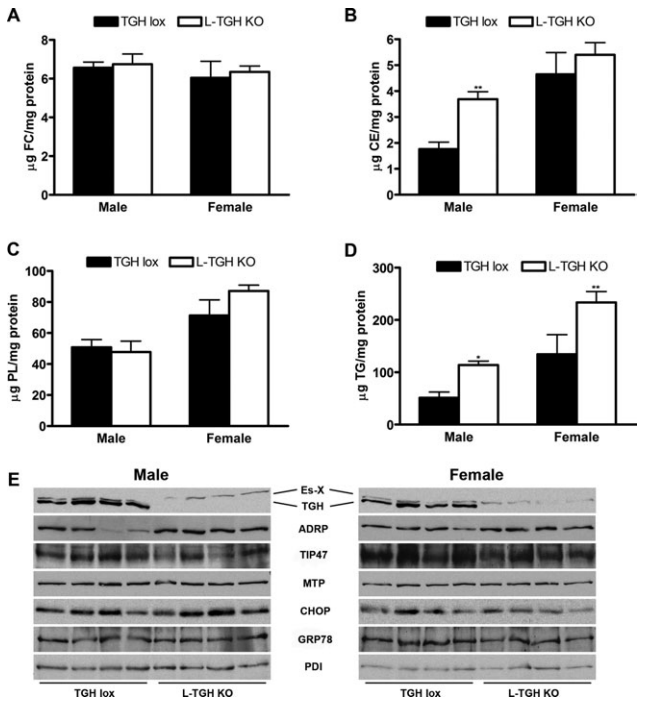

Fig. 3. Increased liver TG accumulation in L-TGH KO mice. Mice were fasted for 16 hours. Lipids were extracted from 0.5 mg liver homogenates and amounts of FC (A), CE (B), PL (C), and TG (D) were determined via gas chromatography. Data are presented as the mean ± SEM (n = 5-9 per group). $*P < 0.05$ and $**P < 0.01$ versus Ces3/TGH lox control group. (E) Immunoblot analysis of liver Ces3/TGH, Es-X, ADRP, TIP47, MTP, CHOP, GRP78, and PDI.

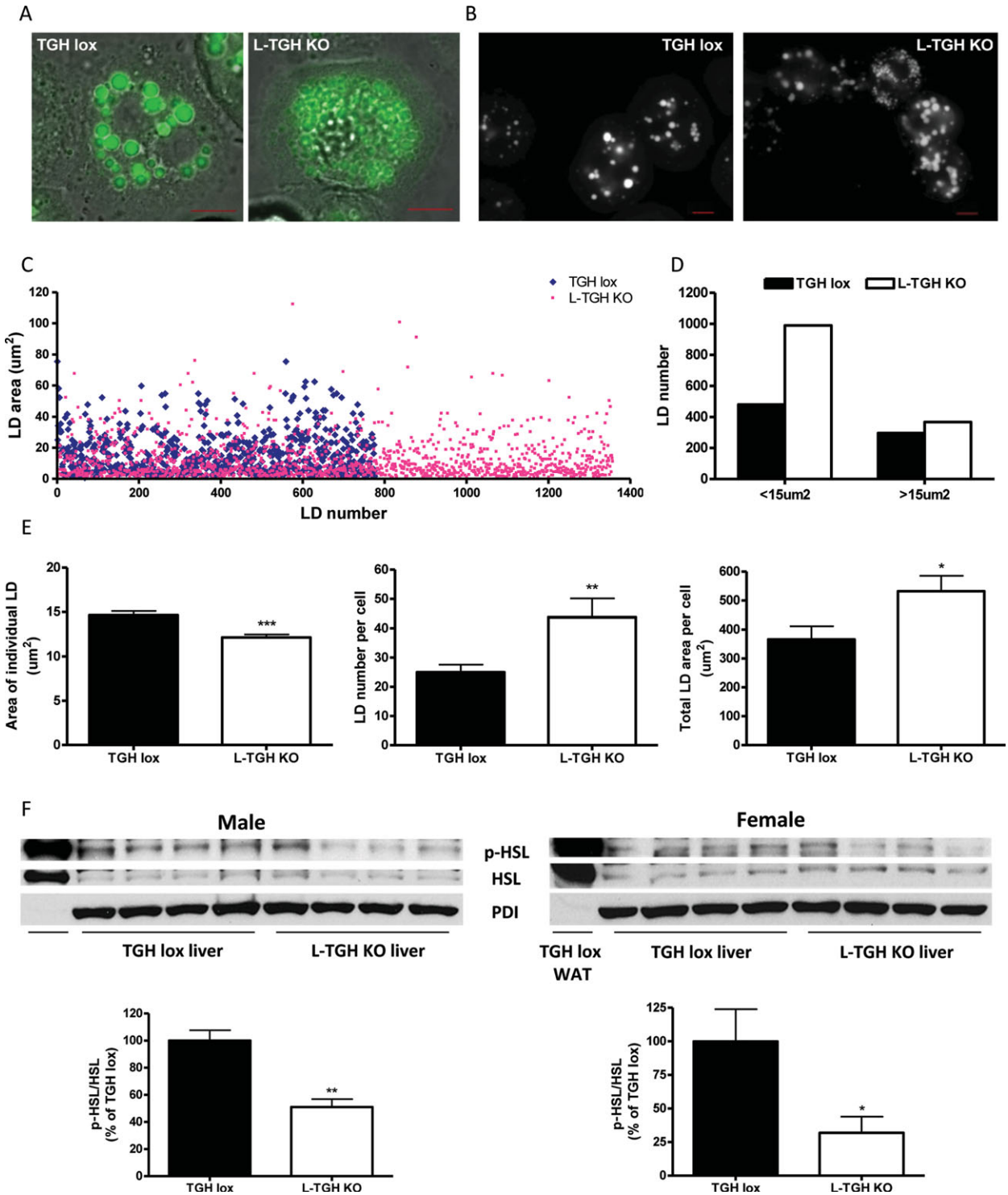

Fig. 4. Liver-specific TGH deficiency affects morphology of LDs. Eleven-week-old male Ces3/TGH lox (control) and L-TGH KO mice were fasted for 16 hours, hepatocytes were isolated and incubated in Dulbecco's modified Eagle's medium + 15% fetal bovine serum for 4 hours without fatty acid supplementation. LDs were stained with BODIPY 493/503 and visualized using florescence microscopy. (A) Morphology of LDs in representative cells of each genotype. Scale bar = 10 μm. (B) Representative fields. Scale bar = 10 μm. (C) Images of 31 cells from each genotype were captured. The semidiameter of LDs was analyzed with AxioVision Rel 4.6. Numbers and areas of LDs from the 31 cells were pooled. Each data point represents an individual LD. (D) Numbers of LDs with areas $\leq$15 μm^2 or $\geq$15 μm^2 in the 31 cells from each genotype. (E) Average area of an individual LD, number of LDs per cell, and total area of LDs per cell in the 31 cells from each genotype. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data are presented as the mean $\pm$ SEM. (F) Liver-specific TGH deficiency affects hepatic p-HSL on Ser^{660} but not HSL protein levels. Mice were fasted for 16 hours before sacrifice. Immunoblots of liver p-HSL, HSL, and PDI (loading control) in Ces3/TGH lox and L-TGH KO mice of each sex are shown. WAT from Ces3/TGH lox mice were used as a positive control for HSL immunoreactivity.

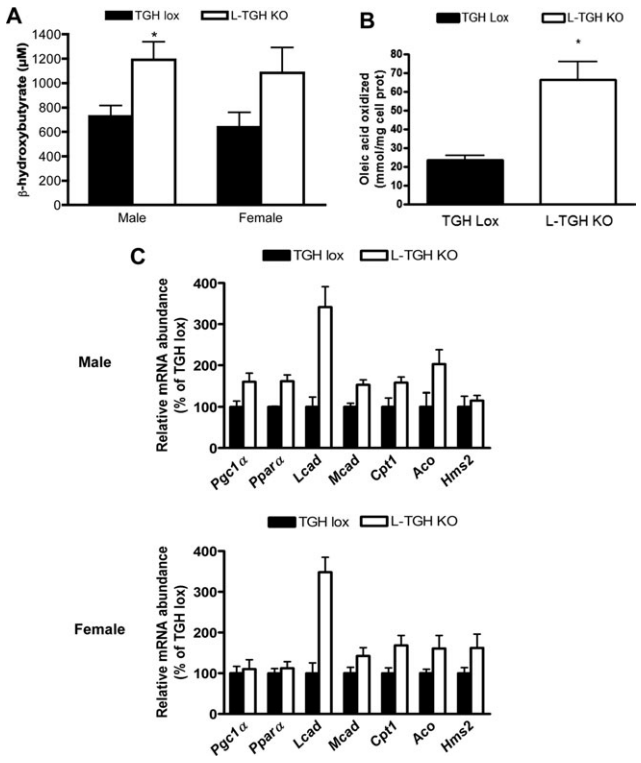

Fig. 5. Induced β-oxidation in L-TGH KO mice. (A) Plasma β-hydroxybutyrate from 16-hour fasted mice. (B) FA oxidation in primary hepatocytes isolated from male mice. (C) The messenger RNA abundance of genes related to fatty acid oxidation and ketogenesis in liver samples from 16-hour fasted mice was analyzed via quantitative real-time polymerase chain reaction. Data are presented as the ratio of expression of a given gene to cyclophilin (*Cyc*). Aco, acyl-CoA oxidase; Cpt1, carnitine palmitoyltransferase-1; Hms2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; Lcad, long-chain acyl-CoA dehydrogenase; Mcad, medium-chain acyl-CoA dehydrogenase; Pgc1α, peroxisome proliferator-activated receptor α coactivator-1α; Pparα, peroxisome proliferator-activated receptor α. Values are normalized to Ces3/TGH lox mice where the gene/*Cyc* ratio was set as 100%. Data are presented as the mean $\pm$ SEM (n = 5-9 per group). $^*P < 0.05$ and $^{***}P < 0.001$ versus Ces3/TGH lox control group.

number of LDs was increased by 80% (Fig. 4E), which is in agreement with increased neutral lipid and ADRP levels (Fig. 3). Increased neutral lipid (TG and CE) storage might be due to lower levels of activated hormone-sensitive lipase (phosphorylated HSL [p-HSL]) in L-TGH KO liver homogenates compared with control (Fig. 4F). No differences were observed in total HSL protein (Fig. 4F) or in adipose triglyceride lipase (data not shown).

Increased FA Oxidation in L-TGH KO Hepatocytes. Plasma β-hydroxybutyrate levels were increased by 64.1% ($^*P < 0.05$) and 70.0% ($P = 0.10$) in male and female L-TGH KO mice, respectively (Fig. 5A). FA oxidation in isolated hepatocytes from male L-TGH KO mice was also significantly augmented (Fig. 5B). The increase in plasma β-hydroxybutyrate and FA oxidation was not associated with significantly augmented expression of mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*Hms2*), the rate-limiting enzyme in ketogenesis, or with increased expression of peroxisome proliferator-activated receptor α (*Ppara*), carnitine acyltransferase-1 (*Cpt1*), medium-chain acyl-CoA dehydrogenase (*Mcad*) and acyl-CoA oxidase (*Aco*) (Fig. 5C). On the other hand, the expression of long-chain acyl-CoA dehydrogenase (*Lcad*), a mitochondrial target of peroxisome proliferator-activated receptor α, was 3.5- and 3.4-fold higher in male and female L-TGH KO mice, respectively, when compared to control mice (Fig. 5C).

Hepatic Insulin Signaling in L-TGH KO Mice. Levels of phosphorylated Akt in response to exogenous insulin administration were not altered by Ces3/TGH deficiency (Fig. 6A), suggesting unaffected hepatic insulin signaling. Furthermore, hepatic expression of key gluconeogenic genes phosphoenolpyruvate carboxykinase (*Pepck*) and glucose-6-phosphatase (*G6p*) were maintained at high levels in the fasted state as expected and were drastically reduced to similar extent in the refed state in both L-TGH KO and control mice (Fig. 6B,C), suggesting a normal ability of endogenous insulin to shut down fasting-induced gluconeogenesis. Male L-TGH KO mice exhibited comparable plasma glucose concentration during a pyruvate tolerance test (Fig. 6D), and no differences between L-TGH KO and controls were observed in glucose production from glycerol or glucose output from isolated primary hepatocytes (Supporting Fig. 1). Female L-TGH KO mice presented with a marginally higher glucose output from pyruvate (Fig. 6D).

No difference of blood glucose and fasted plasma insulin levels were observed in L-TGH KO mice (Supporting Table 1). Plasma insulin levels in the fed state were decreased in male but not female L-TGH KO mice (Supporting Table 1). Both male and female L-TGH KO mice displayed a normal oral glucose tolerance (Fig. 7A). Whereas male L-TGH KO mice exhibited similar decline of blood glucose levels in insulin tolerance tests (Fig. 7B), the decline of blood glucose levels was less pronounced in female L-TGH KO mice than in control mice, suggesting impaired insulin tolerance in female L-TGH KO mice.

L-TGH KO mice challenged with a WTD for 2 weeks presented with decreased plasma lipid levels (Fig. 8A). Although the WTD regimen increased hepatic TG content in control mice 4-fold compared with chow-fed animals (Fig. 8B versus Fig. 3D), hepatic TG concentrations in L-TGH KO mice remained essentially similar in WTD-fed mice as in chow-fed mice (Fig. 8B versus Fig. 3D).

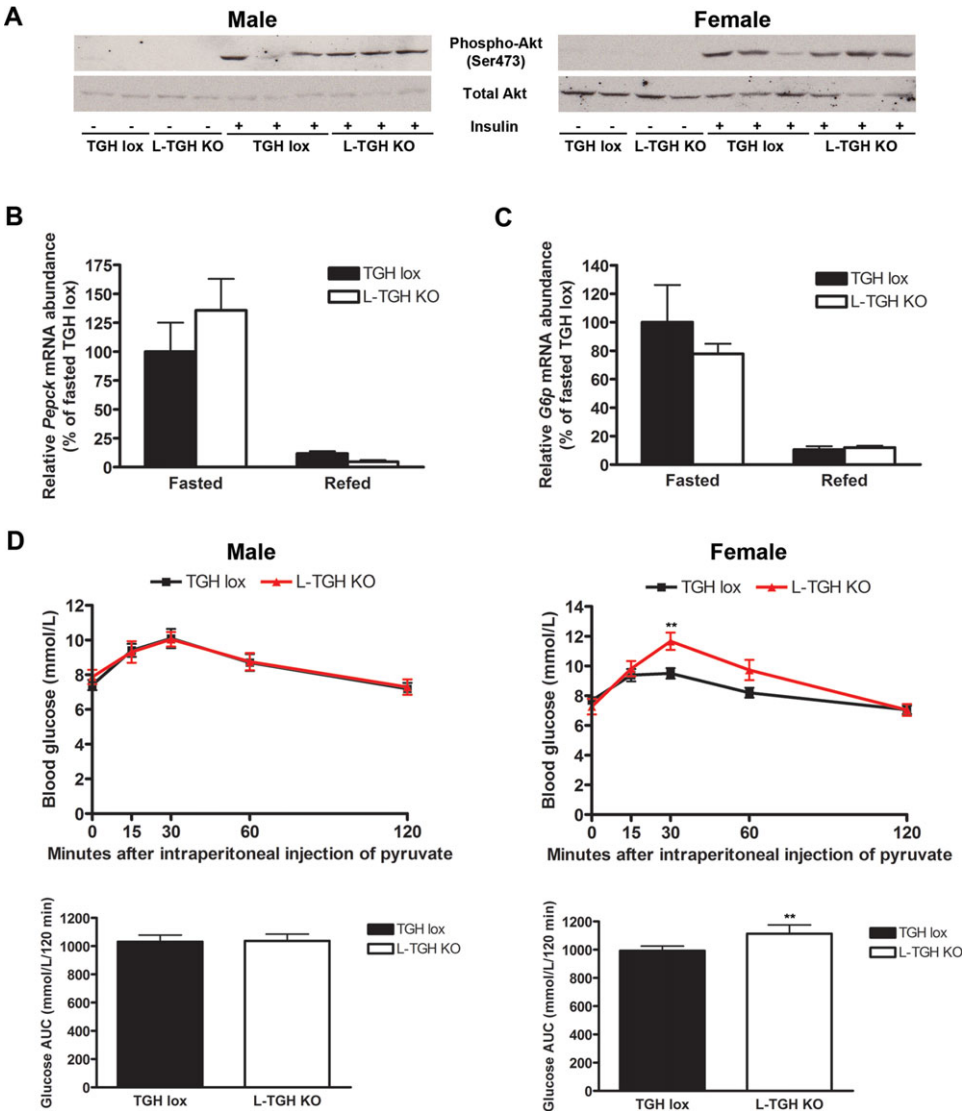

Fig. 6. Hepatic insulin signaling and gluconeogenesis in L-TGH KO mice. (A) Western blot of phosphorylated Akt (Ser473) and total Akt in livers from 16-hour fasted mice after intravenous injection of PBS (−) or insulin (+). Sixteen-hour fasted female mice were sacrificed (Fasted) or refed a standard chow diet for 6 hours before sacrifice (Refed). The messenger RNA abundance of *Pepck* (B) and *G6p* (C) was analyzed by quantitative real-time polymerase chain reaction. Values were normalized to fasted Ces3/TGH lox mice where the gene/*Cyc* ratio was set as 100%. Data are presented as the mean ± SEM (n = 4-6 per group). (D) Pyruvate tolerance test. Blood glucose levels were measured at the indicated times after an intraperitoneal injection of sodium pyruvate. The bottom panel shows the glucose area under the curve. Values represent the mean ± SEM (n = 10-14 per group). $^{**}P < 0.01$ versus TGH lox control mice.

Discussion

The assembly and secretion of VLDL requires the formation of neutral lipid core consisting mainly of TG.[5,8,11,12] During fasting, FA for hepatic TG synthesis are provided from lipolysis of TG stores in WAT. Because global inactivation of Ces3/TGH also decreases the release of FA from WAT, it was not clear whether the mechanism of blood lipid-lowering in global Ces3/TGH deficiency was due to diminished lipolysis in adipose tissue in addition to the liver. To investigate the specific contribution of hepatic Ces3/TGH to lipid and glucose homeostasis, we developed L-TGH KO mice. Liver-specific Ces3/TGH deficiency decreased plasma TG levels by 32.8%-35.4%, which is comparable to the reduction observed in liver-specific MTP-deficient mice.[9,20,38,39] MTP protein levels were not affected by the ablation of *Ces3/Tgh* expression, confirming that the Ces3/TGH-mediated lipolysis represents an important step in hepatic VLDL assembly and secretion. Furthermore, similar reduction in plasma TG levels was observed in global Ces3/TGH KO mice,[27] suggesting that the absence of hepatic Ces3/TGH activity is the primary cause for decreased plasma TG concentration.

In agreement with increased lipid storage in L-TGH KO mice, ADRP protein that is specifically localized at the surface of mature LDs was increased in L-TGH KO livers, whereas the levels of Tip47, a marker for newly formed LDs[40,47] were not affected. Importantly, ablation of liver TGH had a far milder effect on hepatic TG accumulation than the loss of liver MTP, which resulted in a 2.5- to 7-fold increase of TG mass in various studies.[9,21,39] We reported previously that the ablation of *Ces3/Tgh* expression delayed the

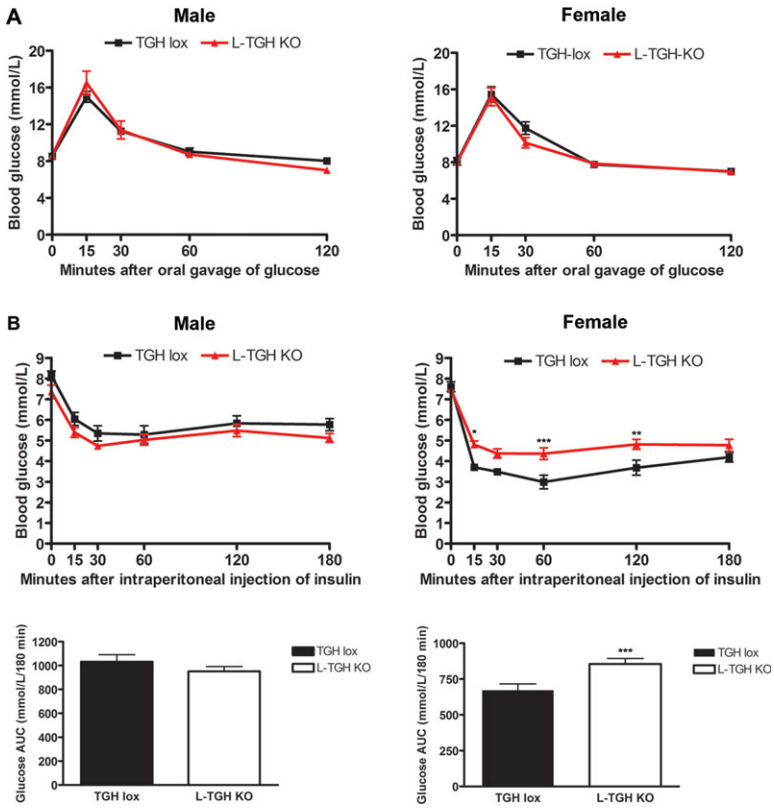

Fig. 7. Oral glucose tolerance test and insulin tolerance test in L-TGH KO mice. (A) Oral glucose and (B) insulin tolerance tests were performed in mice (n = 10-14 per group) fasted for 4 hours. Bars represent the mean ± SEM. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus Ces3/TGH lox control mice.

maturation of LDs.[42] LDs in primary hepatocytes isolated from fasted L-TGH KO mice also showed a decrease in the average area of an individual LD and an increase in LD number. FA oxidation and plasma ketone bodies were increased in L-TGH KO mice. Importantly, loss of hepatic Ces3/TGH appeared to be protective against high-fat diet–induced steatosis.

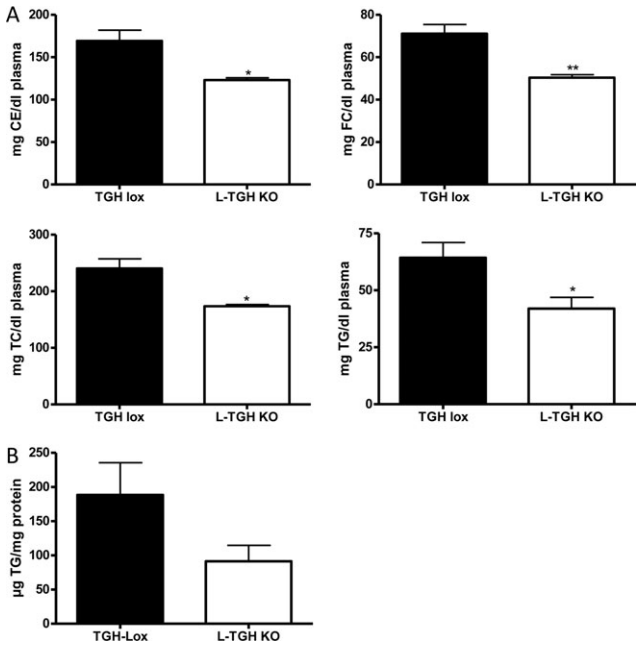

Fig. 8. Decreased plasma and hepatic lipids in male L-TGH-KO mice fed a WTD. PL (A) and liver TG (B) after 2 weeks of WTD are shown. Data are presented as the mean ± SEM [n = 6 (A), n = 4 (B) per group]. *$P < 0.05$ versus TGH lox control group.

Although hepatic steatosis is widely believed to result in insulin resistance, several rodent models with steatosis remain insulin sensitive.[21-24,43,44] Data from genetically engineered mice indicated that the source of accumulated TG plays a more important role in determining the metabolic outcome than hepatic steatosis itself.[21,25] Minehira et al.[21] reported that blocking VLDL secretion by deleting MTP caused hepatic steatosis but did not affect hepatic and peripheral insulin sensitivity, suggesting that lipids destined for VLDL secretion may exist in a separate cellular pool and their accumulation that does not affect insulin signaling. L-TGH KO mice exhibited normal hepatic insulin signaling and glucose tolerance, although female (but not male) L-TGH KO mice exhibited insulin resistance.

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