Ces3/TGH Deficiency Improves Dyslipidemia and Reduces Atherosclerosis in Ldlr\(-/\)- Mice

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ABSTRACT

**Rationale:** Carboxylesterase 3/triacylglycerol hydrolase (TGH) has been shown to participate in hepatic very-low density lipoprotein (VLDL) assembly. Deficiency of TGH in mice lowers plasma lipids and atherogenic lipoproteins without inducing hepatic steatosis.

**Objective:** Investigate the contribution of TGH to atherosclerotic lesion development in mice that lack low-density lipoprotein receptor (LDLR).

**Methods and Results:** Mice deficient in LDL receptor (*Ldlr<sup>−/−</sup>*) and mice lacking both TGH and LDLR (*Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup>*) were fed with Western-type diet for 12 weeks. Analysis of *Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup>* plasma showed an atheroprotective lipoprotein profile with decreased cholesterol in the VLDL and the LDL fractions, concomitant with elevated high density lipoprotein (HDL)-cholesterol. Significantly reduced plasma apolipoprotein B levels were also observed in *Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup>* mice. Consequently, *Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup>* mice presented with a significant reduction (54%, P<0.01) of the high-fat, high-cholesterol diet induced atherosclerotic plaques when compared with *Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup>* mice in the cross-sectional aortic root analysis. TGH deficiency did not further increase liver steatosis despite lowering plasma lipids, mainly due to reduced hepatic lipogenesis. The ameliorated dyslipidemia in *Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup>* mice was accompanied with significantly improved insulin sensitivity.

**Conclusions:** Inhibition of TGH activity ameliorates atherosclerosis development and improves insulin sensitivity in *Ldlr<sup>−/−</sup>* mice.

**Keywords:**
carboxylesterase, apolipoprotein B, cholesterol, atherosclerosis, insulin sensitivity, lipase, lipoproteins

**Non-standard Abbreviations:**
ApoB apolipoprotein B
CE cholesteryl ester
Ces carboxylesterase
HDL high-density lipoprotein
ITT insulin tolerance test
LDL low-density lipoprotein
FAS Fatty acid synthase
SREBP sterol regulatory element binding protein
TG triacylglycerol
TGH triacylglycerol hydrolase
VLDL very-low density lipoprotein
WTD Western-type diet
Introduction

The development of atherosclerosis and coronary heart disease is accelerated in patients with metabolic syndrome characterized by dyslipidemia and insulin resistance. Elevated circulating levels of apolipoprotein B (ApoB)-containing lipoproteins, such as low-density lipoproteins (LDL) and very-low density lipoproteins (VLDL), are recognized independent risk factors for the development of atherosclerosis.\(^1,2\) Hepatic secretion of VLDL is one of the major determining factors of plasma ApoB concentration. The majority of VLDL-TG secreted from hepatocyte is derived from preformed stored triacylglycerol (TG) through a process of lipolysis and re-esterification.\(^3-6\) TG hydrolase (TGH), also termed carboxylesterase 3 (Ces3) or carboxylesterase Ces1d in mice and carboxylesterase 1 (CES1) in humans, has been shown to participate in the provision of substrates for VLDL assembly.\(^7-13\) Overexpression of TGH in McArdle RH7777 cells augmented TG and ApoB100 secretion in the VLDL density range.\(^7\) Liver-specific overexpression of human TGH in mice resulted in increased ApoB secretion and plasma TG concentration in vivo,\(^12\) while inhibition of TGH activity decreased ApoB secretion both in vitro\(^8\) and in vivo.\(^13\) Furthermore, TGH knockout mice presented with reduced plasma lipids and improved insulin sensitivity and glucose homeostasis.\(^13\)

LDL receptor knockout (Ldlr\(^{-/-}\)) mouse is a well-established murine model for the study of atherosclerosis. LDL receptor plays a major role in the clearance of atherogenic ApoB-containing lipoproteins from the circulation. Due to the prolonged plasma half-life of VLDL and LDL, after high-fat, high-cholesterol diet, the Ldlr\(^{-/-}\) mouse shows severe hyperlipidemia and dramatically elevated circulating atherogenic lipoproteins, and consequently, develops atherosclerotic lesions\(^14-16\). To evaluate whether TGH deficiency would improve dyslipidemia and show protective effect against atherosclerosis development in vivo, we crossed the Tgh\(^{-/-}\) mice with Ldlr\(^{-/-}\) mice to generate Tgh\(^{-/-}\)/Ldlr\(^{-/-}\) mice. In the current study, we show that TGH deficiency in Ldlr\(^{-/-}\) mice improves plasma lipid and lipoprotein profiles, reduces atherosclerotic lesions and improves insulin sensitivity.

METHODS

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Mice.**

All animal protocols were approved by the University of Alberta Animal Policy and Welfare Committee. Tgh\(^{-/-}\) mice generated previously\(^13\) were backcrossed into C57BL/6J background for 10 generations and were bred with Ldlr\(^{-/-}\) mice (C57BL/6J background, Jackson Laboratory, Bar Harbor, ME) to produce Tgh\(^{-/-}\)/Ldlr\(^{-/-}\) mice. All mice were maintained on a chow diet (LabDiet, PICO laboratory Rodent diet 20). At 10 to 12 weeks of age, age matched Tgh\(^{-/-}\)/Ldlr\(^{-/-}\) and Tgh\(^{+/+}\)/Ldlr\(^{-/-}\) mice were fed with the high-fat, high-cholesterol diet (TD 88137, Harlan Teklad) containing 21% fat, 0.2% cholesterol by weight, which is usually referred to as Western-type diet (WTD), for 12 weeks. Male mice were used except where indicated.
Lipid and lipoprotein analysis.
Lipid levels and lipoprotein profiles were determined before and after 12 weeks of WTD. Mice were fasted for 5 hours before blood and tissue collection. Plasma lipoprotein profiles were analyzed by Fast Protein Liquid Chromatography. Plasma and liver lipid concentrations were determined by High-Performance Liquid Chromatography.

Immunoblot analysis of plasma ApoB.
One ul plasma was electrophoresed on SDS - 5% polyacrylamide gels. ApoB immunoreactivity was detected by ECL (Amersham-Pharmacia, ON, CA).

Chylomicron secretion rate.
Mice fasted for 12 hours received intraperitoneal injection of 1g/kg body weight of Poloxamer-407. Mice were then gavaged with 150 ul of olive oil containing 10 µCi of [3H]triolein. Blood was collected at 1h, 2h, and 4h after gavage. Lipids were extracted and separated by thin-layer chromatography (TLC) with the solvent system hexane:isopropyl ether:acetic acid (15:10:1). Lipids were visualized by exposure to iodine, and radioactivity in each lipid class was determined by scintillation counting.

Metabolic labeling studies.
Primary hepatocytes prepared from the mice were incubated overnight with serum-free DMEM. For incorporation of exogenous fatty acids into lipids, hepatocytes were incubated for 4 hours in 2 mL of DMEM containing 5 µCi [3H]oleic acid (OA) dissolved in 0.4 mM OA/ 0.5% fatty acid-free bovine serum albumin. Media and cells were harvested, lipids were extracted, resolved by TLC, and radioactivity in various lipid classes was determined. To assess hepatic de novo lipogenesis, primary hepatocytes were incubated for 4 hours in 2 mL of DMEM containing 10 µCi [3H]acetic acid dissolved in 250 mM non-radiolabeled acetic acid. Cells were collected for analysis as described above. Oxidation of [3H]OA was determined in isolated hepatocytes.

In vivo insulin signaling.
Animals were fasted for 12 hours then 1 unit/kg body weight of human insulin or phosphate-buffered saline was injected intraperitoneally. Fifteen min after injection, liver, skeletal muscle and white adipose tissue were collected. Phospho-Akt and total Akt levels in the tissues were detected by immunoblotting.

mRNA expression analysis.
Total RNA was isolated by the Trizol reagent (Life Technologies, CA, US). Complementary DNA was synthesized with the Superscript III reverse transcriptase (Invitrogen, CA, USA). Real-time qPCR was performed with the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) in the Rotor-Gene 3000 instrument (Montreal Biotech, Quebec, CA).

Analysis of aortas.
Samples for cross-sectional aortic root analysis were processed as described previously.17,18
**Blood glucose and insulin concentrations, and insulin tolerance test.**

Blood glucose concentrations were determined in 16 hours fasted mice using glucose strips (Roche Diagnostics, Vienna, Austria). Serum insulin concentration was analyzed in 16 hours fasted mice with rat/mouse insulin ELISA kit (Millipore, MA, US). For insulin tolerance tests (ITT), mice were fasted for 6 hours and then intraperitoneally injected with human insulin (0.75U/kg body weight). Blood glucose concentrations were measured before the insulin injection and 15, 30, 60, 120 min after the injection.

**Statistical analysis.**

Data are presented as the mean ± SEM. Analysis was performed using the GraphPad PRISM® 4 software. Significant differences between groups were determined by unpaired two-tailed *t* test. ITT and fat tolerance test (chylomicron secretion) were analyzed by two-way ANOVA followed by Bonferroni post-tests. *P*<0.05 was interpreted as significantly different.

**RESULTS**

*TGH deficiency improves atherogenic lipoprotein profiles in Ldlr−/− mice.*

To investigate whether TGH deficiency would improve dyslipidemia and prevent atherosclerotic lesion formation in *Ldlr−/−* mice, we generated *Tgh−/−/Ldlr−/−* mice. WTD-mediated hypertriglyceridemia in *Ldlr−/−* mice is dramatically decreased in the absence of *Tgh* expression (Figure 1A, B). In the mice fed chow diet, TGH deficiency did not affect plasma TG concentration in *Ldlr−/−* male mice (Figure 1B), but decreased TG levels were observed in female *Tgh−/−/Ldlr−/−* mice compared to the *Ldlr−/−* controls (Online Figure I A). Similar to male mice, plasma TG concentration was reduced by 50% in WTD-fed female *Tgh−/−/Ldlr−/−* mice, when compared with *Ldlr−/−* mice (Online Figure IA). Plasma cholesteryl ester (CE) and free (unesterified) cholesterol levels were higher in both male and female *Tgh−/−/Ldlr−/−* mice before WTD challenge when compared with *Tgh+/+/*Ldlr−/−* mice. However, after 12 weeks WTD feeding, reduction of plasma cholesteryl ester and free cholesterol levels by 30% was observed in male *Tgh−/−/Ldlr−/−* mice compared to *Ldlr−/−* controls (Figure 1B). This decreased trend was also observed in female *Tgh−/−/Ldlr−/−* mice but did not reach to the statistical significance (Online Figure IA).

Decreased cholesterol levels in VLDL and LDL fractions, and increased HDL-cholesterol were observed in chow-fed male (Figure 1C) and female (Online Figure IB) *Tgh−/−/Ldlr−/−* mice. This lipoprotein profile suggested that the increased plasma cholesterol in *Tgh−/−/Ldlr−/−* mice on chow diet was derived from the HDL-cholesterol fraction. Furthermore, after WTD feeding, the lipoprotein profile in *Tgh−/−/Ldlr−/−* mice was more “atheroprotective”, with lower VLDL/LDL-cholesterol and higher HDL-cholesterol than in *Tgh+/+/*Ldlr−/−* mice (Figure 1C and Online Figure IB).

Because there is only one ApoB molecule per VLDL particle19, 20 plasma ApoB concentrations reflect the number of circulating lipoprotein particles. Chow-fed *Tgh−/−/Ldlr−/−* mice contained 47% fewer plasma ApoB100 particles than *Ldlr−/−* mice, whereas plasma ApoB48 concentration was not
significantly changed (Figure 2A,B). After 3 months of WTD challenge, TGH deficiency decreased plasma ApoB100 concentration by 69% in \( Ldlr^{-/-} \) mice. At the same time, plasma ApoB48 was also decreased by 32% in \( Tgh^{-/-}/Ldlr^{-/-} \) mice compared with \( Tgh^{+/+}/Ldlr^{-/-} \) mice (Figure 2C,D). To specifically assess hepatic secretion of ApoB and TG, primary hepatocytes were isolated from chow and WTD fed \( Tgh^{+/+}/Ldlr^{-/-} \) and \( Tgh^{-/-}/Ldlr^{-/-} \) mice. In the absence of TGH, secretion of ApoB100 and TG from hepatocytes was decreased in \( Tgh^{-/-}/Ldlr^{-/-} \) mice (Online Figure IIA). However, unlike what we found in plasma, the secretion of ApoB48 was not significantly affected in \( Tgh^{-/-}/Ldlr^{-/-} \) hepatocytes (Online Figure IIA). To investigate contribution of intestinal apoB48-containing lipoproteins to the improved lipid profile in \( Tgh^{-/-}/Ldlr^{-/-} \) mice, chylomicron secretion rate was analyzed in animals fed WTD for 3 months. Chylomicron secretion was significantly reduced in \( Tgh^{-/-}/Ldlr^{-/-} \) mice (Online Figure IIB).

**Absence of exacerbated hepatic steatosis in \( Tgh^{-/-}/Ldlr^{-/-} \) mice.**

We have shown that reducing VLDL secretion in \( Tgh^{-/-} \) mice fed chow diet did not result in steatosis. After feeding \( Tgh^{-/-}/Ldlr^{-/-} \) mice with WTD for 3 months, the liver weight did not increase with respect to control \( Ldlr^{-/-} \) mice (Table 1). Hepatic TG and CE contents in the \( Tgh^{-/-}/Ldlr^{-/-} \) mice were also similar to the \( Ldlr^{-/-} \) mice (Figure 3). Furthermore, hepatic free cholesterol levels in \( Tgh^{-/-}/Ldlr^{-/-} \) mice were significantly lower than in \( Ldlr^{-/-} \) mice (Figure 3).

**Decreased hepatic lipogenesis in \( Tgh^{-/-}/Ldlr^{-/-} \) mice.**

To evaluate the effect of TGH deficiency on lipid anabolism, hepatocytes were isolated from \( Tgh^{-/-}/Ldlr^{-/-} \) and \( Tgh^{+/+}/Ldlr^{-/-} \) mice fed chow and WTD for metabolic lipid labeling studies. Incorporation of \(^{1}H\)acetic acid into fatty acids, TG and total cholesterol was significantly decreased in \( Tgh^{-/-}/Ldlr^{-/-} \) mice (Figure 4A), indicating reduced \textit{de novo} lipogenesis. The expression of important lipogenic and sterol synthesis genes was either significantly decreased or was trending toward reduction in \( Tgh^{-/-}/Ldlr^{-/-} \) mice when compared with \( Tgh^{+/+}/Ldlr^{-/-} \) controls after 3 months WTD (Figure 4B). Sterol-regulatory element binding protein-2 (SREBP2) is a key transcription factor regulating cholesterol biosynthesis including the expression of the rate-limiting enzyme HMG-CoA reductase. The expression of HMG-CoA reductase was found to be lower in \( Tgh^{-/-}/Ldlr^{-/-} \) mice, which may have contributed to the reduced hepatic cholesterol levels. Although hepatic gene expression of SREBP1c did not change, fatty acid synthase (FAS), a key enzyme in \textit{de novo} lipogenesis, showed reduced protein levels in \( Tgh^{-/-}/Ldlr^{-/-} \) livers, which might have contributed to the reduced hepatic fatty acid synthesis in these animals. On the other hand, although mRNA expression of SCD1 was significantly reduced in \( Tgh^{-/-}/Ldlr^{-/-} \) liver, the protein expression was not different when compared with \( Tgh^{-/-}/Ldlr^{-/-} \) mice (data not shown). Decreased incorporation of exogenously-supplied radiolabeled OA into TG and CE was observed \( Tgh^{-/-}/Ldlr^{-/-} \) hepatocytes (Figure 4C), indicating an overall reduction in hepatic neutral lipid synthesis.

**Effect of TGH deficiency on hepatic fatty acid oxidation.**

Our previous work showed increased hepatic fatty acid oxidation in chow-fed TGH deficient mice, which could exert protective effect against hepatic lipid accumulation. In the \( Ldlr^{-/-} \)
background, TGH deficiency also augmented fatty acid oxidation in hepatocytes isolated from chow-fed mice (Figure 4D). However, after 3 months of WTD feeding, there was no difference in fatty acid oxidation between $Tgh^{+/+}/Ldlr^{-/-}$ and $Tgh^{-/-}/Ldlr^{-/-}$ hepatocytes (Figure 4D).

**Decreased atherosclerosis in $Tgh^{-/-}/Ldlr^{-/-}$ mice without changes in lesion morphology.**

To determine whether TGH deficiency has a protective effect on atherosclerosis, we examined the extent of atherosclerotic lesions in the aortic root. In the cross-sectional aortic root analysis, the Oil-Red-O stained plaque area was significantly reduced by 54% in $Tgh^{-/-}/Ldlr^{-/-}$ mice when compared with $Tgh^{+/+}/Ldlr^{-/-}$ mice after 12 weeks of the WTD (Figure 5A). Because some lesion area in $Tgh^{-/-}/Ldlr^{-/-}$ mice was still observed, TGH deficiency significantly decreased but did not completely prevent atherosclerosis development in $Ldlr^{-/-}$ mice.

Lesion morphology was analyzed by immunostaining for smooth muscle cells (SMCs) marker α-actin, macrophage marker MOMA-2, and by Trichrome staining for collagen content in aortic plaques. There were no differences observed in the percentage of any of these markers in the lesion area between $Tgh^{+/+}/Ldlr^{-/-}$ and $Tgh^{-/-}/Ldlr^{-/-}$ mice (Online Figure III).

**Improved insulin sensitivity in $Tgh^{-/-}/Ldlr^{-/-}$ mice.**

Lower fasting blood glucose concentration was observed in $Tgh^{-/-}/Ldlr^{-/-}$ mice after 3 months of WTD feeding (Figure 5B). Fasting insulin concentration in $Tgh^{-/-}/Ldlr^{-/-}$ mice also tended to be lower than in $Tgh^{+/+}/Ldlr^{-/-}$ mice on WTD (Figure 5B). Increased concentration of plasma free fatty acid (FFA) is associated with insulin resistance. Tgh$^{-/-}/Ldlr^{-/-}$ mice on WTD showed reduced circulating fasting FFA when compared with $Tgh^{+/+}/Ldlr^{-/-}$ mice (Figure 5B). $Tgh^{-/-}/Ldlr^{-/-}$ mice also showed markedly improved insulin sensitivity when compared with the control $Ldlr^{-/-}$ mice (Figure 5C). Phosphorylation of Akt in response to insulin showed increased trend in the liver, muscle, and white adipose tissue (WAT) of $Tgh^{-/-}/Ldlr^{-/-}$ mice, suggesting improved insulin signaling (Figure 5D). However, no difference was observed in oral glucose tolerance test (OGTT) between $Tgh^{+/+}/Ldlr^{-/-}$ and $Tgh^{-/-}/Ldlr^{-/-}$ mice after 3 months of WTD (Online Figure IV).

Both $Tgh^{+/+}/Ldlr^{-/-}$ and $Tgh^{-/-}/Ldlr^{-/-}$ mice presented with WTD-induced increase in adipose tissue mass. $Tgh^{-/-}/Ldlr^{-/-}$ mice did not show any difference in WAT and brown adipose tissue (BAT) weights (Table 1) compared with $Tgh^{+/+}/Ldlr^{-/-}$ even though plasma lipid levels and atherosclerosis were decreased.

**DISCUSSION**

In the absence of sufficient TG supply for VLDL assembly, ApoB is targeted for degradation instead of secretion. TGH was demonstrated to play an important role in lipidation of ApoB100. Therefore we hypothesized that ablation of Tgh expression in LDLR deficient mice might improve dyslipidemia and afford protection against development of atherosclerosis. Our study has shown that TGH deficiency results in decreased concentration of cholesterol in the VLDL/LDL fractions and in
increased plasma HDL-cholesterol, reflecting an atheroprotective plasma lipid profile. Inverse relationship between plasma HDL-cholesterol and cardiovascular disease including atherosclerosis has been demonstrated. HDL may exert antiatherogenic effects through its role in the reverse cholesterol transport, and by its antioxidative and anti-inflammatory properties. HDL particles in Tgh+/+Ldlr−/− mice appear to be slightly larger based on their elution profile from the gel filtration column, suggesting increased CE content. The elevated HDL-cholesterol combined with lower ApoB-lipoprotein concentrations in Tgh+/+Ldlr−/− mice contributed to the reduced atherosclerotic lesions in these mice. Thus, these data provide evidence that attenuation of TGH activity improves diet-induced dyslipidemia and associated atherosclerosis.

One major concern in inhibiting VLDL secretion is that such approach might lead to severe hepatic lipid accumulation. For instance, inhibition of microsomal triglyceride transfer protein activity, an activity required for basal lipidation and folding of ApoB, decreased VLDL secretion and plasma lipid concentration at the expense of hepatic steatosis. Importantly, ablation of TGH in Ldlr−/− mice did not provoke further hepatic lipid accumulation after WTD challenge. Moreover, hepatic free (unesterified) cholesterol concentration in Tgh+/+Ldlr−/− mice was significantly decreased compared to Tgh+/+Ldlr−/− mice. Free cholesterol is known to be highly cytotoxic and has been implicated in liver injury and pathogenesis of non-alcoholic fatty liver disease (NAFLD). Because the hepatic expression levels of CYP7A1, the key enzyme of bile acid synthesis, ABCG5, an ATP-binding cassette transporter which promotes biliary sterol excretion, and ABCA1, an ABC transporter plays an important role in RCT, were similar between Tgh+/+Ldlr−/− and Tgh+/+Ldlr−/− mice (data not shown), the lower hepatic free cholesterol levels in Tgh+/+Ldlr−/− mice were not likely due to increased cholesterol excretion or decreased cholesterol uptake. Rather, hepatocytes isolated from Tgh+/+Ldlr−/− mice showed decreased cholesterol synthesis, which may be due to the down-regulated expression of SREBP2 and HMG-CoA reductase.

Insulin resistance and type 2 diabetes are generally accompanied by dyslipidemia and this condition is highly associated with cardiovascular disease. Tgh+/− mice present with improved glucose tolerance and insulin sensitivity. Ablation of Tgh expression in the Ldlr−/− background resulted in lower blood glucose levels and improved insulin sensitivity, which benefits energy homeostasis and exerts protective effect against the development of atherosclerosis. Insulin resistance has been demonstrated to affect the activities of lipases involved in HDL metabolism in vivo thereby decreasing circulating HDL-cholesterol. The improved insulin sensitivity in Tgh+/+Ldlr−/− mice may therefore also contribute to the elevated HDL-cholesterol levels observed in these mice.

It has been demonstrated that FAS expression is upregulated in high-fat diet induced hepatic steatosis and insulin resistance. In Tgh+/+Ldlr−/− mice fed with WTD for 3 months, hepatic FAS expression was significantly decreased when compared with Tgh+/+Ldlr−/− mice, which may have contributed to decreased fatty acid synthesis in TGH deficient Ldlr−/− mice. Reduced FAS expression in Tgh+/+Ldlr−/− mice is probably the consequence of decreased blood insulin concentration and improved insulin sensitivity in these mice. Attenuated FAS expression could lead to augmented fatty acid oxidation in the liver due to reduction of malonyl-CoA and subsequent removal of inhibition of fatty acid import into mitochondria by CPT-1. However, fatty acid oxidation was not affected in Tgh+/+Ldlr−/− hepatocytes after WTD, suggesting that reduction of lipid synthesis and delivery of lipids
to the liver from intestinal lipoproteins might be the main mechanism responsible for the absence of further steatosis in Tgh−/−/Ldlr−/− mice despite decreased VLDL secretion.

In conclusion, we showed that the decreased ApoB secretion caused by TGH deficiency reduced the amount of circulating atherogenic lipoproteins and lipid levels in Ldlr−/− background after WTD feeding. The atheroprotective lipid profile in Tgh−/−/Ldlr−/− mice is presumably the major reason for the observed reduction of atherosclerotic lesions induced by WTD. Importantly, TGH deficiency did not induce further liver steatosis in Ldlr−/− mice. The decreased blood glucose concentrations and increased insulin sensitivity in Tgh−/−/Ldlr−/− mice also implies improvements in metabolic control.

ACKNOWLEDGMENTS

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DISCLOSURES

None

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Table 1. Characteristics of Tgh+/+ /Ldlr−/− and Tgh−/− /Ldlr−/− mice

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<td>Liver weight (g)</td>
<td>2.90±0.44</td>
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Data are mean ± SEM, n=6 each group. **P < 0.01 vs Tgh+/+ /Ldlr−/− mice, *P < 0.05 vs Tgh+/+ /Ldlr−/− mice.
FIGURE LEGENDS

**Figure 1. TGH deficiency improves plasma lipid profile in Ldlr<sup>−/−</sup> mice.** Plasma lipid levels and lipoprotein profiles were determined in Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> and Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice (n≥6) before WTD (Chow) and after 3 months of WTD. Plasma was collected from mice fasted for 5 hours. (A) Photograph of representative plasma from 3 months WTD fed Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> and Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice. (B) Plasma TG, cholesteryl esters, and free cholesterol levels. Values are mean ± SEM. *P<0.05, **P<0.01 vs Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> mice. (C) Pooled plasma from 6 mice fed chow or WTD for 3 months was subjected to gel filtration chromatography and in-line cholesterol measurement.

**Figure 2. TGH deficiency decreases plasma ApoB levels in Ldlr<sup>−/−</sup> mice.** Blood was collected from chow and WTD-fed (3 months) mice that were fasted for 5 hours (n=3). (A and C) Plasma ApoB was detected by immunoblot (upper panels); Ponceau S staining of PVDF membranes (lower panels) after transfer was used as the protein loading control. Immunoblots of plasma ApoB from mice before (B) and after (D) WTD were quantified by densitometric analysis with the Quantity One<sup>®</sup> software. Values are mean ± SEM. *P<0.05, **P<0.01 vs Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> mice.

**Figure 3. TGH deficiency does not lead to increased hepatic steatosis in Ldlr<sup>−/−</sup> mice.** Liver lipid concentrations were determined in Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> and Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice (n=6) after 3 months of WTD. CE, cholesteryl ester; FC, free cholesterol. Values are mean ± SEM. *P<0.05,

**Figure 4. Effect of TGH deficiency on hepatic lipid metabolism in Ldlr<sup>−/−</sup> mice.** (A) Decreased hepatic de novo lipogenesis in Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice. Incorporation of [³H]acetic acid was employed to evaluate de novo lipogenesis in hepatocytes isolated from Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> and Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice fed chow diet or WTD for 3 months. (B) Hepatic mRNA expression analysis in Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> and Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice (n=6) was performed after 3 months of WTD. Gene expression was determined by qRT-PCR with cyclophilin as the control gene. Hepatic fatty acid synthase (FAS) protein expression was detected by immunoblotting. Protein disulfide isomerase (PDI) served as a loading control. Livers were harvested from mice fasted for 5 hours. (C) Incorporation of [³H]OA into lipids in isolated hepatocytes. TG mass was determined in the same hepatocytes. (D) Fatty acid oxidation in hepatocyte isolated from Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> and Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice fed either chow or WTD for 3 months. Values are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> mice.

**Figure 5. TGH deficiency decreases atherosclerosis and improves insulin sensitivity in Ldlr<sup>−/−</sup> mice.** (A) Representative aortic root sections in Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> and Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice after 3 months of WTD and quantitation of aortic lesion areas. (B) Fasting blood glucose, insulin, and free fatty acid (FFA) concentrations in Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> mice (n=6) fed WTD for 3 months. (C) Insulin tolerance tests (ITT) performed in WTD fed (3 months) Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> and Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice (n=6) after 6 hour fast and area under the curve (AUC) of ITT. (D) In vivo insulin signaling evaluated by measurements of Akt phosphorylation in WTD fed (3 months) Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> and Tgh<sup>−/−</sup>/Ldlr<sup>−/−</sup> mice (n=5) after 12 hour fast. Values are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs Tgh<sup>+/+</sup>/Ldlr<sup>−/−</sup> mice.

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Novelty and Significance

What Is Known?

- Hyperlipidemia and elevated circulating levels of apolipoprotein B (ApoB)-containing lipoproteins are independent risk factors for the development of atherosclerosis.

- Insulin resistance is highly associated with dyslipidemia and cardiovascular disease.

- Carboxylesterase 3 (Ces3)/triacylglycerol hydrolase (TGH) has been shown to participate in hepatic very-low density lipoprotein (VLDL) assembly, which is one of the major determining factors of plasma ApoB concentration.

- TGH knockout mice showed reduced plasma lipid and circulating (ApoB)-containing lipoproteins, and improved insulin sensitivity.

What New Information Does This Article Contribute?

- $Tgh^{-/-}/Ldlr^{-/-}$ mice were generated to study the effect of TGH deficiency on dyslipidemia and atherosclerosis development.

- Loss of TGH attenuated hyperlipidemia and improved insulin sensitivity in $Ldlr^{-/-}$ mice after high-fat, high-cholesterol western-type diet (WTD) feeding.

- TGH deficiency decreased secretion and circulating level of (ApoB)-containing lipoproteins in $Ldlr^{-/-}$ mice.

- Lack of TGH decreased lipid synthesis in $Ldlr^{-/-}$ hepatocytes.

- TGH deficiency in $Ldlr^{-/-}$ mice attenuated atherosclerosis induced by WTD.

To study the effect of TGH deficiency on atherosclerosis development, we crossed TGH knockout mice with $Ldlr^{-/-}$ mice, which is a well-established atherosclerotic animal model that develops obvious atherosclerotic plaque and hyperlipidemia after WTD feeding. $Tgh^{-/-}/Ldlr^{-/-}$ mice fed with WTD presented with significantly decreased atherosclerotic lesion in aorta root when compared with the $Ldlr^{-/-}$ controls. The protective effect of TGH deficiency on atherosclerosis was attributed to the improved plasma lipid profile and insulin sensitivity. Loss of TGH reduced both hepatic and intestine (ApoB)-containing lipoprotein secretion in $Ldlr^{-/-}$ mice. Importantly, $Tgh^{-/-}/Ldlr^{-/-}$ mice did not show further hepatic lipid accumulation despite decreased VLDL secretion when compared with $Tgh^{+/+}/Ldlr^{-/-}$ mice. This has been attributed to reduced hepatic lipid synthesis. This work provided important information that pharmacological inhibition of Ces3/TGH might offer protection against dyslipidemia and atherosclerosis.
Figure 1

A

B

C
Figure 2

A

Tgh<sup>+/+</sup>/Ldlr<sup>-/-</sup>  Tgh<sup>-/-</sup>/Ldlr<sup>-/-</sup>

ApoB 100

ApoB 48

Ponceau S staining

B

Tgh<sup>+/+</sup>/Ldlr<sup>-/-</sup>  Tgh<sup>-/-</sup>/Ldlr<sup>-/-</sup>

ApoB % of Tgh<sup>+/+</sup>/Ldlr<sup>-/-</sup>

**

Tgh<sup>-/-</sup>/Ldlr<sup>-/-</sup>

**

ApoB100  ApoB48

C

Tgh<sup>+/+</sup>/Ldlr<sup>-/-</sup>  Tgh<sup>-/-</sup>/Ldlr<sup>-/-</sup>

D

Tgh<sup>+/+</sup>/Ldlr<sup>-/-</sup>  Tgh<sup>-/-</sup>/Ldlr<sup>-/-</sup>

ApoB % of Tgh<sup>+/+</sup>/Ldlr<sup>-/-</sup>

*

Tgh<sup>-/-</sup>/Ldlr<sup>-/-</sup>

*

ApoB100  ApoB48
Figure 3

- **TG per mg protein:**
  - Tgh^+/+ /Ldlr^-/-
  - Tgh^-/- /Ldlr^-/-
  - Tgh^-/- /Ldlr^-/-

- **CE per mg protein:**
  - Tgh^+/+ /Ldlr^-/-
  - Tgh^-/- /Ldlr^-/-

- **FC per mg protein:**
  - Tgh^+/+ /Ldlr^-/-
  - Tgh^-/- /Ldlr^-/-

*Significant difference.*
Figure 4

A

B

C

D

Figure 4

Chow WTD

Chow WTD

Chow WTD

Chow WTD
Figure 5

**A**

*Tgh+/+Ldlr−/−*  *Tgh−/−/Ldlr−/−*

**Atherosclerotic lesion area (um X 10^5)**

**B**

**Fasting blood glucose (mM)**

**Fasting insulin (ng/ml)**

**Plasma FFA (mM)**

**C**

% Blood glucose vs Time (min)

**ITT-AUC ( × 10^3)**

**D**

Liver

Muscle

WAT
Ces3/TGH Deficiency Improves Dyslipidemia and Reduces Atherosclerosis in Ldlr⁻/⁻ Mice
Jihong Lian, Ariel D. Quiroga, Lena Li and Richard Lehner

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SUPPLEMENTAL MATERIAL

Ces3/TGH Deficiency Improves Dyslipidemia and Reduces Atherosclerosis in Ldlr<sup>-/-</sup> Mice

Short title: Lian et al TGH Deficiency Reduces Atherosclerosis

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Supplemental Materials and Methods

**Lipid and lipoprotein analysis**

Before WTD, blood was collected from tail veins of 5 hours fasted mice. Plasma triacylglycerol (TG) (Roche Diagnostics GmbH, Mannheim, Germany), cholesteryl esters (CE) and free cholesterol (FC) (WAKO Diagnostics, VA, US) were determined according to the manufacturers’ instructions.

After 3 months of WTD, blood and livers were collected from 5 hours fasted mice. Livers were homogenized in 50 mM Tris-HCl (pH7.4), 250mM sucrose and 1mM EDTA by a glass/Teflon homogenizer for 20s. TG was determined in liver homogenates containing 0.4 mg protein. Liver CE and FC were determined in homogenates containing 2 mg protein. Plasma lipids were determined in 5 ul of plasma. Using phosphatidyl dimethylethanolamine (PDME) as an internal standard, lipids were extracted by modified Folch method<sup>1</sup>, and analyzed by high-performance liquid chromatography (HPLC).

To determine plasma lipoprotein levels, plasma prepared from fasted animals was pooled from each group of mice and applied onto a Sepharose 6 fast-protein liquid chromatography column (Pharmacia, Uppsala, Sweden). Eluted lipoprotein fractions were mixed in-line with the Infinity Cholesterol Reagent (Fisher Diagnostics, Middletown, VA, US) using a postcolumn T-connector/Solvent Delivery Module (model 110B; Beckman Coulter, Mississauga, Ontario, Canada) and passed through a CH-30 Column Heater (Eppendorf, Mississauga, Ontario, Canada) set at 37°C. Reaction products were monitored at 500 nm in real time using a Programmable Detector Module (model 166; Beckman Coulter).<sup>2</sup>

**Immunoblot analysis**

For plasma ApoB analysis, 1 ul plasma was heated for 10 min at 95°C in 62.5mM Tris-HCl (pH8.3), 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 1% SDS, and 0.004% Bromophenol Blue. Samples were electrophoresed on SDS - 5% polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were stained with Ponceau S as the loading control before incubated overnight at 4°C with goat polyclonal anti-human ApoB antibody (Dilution 1/10,000, Chemicon international, Temecula, CA) followed by an 1 hour incubation with secondary mouse anti-goat antibody conjugated to HRP (Pierce Biotechnology, Rockford, IL, US).<sup>3</sup> For liver fatty acid synthase (FAS), liver homogenate containing 20 ug protein was separated on 8% polyacrylamide gel, rabbit anti-FAS antibody (Dilution 1/2000, cell signaling, Danvers, MA, US) were used for protein detection.
Protein disulfide isomerase (PDI) was immunoblotted as a loading control (1:5000. Stressgen. Victoria, BC, CA). For insulin signaling, tissue homogenate was separated on 10% polyacrylamide gel. Phospho-Akt (Ser473, dilution 1/1000) and Akt antibodies (dilution 1/1000) were from Cell Signaling. The ratios between Phospho-Akt and Akt were calculated and normalized to PBS treated group to evaluate the fold increase after insulin injection. Immunoreactivity was detected by ECL system (Amersham-Pharmacia, ON, CA) according to the manufacturer’s instructions. Immunoblots were quantified by densitometric analysis with the software Quantity One® (Bio-Rad, Hercules, CA).

RNA isolation and PCR analysis
Livers were homogenized in Trizol Reagent (Life Technologies, CA, US) and total RNA was isolated according to the manufacturer’s instruction. First-strand cDNA was synthesized from 2 µg total RNA using Superscript III reverse transcriptase (Invitrogen, CA, US) primed by oligo (dT)12-18 and random primers (Invitrogen). Real-time qPCR was performed with the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) in the Rotor-Gene 3000 instrument (Montreal Biotech, Quebec, CA) to detect the gene expression. Data were analyzed with the Rotor-Gene 6.0 program. A standard curve was used to calculate mRNA level relative to that of a control gene, cyclophilin. Primers of the various genes are listed in the Table S1. All primers were synthesized at the DNA Core Facility of the University of Alberta.

Secretion of ApoB from hepatocytes
Primary mouse hepatocytes were incubated for 4 hours with DMEM containing 0.4 mM OA complexed to 0.5% BSA in order to increase intracellular TG stores. Cells were then washed and incubated with serum-free DMEM overnight. Media were collected and briefly centrifuged to remove cellular debris. Lipid mass in media was determined by gas chromatography. Proteins in media were collected by Cab-O-Sil® and ApoB levels were analyzed by immunoblotting as described above.

In vitro fatty acid oxidation
Mouse primary hepatocyte were isolated and seeded in 60 mm dishes containing DMEM with 15% FBS for 4 hours to allow attachment. Medium was switched to low glucose serum-free medium for overnight incubation. Hepatocytes were then incubated for 6 hours in 2 ml low glucose DMEM containing 0.5 mM carnitine and 5 µCi [3H]OA dissolved in 0.4 mM OA/0.5% FA-free BSA. Medium was collected and 300 µl was transferred to a 0.5 µl Eppendorf tube and this was placed into a scintillation vial containing 0.5 ml water. The vials were tightly capped and incubated for 24 hours at 50°C. Then vials were placed for 24 hours at 4°C. Finally, tubes were removed from the vials and radioactivity in water was measured by scintillation counting.

Quantification of aortic atherosclerosis and analysis of lesion morphology
Before excision, mice hearts were perfused with cold PBS containing 10 U/ml heparin. After incubated in Kreb-Hanseleit buffer for 30 min, heart tissue was fixed in 10% neutral buffered formalin and embedded in HistoPrep™ (Fisher, NJ, US). Cross-sectional aortic roots analysis was processed as described previously. 5,6 Briefly, serial 10 µm cross-sections were collected from the aortic root and stained with Oil Red O to detect the atherosclerotic plaque and Mayer’s hematoxylin (Sigma-Aldrich, Egham, UK) to assist in tissue visualization. The software ImageJ was used to quantify the lesion area in 14 sections (2 adjacent sets of 80-µm interval serial sections) over 500 µm
aortic root, and the mean lesion area per mouse was calculated. Gimori Trichrome was employed to assess collagen content in lesion area. Collagen was shown as green color. Macrophage was detected by immunostaining with antibody against to marker MOMA-2 (dilution 1:25. AbD Serotec, IL, US). Smooth muscle cells (SMC) were detected by antibody to marker α-actin (1:200, Sigma). HRP labeled secondary antibodies and DAB was used to visualize signal on slides. Sections without incubation with primary antibodies were used as negative staining control. Images were analyzed by ImageJ, Data were expressed as a percentage of positive staining to total lesion area. At least 3 sections of each animal were analyzed.

**Oral Glucose Tolerance Test (OGTT)**

Mice fasted for 6 hours were gavaged with dextrose solution (2mg/kg body weight). Blood glucose levels were tested before and 15, 30, 60, 120 min after gavage with glucose strips (Roche Diagnostics, Vienna, Austria).

**Supplemental References:**

**Online Table I.** Primer sequences used for qRT-PCR

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<thead>
<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td>SREBP-1c</td>
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<td>GTTGCCAGTTTTCTTTCTTG</td>
<td>GGGAAGCCAAGTTTTCTACACA</td>
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<td>TTCCGTACACTCCAGTTAGAG</td>
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<td>ACC</td>
<td>GGCGACTTACGTTCCTAGTTG</td>
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<td>SREBP-2</td>
<td>CAGGCGACCAGGAAGAAGA</td>
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<td>HMG-CoA reductase</td>
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<td>HMG-CoA synthase</td>
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<td>Cyclophilin</td>
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<td>TCTTCTTGGCTGGTCTTGCATTCC</td>
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F, Forward; R, Reverse.
Online figure legends:

**Online Figure I.** Plasma lipid levels were measured in female Tgh^{+/+}/Ldlr^{-/-} and Tgh^{-/-}/Ldlr^{-/-} mice (n≥6) before WTD (Chow) and after 3 months of WTD. Blood was collected from 5 hours fasted mice. (A) Plasma TG, CE, and FC levels were analyzed by HPLC. *P<0.05, **P<0.01, ***P<0.001 vs Tgh^{+/+}/Ldlr^{-/-} mice. TG, triglyceride; CE, cholesteryl ester; FC, free cholesterol. (B) Blood was collected from 5 hours fasted mice before (Chow) and after 3 months WTD (n=6). Total cholesterol in lipoprotein fractions was analyzed by FPLC.

**Online Figure II.** Lipoprotein secretion in Tgh^{+/+}/Ldlr^{-/-} and Tgh^{-/-}/Ldlr^{-/-} mice. (A) ApoB and TG secretion by hepatocytes isolated from chow and WTD fed Tgh^{+/+}/Ldlr^{-/-} and Tgh^{-/-}/Ldlr^{-/-} mice. (B) Chylomicron secretion rate from Tgh^{+/+}/Ldlr^{-/-} and Tgh^{-/-}/Ldlr^{-/-} mice after 3 months WTD feeding. *P<0.05, ***P<0.001 vs Tgh^{+/+}/Ldlr^{-/-} mice.

**Online Figure III.** Lesion morphology in Tgh^{+/+}/Ldlr^{-/-} and Tgh^{-/-}/Ldlr^{-/-} mice aortic root. (A) Representative images for SMC α-actin, macrophage MOMA-2, and Trichrome stained collagen on aortic sections. Arrows indicated positive staining for α-actin. (Lu) indicated the direction of aortic sinus lumen. Slides without primary antibody incubation were used as negative staining control. (B) The α-actin, MOMA-2, and collagen positive stained area was expressed as a percentage of lesion area.

**Online Figure IV.** Oral glucose tolerance tests (OGTT) were performed in WTD fed (3 months) Tgh^{+/+}/Ldlr^{-/-} and Tgh^{-/-}/Ldlr^{-/-} mice (n=5) after 6 hours fasting. Area under the curve (AUC) of OGTT.
Online Figure II

A

<table>
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<tr>
<th></th>
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<th>$Tgh^{-/-}/Ldlr^{-/-}$</th>
<th>$Tgh^{+/+}/Ldlr^{-/-}$</th>
<th>$Tgh^{-/-}/Ldlr^{-/-}$</th>
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<tbody>
<tr>
<td>ApoB 100</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
<td>[Image]</td>
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<tr>
<td>ApoB 48</td>
<td>[Image]</td>
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<td>[Image]</td>
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</tr>
</tbody>
</table>

Medium TG

![Bar graph showing ug/mg cell protein vs Chow and WTD](image)

B

![Line graph showing 10^3 DPM in TG/mL plasma vs Time (h)](image)
Online Figure III

A

<table>
<thead>
<tr>
<th>Tgh^{+/+}/Ldlr^{-/-}</th>
<th>Tgh^{-/-}/Ldlr^{-/-}</th>
<th>Negative staining control</th>
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</table>

B

<table>
<thead>
<tr>
<th>SMC (% of lesion area)</th>
<th>Macrophages (% of lesion area)</th>
<th>Collagen (% of lesion area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tgh^{+/+}/Ldlr^{-/-}</td>
<td>Tgh^{-/-}/Ldlr^{-/-}</td>
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</tr>
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</table>
Online Figure IV

The figure shows two graphs. The left graph depicts blood glucose levels over time in different mouse genotypes: Tgh\(^{+/+}\)/Ldl\(^{-/-}\) and Tgh\(^{-/-}\)/Ldl\(^{-/-}\). The y-axis represents blood glucose levels in mM, and the x-axis represents time in minutes (0 to 120). The red line represents Tgh\(^{+/+}\)/Ldl\(^{-/-}\), and the black line represents Tgh\(^{-/-}\)/Ldl\(^{-/-}\).

The right graph shows OGT-AUC (OGTT area under the curve) in different genotypes: Tgh\(^{+/+}\)/Ldl\(^{-/-}\) and Tgh\(^{-/-}\)/Ldl\(^{-/-}\). The y-axis represents OGT-AUC in units of 10\(^3\), and the x-axis has a range from 0.0 to 2.0. The data is represented by bars, with the red bar for Tgh\(^{+/+}\)/Ldl\(^{-/-}\) and the black bar for Tgh\(^{-/-}\)/Ldl\(^{-/-}\).