Lipolysis, and Not Hepatic Lipogenesis, Is the Primary Modulator of Triglyceride Levels in Streptozotocin-Induced Diabetic Mice


Objective—Diabetic hypertriglyceridemia is thought to be primarily driven by increased hepatic de novo lipogenesis. However, experiments in animal models indicated that insulin deficiency should decrease hepatic de novo lipogenesis and reduce plasma triglyceride levels.

Approach and Results—To address the discrepancy between human data and genetically altered mouse models, we investigated whether insulin-deficient diabetic mice had triglyceride changes that resemble those in diabetic humans. Streptozotocin-induced insulin deficiency increased plasma triglyceride levels in mice. Contrary to the mouse models with impaired hepatic insulin receptor signaling, insulin deficiency did not reduce hepatic triglyceride secretion and de novo lipogenesis-related gene expression. Diabetic mice had a marked decrease in postprandial triglycerides clearance, which was associated with decreased lipoprotein lipase and peroxisome proliferator-activated receptor α mRNA levels in peripheral tissues and decreased lipoprotein lipase activity in skeletal muscle, heart, and brown adipose tissue. Diabetic heterozygous lipoprotein lipase knockout mice had markedly elevated fasting plasma triglyceride levels and prolonged postprandial triglycerides clearance.

Conclusions—Insulin deficiency causes hypertriglyceridemia by decreasing peripheral lipolysis and not by an increase in hepatic triglycerides production and secretion. (Arterioscler Thromb Vasc Biol. 2015;35:102-110. DOI: 10.1161/ATVBAHA.114.304615.)

Key Words: diabetes mellitus ■ hypertriglyceridemia ■ lipoprotein lipase

Dyslipidemia is a major risk factor for cardiovascular disease in both type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM).1,2 Diabetic dyslipidemia is associated with high plasma triglycerides, low high-density lipoprotein cholesterol and increased small dense low-density lipoprotein-cholesterol particles.3 According to a National Health and Nutrition Examination Survey, >30% of people with T2DM have triglyceride levels >2.258 mmol/L (200 mg/dL).4 Patients with T1DM also have increased triglyceride levels, especially with poor glucose management.1 Possible causes of hypertriglyceridemia in patients with diabetes mellitus are increased hepatic very-low-density lipoprotein (VLDL) production or defective removal of triglyceride-rich lipoproteins (chylomycins and VLDL).

Several rodent models have been developed to explain the relationship between insulin actions and triglycerides. It has been postulated that hyperinsulinemia associated with T2DM drives hepatic de novo triglyceride synthesis via induction of sterol response element-binding protein-1c.5 Consistent with this hypothesis, lack of insulin action in the liver because of ablation of hepatic insulin receptors and Akt deficiency in mice prevented hepatic triglycerides production, reduced liver triglycerides secretion and led to low circulating triglyceride levels.6-8 According to this hypothesis, humans with poorly managed T1DM should show reduced hepatic triglycerides production and plasma triglyceride levels. Likewise, insulin therapy in T2DM should also drive greater liver triglycerides production. However, the opposite has been found: in fact, plasma triglyceride concentrations are increased in patients with T1DM.9,10 Moreover, treatment of T2DM patients with insulin results in systemic hyperinsulinemia, but reduced triglyceride levels and decreased hepatic lipid accumulation.11 Studies in diabetic rodents also conflict with conclusions derived from mice with genetic modifications in the
insulin-signaling pathway. Viral destruction of pancreatic islet cells in mice leads to hypertriglyceridemia\(^1\) and refeeding of insulin-deficient mice increased lipogenic gene expression, suggesting that regulation of de novo synthesis is independent of insulin.\(^2\) These data suggest that diabetic hypertriglyceridemia is not primarily caused by defective insulin signaling leading to increased hepatic fatty acid synthesis. The objective of this study was specifically to determine whether the effects of impaired insulin signaling on hepatic triglycerides production found with genetic modifications were also evident in mice with insulin deficiency.

In this report, we show that insulin deficiency in mice leads to increased plasma triglyceride levels and defective removal of postprandial triglycerides. This type of diabetic hypertriglyceridemia was not associated either with reduced mRNA levels of de novo triglyceride synthesis-related genes or with decreased hepatic triglycerides production. Lipoprotein lipase (LpL) mRNA was significantly reduced in skeletal muscle, white adipose tissue (WAT), and heart. Furthermore, LpL activity was decreased in skeletal muscle, brown adipose tissue, and heart. In addition, diabetes mellitus further increased plasma triglycerides in animals with a genetic LpL defect. Our data support human studies and suggest that significant hypertriglyceridemia in insulin-deficient diabetes mellitus is primarily because of changes in lipolysis and substrate return to the liver signaling.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

**Streptozotocin-Induced Diabetes Mellitus Causes Hypertriglyceridemia in Mice**

Two weeks after induction of insulin deficiency by intraperitoneal streptozotocin administration, diabetic mice displayed marked hyperglycemia (6.66±0.5 versus 25.55±0.72 mmol/L; Table 1A). Concomitantly, these mice had significantly elevated plasma triglyceride levels (1.42±0.09 versus 0.82±0.03 mmol/L in nondiabetic mice). Hypertriglyceridemia persisted after 6 weeks of streptozotocin diabetes mellitus (1.99±0.18 versus 0.91±0.06 mmol/L). In contrast, total plasma cholesterol levels and high-density lipoprotein cholesterol did not change at either time point. As expected, streptozotocin-diabetic mice lost weight compared with nondiabetic control animals. Changes in triglycerides were largely caused by increased VLDL triglycerides (1.33±0.09 versus 0.71±0.02 mmol/L; Table 1B). Plasma free fatty acid (FFA) were increased at both 3 and 6 weeks. Note that the baseline plasma FFA levels were higher in older mice. Plasma FFA showed a positive correlation with plasma triglyceride levels in streptozotocin-diabetic mice, whereas plasma FFA and triglycerides did not significantly correlate with body weight (online-only Data Supplement IA–IC).

**Insulin Deficiency Does Not Change Hepatic De Novo Lipogenesis and Hepatic Triglyceride Secretion**

To assess whether insulin deficiency and circulating glucose levels affect hepatic triglycerides production, we quantified triglycerides secretion in mice treated with streptozotocin and a lipase inhibitor, P407. Streptozotocin-induced diabetes mellitus did not alter triglycerides secretion compared with nondiabetic wild-type controls (Figure 1A). In line with this, hepatic gene expression of de novo lipogenesis genes (Fasn, Lpl) was decreased in skeletal muscle, brown adipose tissue, and heart. Furthermore, LpL activity was decreased in skeletal muscle, brown adipose tissue, and heart. In addition, diabetes mellitus further increased plasma triglycerides in animals with a genetic LpL defect. Our data support human studies and suggest that significant hypertriglyceridemia in insulin-deficient diabetes mellitus is primarily because of changes in lipolysis and substrate return to the liver signaling.

### Table 1. Metabolic Parameters in Control and Streptozotocin-Administered Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2nd Week</th>
<th>6th Week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-Diabetic Control</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>6.66±0.5</td>
<td>25.25±0.72</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>0.82±0.03</td>
<td>1.42±0.09</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>2.56±0.13</td>
<td>2.79±0.1</td>
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<tr>
<td>FFA, mmol/L</td>
<td>0.88±0.08</td>
<td>1.31±0.01</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>27.5±0.5</td>
<td>23.5±0.5</td>
</tr>
<tr>
<td>n</td>
<td>24</td>
<td>22</td>
</tr>
</tbody>
</table>

Student t test. FFA indicates free fatty acid.
*P<0.0001.
†P<0.001.
‡P<0.01 vs nondiabetic control.

### Table 2. Cholesterol and Triglycerides Subfractions in Control and Streptozotocin-Administered Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Triglycerides</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very-low-density lipoprotein (mmol/L)</td>
<td>0.71±0.02</td>
<td>0.36±0.03</td>
</tr>
<tr>
<td>Low-density lipoprotein (mmol/L)</td>
<td>0.26±0.02</td>
<td>0.6±0.08</td>
</tr>
<tr>
<td>High-density lipoprotein (mmol/L)</td>
<td>0.20±0.02</td>
<td>1.50±0.08</td>
</tr>
</tbody>
</table>

Student t test.
*P<0.0001.
‡P<0.01 vs nondiabetic control.
"Acc1, Dgat2, Scd1, Elovl6) showed no significant difference between the diabetic mice and healthy controls after 3 and 8 weeks (Figure 1B; online-only Data Supplement ID). Triglycerides content in the liver was decreased (Figure 1C; online-only Data Supplement IE), which was likely because of increased β-oxidation as indicated by increased gene expression of the rate limiting enzyme CPT1α (Figure 1B; online-only Data Supplement ID). Thus, hypertriglyceridemia in insulin-deficient mice is not caused by increased de novo lipogenesis and hepatic triglycerides secretion.

Hyperglycemia Does Not Drive Hypertriglyceridemia
Excess glucose in diabetes mellitus can be a substrate for hepatic de novo lipogenesis, thereby contributing to diabetic hypertriglyceridemia.14 To study if hyperglycemia drives hypertriglyceridemia, we treated control and streptozotocin-diabetic mice with the glucose-lowering agent dapagliflozin for 3 weeks. Dapagliflozin selectively inhibits the sodium glucose cotransporter 2 in the kidney, thereby increasing urinary glucose excretion and decreasing plasma glucose levels.

Figure 1. Insulin deficiency does not change hepatic de novo lipogenesis and hepatic triglycerides secretion. A. Triglycerides secretion was measured in streptozotocin-diabetic and nondiabetic C57BL/6 mice after IP injection of Poloxamer 407 (P407; n=5 per group). B. Hepatic gene expression of Fasn, Dgat2, Scd1, Acc1, Elovl6, Cpt1α, and Acc2 was assessed using quantitative real-time polymerase chain reaction. Gene expression is expressed relative to fed nondiabetic control mice (n=5 per group). C. Hepatic triglycerides, free fatty acid, and cholesterol content (n=5 per group). *P≤0.05, **P≤0.01. Results are presented as mean±SEM.

Figure 2. Hyperglycemia does not drive hypertriglyceridemia. A. streptozotocin-diabetic and control C57BL/6 mice were treated with the sodium glucose cotransporter 2 inhibitor dapagliflozin or vehicle and plasma glucose was measured at indicated time points (n=10 per group). ***P≤0.001 vs streptozotocin+vehicle. B. Four-hour fasting plasma triglyceride levels over time (n=10 per group). ***P≤0.001 vs nondiabetic controls. C. Hepatic triglycerides secretion was measured by IP injection of Poloxamer 407 (P407; n=5 per group). D. Plasma insulin levels after 4-hour fasting (n=10 per group). *P≤0.05, ***P≤0.001 vs control+vehicle. Results are presented as mean±SEM.
without affecting insulin levels in streptozotocin-diabetic mice as shown before15 and in our study (Figure 2D). Insulin levels in dapagliflozin-treated control mice are reduced likely because of the reduction in circulating glucose levels. Despite a significant reduction in plasma glucose levels in streptozotocin-diabetic mice neither plasma triglyceride levels (Figure 2A & 2B) nor triglycerides secretion (Figure 2C) decreased. Similar to the pharmacological approach, an antisense oligonucleotide against sodium glucose cotransporter 2 did not decrease plasma triglyceride levels in both control and streptozotocin-diabetic mice over the course of 4 weeks (online-only Data Supplement II). These studies suggest that diabetic hypertriglyceridemia is not driven by excess glucose.

Insulin Deficiency Leads to Increased Postprandial Lipemia

As hepatic de novo lipogenesis and triglycerides secretion did not seem to contribute substantially to hypertriglyceridemia, we explored whether defective peripheral lipolysis is the culprit for increased plasma triglyceride levels. After an olive oil gavage, streptozotocin-diabetic mice displayed markedly higher plasma triglyceride levels followed by delayed clearance of plasma triglycerides compared with that of controls (Figure 3A). LpL mRNA levels were significantly reduced in skeletal muscle, WAT, and heart after 3 weeks of diabetes mellitus (Figure 3B). At 8 weeks, skeletal muscle LpL was reduced and tended to decrease in the WAT and heart of these mice (online-only Data Supplement IIIA). However, mRNA levels of LpL do not always reflect LpL activity because LpL is regulated at transcriptional, translational, and posttranslational levels.15,16 Heparin-releasable LpL activity in the heart, brown adipose tissue, and skeletal muscle was significantly reduced in streptozotocin-diabetic mice (Figure 3C); there was no change in heparin-releasable activity of hepatic lipase and hepatic lipase mRNA in the liver (online-only Data Supplement IIIB and IV). Hepatic mRNA expression of the LpL-regulating proteins angiopoietin-like 3, 4, and 8 (Angptl 3, 4, and 8) and apolipoproteins apoA-V, ApoB, and ApoC-III were not significantly altered at both 3 and 8 weeks. We also did not detect significant changes of Angptl4, lipase maturation factor 1 and glycosylphosphatidylinositol-anchored high-density-binding protein 1 in the skeletal muscle (Figure IV in the online-only Data Supplement). However, we did find an increase in apoC-III in the plasma of streptozotocin-diabetic mice.

Figure 3. Increased postprandial lipemia with insulin deficiency. A, triglycerides turnover in mice after gavage of 10 mL/kg BW olive oil. Plasma triglycerides level were measured after 2, 4, and 6 hours. B, LpL mRNA analysis by quantitative polymerase chain reaction (PCR) in skeletal muscle, white adipose tissue, and heart at 3 weeks. C, Heparin-releasable lipoprotein lipase activity from freshly isolated heart, brown adipose tissue, and skeletal muscle using a radiolabeled triglyceride substrate. D, Immunoblot of plasma for ApoC-III. E, Gene expression of PPARα, PPARγ, and CD36 in the skeletal muscle after 3 weeks of streptozotocin-diabetes mellitus using quantitative real-time PCR (n=5 per group). *P≤0.05, **P≤0.01, ***P≤0.001. Results are presented as mean±SEM.
mice (Figure 3D). LpL activity in postheparin plasma did not correlate with the changes in mRNA levels or heparin-released muscle LpL activity (online-only Data Supplement IIIIC). Taken together, insulin-deficient diabetes mellitus caused a marked reduction in postprandial triglycerides removal associated with reduced expression and activity of skeletal muscle LpL.

**LpL Deficiency is Associated With Decreased Expression of PPARα and PPARδ**

To explore how insulin deficiency affects LpL expression and activity, we studied LpL regulating factors. LpL expression in several tissues has been linked to peroxisome proliferator-activated receptor (PPAR) expression and activation. In our study, reduced LpL mRNA levels were associated with markedly lower PPARα mRNA in the skeletal muscle of diabetic mice after 3 weeks (Figure 3E). PPARδ mRNA was also decreased, whereas PPARγ was unchanged. We detected similar expression patterns of PPARs in the skeletal muscle after 8 weeks of streptozotocin-diabetes mellitus (online-only Data Supplement V).

**LpL Expression in the Skeletal Muscle Modulates Diabetic Dyslipidemia**

Fasted wild-type mice have a rapid turnover of plasma triglycerides. To study a more human-like model with less robust triglycerides lipolysis, we induced diabetes mellitus in heterozygous LpL knockout mice (Lpl+/−). Our laboratory has previously reported that Lpl+/− mice on the ApoB overexpressing transgenic background have marked hypertriglyceridemia when treated with streptozotocin. Streptozotocin treatment of Lpl+/− mice led to markedly increased plasma triglycerides (2.45±0.26 mmol/L) compared with nondiabetic Lpl+/− mice (1.389±0.316 mmol/L; Figure 4A & B). Similar to wild-type mice, triglycerides secretion was not altered in streptozotocin-diabetic Lpl+/− mice after P407 treatment and hepatic triglycerides content was reduced (Figure 4C & D). Again, after olive oil gavage streptozotocin-diabetic Lpl+/− mice displayed a significant impairment of triglycerides clearance (Figure 4E).

![Figure 4](http://atvb.ahajournals.org/)

The highest postprandial triglyceride levels in streptozotocin-treated mice were identical to those of diabetic wild-type mice, but the rate of reduction between 4 and 6 hours was reduced compared with diabetic wild-type mice (4.2 mmol/L/h versus 7.7 mmol/L per h).

These data suggest that impaired triglycerides clearance is the major cause for hypertriglyceridemia in streptozotocin-diabetic mice. To test whether LpL overexpression would alter these diabetes mellitus-induced triglycerides changes, we studied postprandial lipemia in mice expressing human LpL primarily in skeletal muscle (Lpl+/−). We note that the MCK-LpL transgene also leads to a small amount of LpL expression in the heart. Streptozotocin-diabetic MCK-LpL/Lpl+/− mice had significantly lower fasting triglyceride levels compared with streptozotocin-diabetic muscle creatine kinase (MCK)-LpL mice (Figure 5A). In addition, triglycerides clearance after olive oil gavage was significantly improved in streptozotocin-diabetic MCK-LpL/Lpl+/− mice compared with streptozotocin-diabetic Lpl+/− mice (Figure 5B). Therefore, LpL overexpression corrects diabetic dyslipidemia in insulin-deficient mice.

![Figure 4](http://atvb.ahajournals.org/)

Figure 4. streptozotocin-diabetic heterozygous Lpl+/− mice display increased plasma triglycerides and postprandial hypertriglyceridemia. Four-hour fasting glucose (A) and triglycerides levels in streptozotocin-diabetic and nondiabetic Lpl+/− mice at 4 weeks (B; n=6–10 per group). Hepatic triglycerides secretion in Lpl+/− mice was measured by IP injection of Poloxamer 407 (C; P407; n=6–10 per group). Hepatic triglycerides content in streptozotocin-diabetic and nondiabetic Lpl+/− mice (D; n=5–7 per group). Hepatic triglycerides turnover was quantified in mice after gavage of 10 mL/kg BW olive oil. Plasma triglyceride levels were measured after 2, 4, and 6 hours (E; n=5–7 per group). *P≤0.05, **P≤0.01, vs nondiabetic Lpl+/− mice. Results are presented as mean±SEM.
Discussion

In this report, we show that hypertriglyceridemia in insulin-deficient diabetic mice is primarily caused by changes in peripheral lipolysis and not because of changes in hepatic insulin signaling and hepatic triglycerides secretion. Similar to humans with either T1DM or T2DM, streptozotocin-induced diabetes mellitus in C57BL/6 mice led to a significant increase in plasma triglyceride levels. This observation was similar to that described by others. The primary lipoprotein abnormality in human diabetes mellitus is an increase in VLDL, which was reproduced in our streptozotocin-treated mice. There are 2 possible causes of diabetes mellitus-associated increase in triglycerides: elevated secretion of triglycerides from the liver or decreased clearance of triglycerides. Hepatic overproduction of triglycerides has been attributed to compromised ApoB degradation because of the loss of insulin action, which eventually leads to increased VLDL assembly and secretion. Loss of insulin action also increases FFA flux to the liver and increases hepatic lipogenesis. In addition, it has been suggested that hyperinsulinemia can drive de novo lipogenesis in the presence of increased plasma glucose levels and hepatic insulin resistance. It should be noted that most human kinetic studies on triglycerides production are performed by injection of glycerol, which cannot separate de novo lipogenesis from substrate-driven triglycerides production. Therefore, these studies cannot determine the contribution of hepatic de novo lipogenesis and substrate-driven triglycerides production to hypertriglyceridemia. (2) Diabetic humans exhibit a marked defect in the clearance of postprandial lipemia. These findings are in accordance with reports of decreased LpL activity in postheparin plasma and in skeletal muscle biopsies of diabetic patients. Accordingly, Taskinen et al have shown that >20% of the hypertriglyceridemia in obese men is associated with increases in VLDL, secretion but almost 50% is related to impaired fractional catabolic rate. It is not clear, however, why only some patients develop hypertriglyceridemia with diabetes mellitus. For instance, in the ACCORD trial the average triglycerides level in all diabetic patients was 1.829 mmol/L (162 mg/dL), a level below the clinical threshold of hypertriglyceridemia (2.258 mmol/L [200 mg/dL]). Therefore, it is likely that the minority of patients who develop severe hypertriglyceridemia have a defect in lipolysis.

Our objective was specifically to determine whether the effects of insulin signaling on hepatic triglyceride production found with genetic modifications were evident in mice with insulin deficiency. In our mouse model of streptozotocin-induced diabetes mellitus, insulin deficiency did not lead to a significant reduction in hepatic triglycerides secretion nor did the lack of insulin actions reduce expression of genes involved in de novo lipogenesis. As reported by others, hepatic triglyceride content was significantly reduced and plasma FFA levels were increased in streptozotocin-diabetic mice. The reduced hepatic triglycerides content could be partially caused by increased fatty acid oxidation because mRNA levels of the rate limiting enzyme of β-oxidation CPT1-α were increased. Jourdan et al have also noted an increase in CPT1-α and reported increased β-oxidation of palmitate in the livers of streptozotocin-treated mice. It is possible that without this increase in oxidation, more FFA would be converted to triglycerides leading to greater hepatic triglycerides secretion. Taken together, our data do not support the hypothesis that insulin deficiency reduces triglycerides production and suggests that the hypertriglyceridemia in streptozotocin-diabetic mice is primarily caused by a catabolic defect.

No model of diabetes mellitus is perfect but the streptozotocin model used in our studies is the one recommended by the National Institutes of Health Animal Models of Diabetic Complications Consortium. The low dose streptozotocin leads to partial insulin deficiency that causes hyperglycemia but allows sufficient insulin to prevent ketoadiisis and early death. This model does not lead to obesity and peripheral insulin resistance as would be seen in T2DM. In fact, streptozotocin-treated mice have reduced adipose stores and the effects of insulin deficiency in obese animals are likely to be different. Specifically, greater adipose would be expected to lead to greater FFA release and high plasma FFA levels that are likely to drive greater liver production of triglycerides. Nonetheless, we think that streptozotocin treatment is more likely to reflect human disease than models that lead to nonphysiological modification of gene expression.

Independent of insulin action on hypertriglyceridemia, glucose also generates a signal that may modulate triglycerides production and triglycerides clearance. Glucose controls the expression of key genes involved in energy metabolism through the carbohydrate response element-binding protein. Carbohydrate response element-binding protein binds to the promoter region of glycolysis-associated (eg, pyruvate kinase) and lipogenesis-associated genes and may drive diabetic hypertriglyceridemia by increasing transcription of de novo lipogenesis genes. Glucose may also exert direct effects on triglycerides clearance. Hyperglycemic conditions...
downregulate LpL activity in humans\textsuperscript{42,43} and in cultured human adipocytes.\textsuperscript{44} This suggests that hyperglycemia per se contributes to the decreased LpL activity and subsequent hypertriglyceridemia in poorly controlled diabetes mellitus. Until recently it has been difficult to separate the effects of hyperglycemia versus defective insulin actions on diabetic hypertriglyceridemia because glucose-reducing interventions were achieved by either insulin administration or by improved insulin sensitivity. By inhibiting sodium glucose cotransporter 2 in the kidney, which leads to increase glucose output in the urine, we were able to reduce plasma glucose levels without affecting insulin levels in streptozotocin-diabetic mice, although we cannot rule out changes in insulin signaling.\textsuperscript{45} Both the pharmacological inhibition by dapagliflozin and antisense oligonucleotide-mediated inhibition of sodium glucose cotransporter 2 significantly reduced plasma glucose levels in streptozotocin-diabetic mice, but did not change plasma triglyceride levels. More importantly, hepatic triglycerides secretion was similar in streptozotocin-diabetic mice with normalized glucose levels making it unlikely that increased glucose levels contribute substantially to hypertriglyceridemia in streptozotocin-diabetic mice.

Postprandial lipemia is markedly increased in humans with diabetes mellitus.\textsuperscript{46-48} Accordingly, in our study streptozotocin-diabetic mice displayed increased plasma triglyceride levels and delayed clearance after gavage with olive oil. LpL is the rate-limiting enzyme for the hydrolysis of triglycerides in circulating chylomicrons and VLDL. LpL-mediated hydrolysis products, fatty acids, and monoacylglycerol, are taken up by the tissues and stored as neutral lipids in WAT, or oxidized in skeletal and cardiac muscle.\textsuperscript{15,16} LpL is regulated at the transcriptional, post-transcriptional, and post-translational level in a tissue-specific manner.\textsuperscript{49} Food-intake decreases LpL activity in skeletal muscle and increases it in WAT. Fasting increases LpL activity in the skeletal muscle and heart, and decreases LpL in the WAT. We detected a significant decrease of LpL gene expression in the skeletal muscle, heart, and WAT after 3 and 8 weeks of streptozotocin-diabetes mellitus. Accordingly, heparin-releasable LpL activity in the skeletal muscle, heart, and brown adipose tissue of streptozotocin-diabetic mice was significantly reduced; all these tissues are major sites for LpL synthesis.\textsuperscript{50} We were unable to study heparin-releasable LpL activity in WAT because streptozotocin-diabetic mice were lipodystrophic, but the reduction in total adipose LpL, as we have shown before, likely contributes to the defective lipolysis.\textsuperscript{51} Therefore, these results show that decreased LpL activity in brown adipose tissue, skeletal muscle, and heart correlates with hypertriglyceridemia in streptozotocin-diabetic mice. Increased apoC-III that we detected in plasma might also affect lipolysis or the changes in lipoproteins associated with LpL deficiency and diabetes mellitus might have caused a secondary increase in apoC-III. Perhaps this ApoC-III increase is also responsible for the defect in low-density lipoprotein uptake previously reported in diabetic low-density lipoprotein receptor knockout mice.\textsuperscript{52} LpL activity has been studied previously in diabetic humans and rodents. In patients with T2DM LpL mRNA, protein expression and activity were significantly decreased in skeletal muscle.\textsuperscript{35,36} Conflicting results have been reported in studies of LpL activity in the heart and the skeletal muscles in streptozotocin-treated rodents. Both decreased\textsuperscript{53} and increased\textsuperscript{54} activity levels have been observed. The reported differences might relate to the length as well as severity of diabetes mellitus and methodological differences, for example, measurement of LpL activity in homogenized tissue versus heparin-releasable LpL activity and data obtained from mice versus rats. In fact, although we found reduced heparin-releasable LpL activity in the muscles of diabetic mice, LpL activity measurements of tissue homogenates were not significantly different (data not shown).

In contrast to the decreased LpL activity in the skeletal muscle, postheparin lipase activity in the plasma did not differ between our streptozotocin-diabetic and control mice (2.4±0.16 μM FFA/mL per h versus 2.4±0.10 μM FFA/mL per h, respectively). Similarly, Pollare et al\textsuperscript{56} did not detect differences in postheparin LpL activity in the plasma of T2DM patients but reported decreased LpL activity in the skeletal muscle of these patients. It is questionable whether total postheparin plasma LpL activity accurately reflects changes in total lipolytic activity.

To explain the reduced levels of LpL, we tested gene expression levels of 2 well-known transcriptional regulators of LpL, PPAR\textsubscript{α}, and PPAR\textsubscript{δ}. Schoonjans et al\textsuperscript{57} showed that the expression of LpL is regulated by PPAR\textsubscript{α} (and PPAR\textsubscript{γ}), which interact with a response element in the LpL promoter. PPAR\textsubscript{δ} agonist treatment increases LpL mRNA and protein levels in skeletal muscle cells\textsuperscript{58}; PPAR\textsubscript{δ} also increases expression of the LpL inhibitor Angplt4,\textsuperscript{55} which was not significantly decreased in our studies. Several clinical studies suggested that the triglyceride-lowering action of PPAR\textsubscript{α} agonists is associated with an increase in LpL activity.\textsuperscript{56-58} Others have shown that PPAR\textsubscript{α} and PPAR\textsubscript{γ} agonists enhance LpL expression and activity in human macrophages.\textsuperscript{59} Considering these studies, we hypothesized that decreased LpL expression in the skeletal muscle of streptozotocin-diabetic mice was because of a decreased transcription of PPAR\textsubscript{α}. Insulin enhances the transcriptional activity of PPAR\textsubscript{α} in vitro.\textsuperscript{60} We found reduced expression of PPAR\textsubscript{α} and PPAR\textsubscript{δ} with insulin deficiency in skeletal muscle of streptozotocin-diabetic mice. We hypothesize that the lack of insulin leads to a decrease primarily of PPAR\textsubscript{α} followed by a decrease of LpL expression in the skeletal muscle. Surprisingly, we noted reduced CD36 expression in skeletal muscle of diabetic mice. Although one might expect that myocytes would shift to greater fatty acid oxidation with insulin deficiency, we suspect that muscle metabolism in our animals was heavily dependent on glucose, but with glucose uptake primarily via noninsulin-dependent and non-GLUT4-dependent pathways.

To study if changes in LpL expression would actually modify diabetes mellitus-induced hypertriglyceridemia, we studied mice with a heterozygous deletion of the LpL gene (as homozygous LpL knockout mice are not viable). LpL\textsuperscript{−/−} mice exhibited marked hypertriglyceridemia after induction of streptozotocin-diabetes mellitus. More importantly, triglycerides secretion was again not significantly different
strepotzotocin-diabetic Lpl+/− mice but triglycerides clearance was delayed after gavage with olive oil. Similar to our diabetic Lpl+/− mice, human diabetic carriers of dysfunctional LpL alleles are at risk for severe lipemia.60 We hypothesized that increasing LpL expression would ameliorate diabetic hypertriglyceridemia in our animal model. Constitutive skeletal muscle-specific expression of human LpL eliminated hypertriglyceridemia in the streptozotocin-diabetic Lpl+/− mice and triglycerides clearance after olive oil gavage was reduced to almost normal nondiabetic levels. We presume that the constitutive expression of LpL increased lipolysis and VLDL remnant removal. Similarly, Shimada et al61 reported that diabetes mellitus-mediated increases in triglycerides were eliminated by overexpression of LpL in streptozotocin-diabetic C57BL/6 mice. Thus, a major regulator of circulating triglycerides in diabetes mellitus is LpL action regardless of changes in liver triglycerides production.

In conclusion, hypertriglyceridemia in insulin-deficient mice is primarily caused by a defect in peripheral lipolysis and not by an increase in hepatic triglycerides secretion as suggested by mouse models of impaired hepatic insulin signaling. Most humans with diabetes mellitus do not have marked hypertriglyceridemia. However, humans, like mice, that have a defect in LpL regulation are most likely to develop severe hypertriglyceridemia regardless of their degree of insulin deficiency or resistance. Therefore, methods to augment peripheral triglycerides lipolysis are likely to be most efficacious in the treatment of diabetes mellitus-induced hypertriglyceridemia.

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Disclosures

None.

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mellitus is primarily because of changes in lipolysis and not changes in hepatic insulin signaling. Regulation of mitochondrial biogenesis by lipoprotein lipase in muscle of insulin-resistant offspring of parents with type 2 diabetes. Shulman GI. Regulation of mitochondrial biogenesis by lipoprotein lipase. Clin Invest 1996;26:89–108.


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Supplement Material
Supplemental Figure I: Multivariate analysis comparing (A) FFA and body weight, (B) TG and body weight and (C) TG and FFA. (D) Hepatic gene expression of Fasn, Dgat2, Scd1, Acc1, Elovl6, Cpt1α and Acc2 was assessed in mice with 8 weeks of STZ-diabetes using quantitative real-time PCR. Gene expression is expressed relative to fed non-diabetic control mice (n = 5-8/group). (E) TG content in livers of 8 weeks STZ-diabetic mice (n = 5-8/group). *: p ≤ 0.05 vs. control. Results are presented as mean ± SEM.
Supplemental Figure II: Glucose reduction in STZ-diabetic mice by SGLT2-antisense does not reduce plasma TG levels (A) Course of 4 hours fasting glucose levels over 28 days. **: p ≤ 0.01, ***: p ≤ 0.001 vs. STZ + mismatch, #: p ≤ 0.001 vs. control + mismatch. (B) Course of 4 hours fasting TG levels, *: p ≤ 0.05 STZ-diabetic groups vs. respective non-diabetic control groups. (C) Gene expression of SGLT2 in the kidney of the different treatment groups, ***: p ≤ 0.001 vs. respective mismatch group. (D) Glucose levels in the urine of the different treatment groups. *: p ≤ 0.05 SGLT2-ASO mice vs. respective mismatch mice, #: p ≤ 0.001 vs. control + mismatch. (n = 5-8/group for all panels). Results are presented as mean ± SEM.
Supplemental Figure III: (A) Lpl mRNA analysis by quantitative PCR in skeletal muscle, WAT and heart at 8 weeks of diabetes. *: p ≤ 0.05 vs. non-diabetic control (n = 5-8/group). (B) heparin-releasable hepatic lipase (HL) activity from liver tissue. (C) Post-heparin plasma (PHP) LpL and hepatic lipase (HL) activity at 3 weeks of diabetes (n = 5/group). Results are presented as mean ± SEM.
Supplemental Figure IV: Gene expression of apolipoproteins and regulators of lipolysis in liver, skeletal muscle and WAT after 3 (A) and 8 weeks (B) of STZ-diabetes using quantitative real-time PCR (n = 5/group for 3 weeks and 5-8/group for 8 weeks). Results are presented as mean ± SEM.
Supplemental Figure V: Gene expression of PPARα, PPARδ, PPARγ and CD36 was assessed in the skeletal muscle after 8 weeks of STZ-diabetes using quantitative real-time PCR (n = 5-8/group). *: p ≤ 0.05, **: p ≤ 0.01. Results are presented as mean ± SEM.
## Supplemental Table I: Primer sequences used for RT-PCR

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Detailed Methods and Material

Mouse breeding and husbandry: All procedures were in accordance with current National Institutes of Health guidelines and were approved by the Columbia University Institutional Animal Care and Use Committee. Wild type C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Muscle-specific LpL-expressing transgenic mice (MCK-LpL) were generated as described\(^1\). Heterozygous LpL knockout mice (LpL\(^{+/-}\)) were generated by crossing LpL knockout mice rescued with LpL expression in skeletal muscle (MCK-LpL/LpL\(^{-/-}\) mice) with wild type C57BL/6 mice. Mice were maintained in a temperature-controlled (25°C) facility with a 12-h light/dark cycle and given free access to water and food, except when fasting blood specimens were obtained. Mice were fed a laboratory rodent chow diet. After 4 hour-fasting blood samples were collected using heparinized capillary tubes and then placed into 1 mM EDTA-containing Eppendorf tubes. Mice were allowed 7 days to recover between phlebotomies.

Genotyping: LpL knockout mice were screened with primers 5'- GCCGGGGGCGGGGGGAATCTCCTGACTAGGGG-3', 5'-CTCGCTGGCACTAGGGG-3' and 5'-ACTGGAGCGCGGTGGAGCGCCGTAGGGCA-3'. PCR amplification was performed using 30 cycles at 94°C for 1 min and 60°C for 2 min and 72°C for 3 min. Genotypes for MCK–human LpL mice were determined by PCR as described below. Human LpL specific primers used were as follows: 5'- CAC ATG GCC AGA GTC AGC AC - 3' . The protocol involved 20 cycles of 94°C for 30 sec, 68°-0.5°C/cycle for 1 min, and 72°C for 1 min followed by 10 cycles of 94°C for 30 sec, 58°C for 1 min and 72°C for 60 sec. PCR products were resolved by gel electrophoresis, and were visualized by ethidium bromide staining.

Induction of insulin deficiency: Mice were made diabetic by streptozotocin (STZ) treatment similar to that described by Kunjathoor, Wilson, and LeBoeuf\(^2\) and adopted by the Diabetes Complications Consortium\(^3\). Mice were divided into two groups; one group was treated with STZ (Sigma Chemical Co., St. Louis, MO), the other group was treated with vehicle. STZ was dissolved in sterile citrate buffer (pH 4.5) and used within 20 min of preparation. The solution was injected intraperitoneally (i.p.) into mice (50 mg/kg, ~200 µl) for five consecutive days. 10 days after the last STZ injection 4-hours fasting glucose levels were measured. STZ-injected mice with glucose levels >13.9 mmol/l (>250mg/dl) were considered to be diabetic.

Plasma glucose, plasma insulin, lipid, and lipoprotein determinations: Glucose was measured using a glucose meter (OneTouch Ultra2, LifeScan, Milpitas, CA). Plasma insulin levels were measured using the Mouse Insulin ELISA by Merck Millipore (# EZRMI-13K, Billerica, MA). Lipids were measured using Infinity Triglyceride Reagent and Infinity Total Cholesterol Reagent (#TR22321 and #TR13521 respectively, Thermo Scientific, Waltham, MA). Lipoproteins, VLDL (d < 1.006 g/ml), IDL+LDL (d 1.006–1.063 g/ml), and HDL (d 1.063–1.21 g/ml), were separated by sequential density ultracentrifugation of mouse plasma in a TLA 100 rotor (Beckmann Instruments, Palo Alto, CA). Free fatty acids (FFA) were measured using the Wako HR series kit NEFA (#99934691, Wako Life Sciences, Richmond, VA). All of the above parameters were measured after 4 hours of fasting (8am -12 am).

Tissue collection: After 4 hour-fasting mice were anesthetized with xylazine (10 mg/kg) and ketamine (100 mg/kg) and then perfused by heart puncture with 10 ml of PBS or until the livers blanched. Tissues were rapidly excised and snap frozen in liquid nitrogen unless otherwise noted.

Gene expression by quantitative real time PCR: Total RNA was obtained from tissues homogenized in TRIzol reagent (#15596-018, Ambion, Austin, Tx). The PureLink RNA mini kit (#12183018A, Ambion, Austin, Tx) was used for the RNA purification. The RNA was then
reverse-transcribed by ThermoScript RT-PCR System (#11146-057, Invitrogen, Carlsbad, CA), and quantitative real time PCR was performed with Stratagene Mx3005 using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). All genes were normalized to 18s rRNA expression. All primer sequences are listed in Supplement Table I.

**LpL Activity Assay:** Postheparin plasma LpL activity was determined as described by Hocquette et al. Postheparin plasma was obtained from 4h-fasted mice 7 min after femoral vein injection of 100 units of heparin/kg body weight. To measure total lipase activity, plasma was incubated with 10% Intralipid/[^3]H]-triolein (#NET431L005MC, Perkin Elmer, MA) emulsion as substrate and human serum as the source of apoCII. The contribution of hepatic lipase in the plasma was determined by including NaCl (final concentration 1M) in the assay and was subtracted from the total lipase activity to estimate specific LpL activity. Activity was expressed as µmol FFA/ml/h. Aliquots of human plasma were used for a standard curve in all the experiments. Heparin-releasable LpL activity in skeletal muscle, BAT and heart was measured following the method by Haugen et al. Briefly, freshly isolated tissues were minced in Krebs-Ringer phosphate buffer and incubated for 1 hour in a 25 °C water bath in the presence of 5U/ml heparin. 100 µl aliquots of the buffer were used for the lipase assay with 100 µl of 10% Intralipid[^3]H]-triolein (NET431L005MC, Perkin Elmer, MA) emulsion for 1 hour at 25°C.

**Hepatic TG secretion:** To measure hepatic TG production rate, mice were injected intraperitoneally with Poloxamer-407 (#16758, Sigma-Aldrich, St. Louis, MO) at 1 g/kg in saline after 4-h fasting. Immediately prior to injection, and at 1, 2, and 6h following injection, blood samples were drawn in heparin capillary tubes, plasma was prepared, and TG concentrations were determined. The TG production rate was calculated from the difference in plasma TG levels over a given interval following detergent injection.

**Olive oil gavage:** After a 4 hour fast, mice were given olive oil by oral gavage at 10ml/kg BW (~250µl). Blood samples were drawn at baseline 2, 4 and 6 hours after receiving the olive oil gavage. For comparison TG levels were enzymatically measured using Infinity Triglyceride Reagent (#TR22321, Thermo Scientific, Waltham, MA).

**Western Blot Analysis:** Plasma samples of four control and four STZ-diabetic mice were used for Western Blot Analysis of ApoCIII. Twenty micrograms protein of each sample were resolved by SDS-PAGE and transferred onto PVDF membranes. Immunoblotting was carried out using a rabbit anti-ApoCIII antibody (kindly provided by ISIS Pharmaceuticals, Inc., Carlsbad, CA). Albumin was used as a loading control. Bands were quantified by densitometry using Molecular Analysis Software (Bio-Rad).

**Lipid extraction and hepatic TG, total cholesterol and FFA content:** The lipid extraction protocol was adapted from Folch et al. Approximately 100 mg of tissue in 1 mL of PBS were homogenized using stainless steel beads for 1 min in a bead beater homogenizer. From each sample, 50 µL were removed for protein analysis and 3 mL of 2:1 chloroform: methanol was added to the rest and vortexed. Samples were then centrifuged for 10 minutes at 3000 rpm at 4°C. The lower, organic phase was then collected and dried under nitrogen gas. The dried lipid was then dissolved in 500 µL of 1% Triton-X 100 in chloroform, further dried and then dissolved in 100µL of double distilled water. Hepatic TG, total cholesterol and FFA content were measured using Infinity Triglyceride Reagent, Infinity Total Cholesterol (both Thermo Scientific, Waltham, MA) and the Wako HR series kit NEFA (Wako Life Sciences, Richmond, VA), respectively.

**Tissue lipid and protein measurements:** The sample of tissue lysate retained from the lipid extraction protocol was assayed for protein content using Pierce BCA Protein kit (#23227 Thermo Scientific, Rockfort, IL) following the instructions of the manufacturer. Using the
tissue lipid extract, assays for TG were performed as previously described for plasma lipids. Lipid measurements were normalized to protein content.

**Antisense oligonucleotide (ASO) treatment:** In order to inhibit the sodium glucose co-transporter 2 (SGLT2), expression mice were injected with the ASO, ISIS 388625 (sequence 5'-TGTTCCAGCCCA-3') at 20 mg/kg body weight 10 days after the last STZ injection. Control mice were injected with ASO, ISIS-141923 (5'-CCTTCCCTGAAGGTTCCTCC-3'), which does not have perfect complementarity to any known gene in public databases. All ASOs were dissolved in sterile saline for IP injection.

**Statistical analyses:** Data are presented as means ± SEM, where the means are based on at least three independent experiments. Statistical differences were assessed via a paired Student’s t-test or a one-way ANOVA where appropriate. A p value less than 0.05 was considered as significant.
Supplemental References


