Insulin sensitivity of hepatic glucose and lipid metabolism in animal models of hepatic steatosis
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Acute hepatic steatosis in mice by blocking β-oxidation does not reduce insulin sensitivity of very low density lipoprotein production

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Abstract

 Accumulation of triglycerides (TG) in the liver is generally associated with hepatic insulin resistance. We questioned whether acute hepatic steatosis induced by pharmacological blockade of β-oxidation affects hepatic insulin sensitivity, i.e., insulin-mediated suppression of VLDL production and insulin-induced activation of phosphatidylinositol 3-kinase (PI3-kinase) and PKB. Tetradecylglycidic acid (TDGA), an inhibitor of carnitine palmitoyl transferase-1 (CPT1), was used for this purpose. Male C57BL/6J mice received 30 mg/kg TDGA or its solvent intraperitoneally and were subsequently fasted for 12 h. CPT1 inhibition resulted in severe microvesicular hepatic steatosis (19.9 ± 8.3 vs. 112.4 ± 25.2 nmol TG/mg liver, control vs. treated, P < 0.05) with elevated plasma nonesterified fatty acid (0.68 ± 0.25 vs. 1.21 ± 0.41 mM, P < 0.05) and plasma TG (0.39 ± 0.16 vs. 0.60 ± 0.10 mM, P < 0.05) concentrations. VLDL-TG production rate was not affected on CPT1 inhibition (74.9 ± 15.2 vs. 79.1 ± 12.8 µmol TG·kg⁻¹·min⁻¹, control vs. treated) although treated mice secreted larger VLDL particles (59.3 ± 3.6 vs. 66.6 ± 4.5 nm diameter, P < 0.05). Infusion of insulin under euglycemic conditions suppressed VLDL production rate in control and treated mice by 43 and 54%, respectively, with formation of smaller VLDL particles (51.2 ± 2.5 and 53.2 ± 2.8 nm diameter). Insulin-induced insulin receptor substrate (IRS)1- and IRS2-associated PI3-kinase activity and PKB-phosphorylation were not affected on TDGA treatment. In conclusion, acute hepatic steatosis caused by pharmacological inhibition of β-oxidation is not associated with reduced hepatic insulin sensitivity, indicating that hepatocellular fat content per se is not causally related to insulin resistance.
Introduction

The liver is a key player in the control of whole body energy metabolism by its ability to synthesize, oxidize, store, and distribute the major sources of energy, i.e., glucose and fatty acids. In fed conditions, when excess glucose is available from the intestine, plasma insulin levels are high, and glucose is stored in the liver as glycogen or enters the glycolytic pathway. High insulin suppresses hepatic glucose production (HGP), increases hepatic glucose uptake, and stimulates de novo lipogenesis (11). In addition, insulin suppresses the secretion of VLDL particles by the liver (36). The actions of insulin on the liver, initiated by binding of insulin to its receptor, involve signaling pathways that transduce its effects on gene transcription, protein metabolism, and, finally, fluxes of substrates. Of relevance for this work is insulin signaling through the phosphatidylinositol 3-kinase (PI3-kinase) pathway. On insulin binding, the insulin receptor substrate (IRS) is phosphorylated, and, subsequently, PI3-kinase is activated. PI3-kinase-mediated phosphatidylinositol 3,4,5-trisphosphate (PIP3) generation and PIP3-mediated phosphorylation of PKB at Ser473 and Thr308 are key processes in the insulin signaling cascade (34). Both PI3-kinase and PKB are involved in execution of the effects of insulin on hepatic glucose metabolism (34). It has been reported that insulin affects VLDL production via accelerated degradation of apolipoprotein B (apoB) (10), a process mediated by PI3-kinase (7) but thought to be independent of PKB (2).

When insulin levels are low, e.g., during fasting, glucose is mobilized from hepatic glycogen stores and hepatic gluconeogenesis is facilitated. β-Oxidation of fatty acids is considered the primary source of the energy and reducing equivalents (ATP, NADH) needed for gluconeogenesis. β-Oxidation disorders in humans and mice are associated with hypoglycemia and reduced ketogenesis as well as with hepatic triglyceride (TG) accumulation (hepatic steatosis) on fasting (33). For instance, mice deficient for the major regulator of β-oxidation, peroxisome proliferator-activated receptor-α, showed massively increased hepatic TG levels on fasting (3,17). Hepatic steatosis is generally associated with reduced hepatic insulin sensitivity (1,9,24,35), and various animal models have been used to study the effects of hepatic steatosis on insulin sensitivity with regard to glucose and fatty acid homeostasis (9). For instance, the hepatic steatosis in leptin-deficient ob/ob mice is associated of reduced insulin sensitivity of both HGP (16a) and VLDL secretion (44). Moreover, mice that lack the peripheral fatty acid transporter (CD36−/− mice) showed massive hepatic TG accumulation with decreased sensitivity of HGP to insulin (15).

During the β-oxidation process, fatty acyl-CoAs are broken down into shorter chains by a series of dehydrogenases. Long-chain fatty acids are unable to freely pass the mitochondrial membrane and their transfer across the outer mitochondrial membrane is mediated by carnitine palmitoyltransferase-1 (CPT1). CPT1 facilitates coupling of carnitine to fatty acyl-CoA (32,41). The acylcarnitine thus generated can be transferred across the outer membrane by CPT1. Acylcarnitines pass the inner mitochondrial membrane with the help of carnitine acylcarnitine translocase. Finally, inside the mitochondria, carnitine is removed from the acylchain by CPT2. CPT1 activity has been shown to be rate controlling for a major part of hepatic β-oxidation (32). Tetradecylglycidic acid (TDGA) is an inhibitor of CPT1 (22,38,39) and can be used to evaluate the immediate effects of impaired β-oxidation. We used TDGA to address the question of whether hepatic steatosis due to impaired β-oxidation is associated with impaired hepatic insulin sensitivity, i.e., insulin-mediated suppression of VLDL secretion and insulin-stimulated PKB phosphorylation and PI3-kinase activity.
Material and methods

Animals and chemicals
Male C57BL/6J mice (Harlan, Horst, The Netherlands) were housed in a light- and temperature-controlled facility and were fed a commercially available lab chow (cat. no. RMH-B; Hope Farms, Woerden, The Netherlands). All experiments were approved by the Animal Experiments Ethical Committee of the University of Groningen.

TDGA was a kind gift from Dr. P. J. Voshol (TNO, Leiden, The Netherlands) and was suspended in a concentration of 2.0 mg/ml in a vehicle consisting of 90 mg/ml BSA in saline (45).

Collection of basal plasma and liver samples
Mice received either 30 mg/kg TDGA or its solvent by intraperitoneal injection. Food was removed, but the mice still had access to water. Blood glucose levels were measured with a Lifescan EuroFlash glucose meter (Lifescan Benelux, Beersel, Belgium) in a small tail blood sample taken every hour. After 12 h, mice were killed under isoflurane anesthesia. A large blood sample was collected by cardiac puncture and centrifuged. Plasma was stored at –20°C until analyzed. The liver was quickly removed, weighed, and frozen in separate portions for RNA isolation and lipid analyzes. Plasma TG, phospholipid, nonesterified fatty acids (NEFAs), and cholesterol were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany, and Wako Chemicals, Neuss, Germany).

Hepatic TG and cholesterol concentrations were measured using commercial kits (Roche Diagnostics and Wako Chemicals) after lipid extraction according to Bligh and Dyer (5). After lipid extraction, phospholipid content of the liver was determined according to Böttcher et al. (6). Protein concentrations in livers were determined according to Lowry et al. (26) by using BSA (Pierce, Rockford, IL) as standard. Hepatic glycogen and glucose-6-phosphate levels were determined as described previously (18,20). Fatty acid composition was determined by gas chromatography after methylation as described previously (25). Liver histology was examined on 4-µm-thick frozen liver sections after Oil-Red-O staining for neutral lipids by standard procedures. mRNA expression levels in liver was measured by real-time RT-PCR as described previously (16). PCR results were normalized to β-actin and 18S mRNA levels. The sequences of the primers and probes used were listed previously (16,31).

In vivo VLDL-TG production rate
Mice received either 30 mg/kg TDGA or its solvent as described and were subsequently fasted for 9 h. After fasting, mice received an orbital injection of 12.5 mg Triton WR-1339 in 100 µl PBS. Tail blood samples were taken under light isoflurane anesthesia before and every 30 min after Triton injection. A large blood sample was collected by cardiac puncture, 90 min after Triton injection. The collected blood samples were used for TG measurements.

To determine the VLDL-TG production rate under hyperinsulinemic conditions, mice were equipped with a permanent catheter in the right atrium via the jugular vein (23). The two-way entrance of the catheter was attached to the skull with acrylic glue. The mice were allowed a resting period of at least 5 days. Mice then received either 30 mg/kg TDGA or its solvent as described and were subsequently fasted. Mice were kept in metabolic cages during the experiment and the preliminary fasting period, allowing frequent collection of small tail blood samples under conscious and unrestrained conditions (40). After 9 h of fasting, the mice were infused for 3 h with two solutions. The first was a 1% BSA solution containing 40 µg/ml somatostatin (UCB, Breda, The Netherlands). This solution contained insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark), leading to an insulin infusion rate of 20 mU·kg⁻¹·min⁻¹. To prevent too high total infusion rates, this solution contained 200 mg/ml glucose. The solution
was infused at a constant flow rate of 0.135 ml/h. The second infusate was a 30% glucose solution and its infusion rate was adjusted according to measured blood glucose levels to maintain euglycemic conditions. Blood glucose levels were measured in a small tail blood sample taken every 15 min. After 90 min of infusion, mice received an injection of 12.5 mg Triton WR-1339 in 100 µl PBS via the orbita under light isoflurane anesthesia. Orbital blood samples were taken under light isoflurane anesthesia before and every 30 min after Triton WR-1339 injection. A large blood sample was collected by cardiac puncture 90 min after Triton WR-1339 injection. The collected blood samples were used for TG measurements. VLDL-TG production rate was calculated from the slope of the TG concentration vs. time curve (27). The large blood sample was used for isolation of VLDL.

**VLDL isolation and analyses**

Plasma VLDL/intermediate-density lipoprotein (IDL) (density < 1.006 g/ml) was isolated by density gradient ultracentrifugation. Hereo, 300 µl plasma was adjusted to 1,000 µl with a NaCl/KBr solution of density = 1.006 g/ml and centrifuged at 120,000 rpm in a Optima LX tabletop ultracentrifuge (Beckman Instruments, Palo Alto, CA). VLDL was isolated by tube slicing, and the volume was recorded by weight. ApoB100 and apoB48 were determined by Western blot analysis, using antibodies against human apoB raised in rabbit. TG and cholesterol content were determined as described for plasma. Phospholipid content was determined using a commercially available kit (Wako Chemicals), and fatty acid composition as described previously (25).

VLDL size and volume distribution profiles were analyzed by dynamic scattering using a Nicomp model 370 submicron particle analyzer (Nicomp Particle Sizing Systems, Santa Barbara, CA). Particle diameters were calculated from the volume distribution patterns provided by the analyzer.

**Hepatic insulin signaling pathways**

Mice received either 30 mg/kg TDGA or its solvent as described. After 9 h of fasting, the mice were anesthetized and equipped with an infusion line in the vena cava inferior. During 30 min, mice were infused with saline or a 1% BSA solution containing 13.3 µg/ml somatostatin and 100 mg/ml glucose. The latter solution contained insulin resulting in an insulin infusion rate of 10 mU·kg⁻¹·min⁻¹. The solutions were infused at a flow rate of 0.405 ml/h. After 30 min of infusion, the liver was quickly removed and frozen in liquid nitrogen.

Hepatic lysates were made in buffer [in mM: 30 Tris-Cl, pH 7.4, 2.5 EDTA, pH 8.0, 150 NaCl, 0.5 Na₃VO₄, 5 NaF, 5 MgCl₂, and 1.3 M glycerol and protease inhibitors (Complete; Roche Diagnostics)] and cleared by centrifugation. Protein content was determined using BCA-kit (Pierce), and equal amounts of protein were used to determine PKB-Ser473 and PKB-Thr308 phosphorylation by Western blotting, using antibodies against PKB-Ser473P and PKB-Thr308P raised in rabbit (Cell Signaling, Beverly, MA). For PI3-kinase activity, the lysates were immunoprecipitated overnight with antibodies against IRS1 (29) or IRS2 (37) raised in rabbits. Following extensive washing, PI3-kinase activity was determined as described previously (29). The incorporated radioactivity was quantified using a phosphorimager.

**Statistics**

All values represent means ± SD for the number of animals indicated. Statistical analysis was assessed by the Mann-Whitney U-test. The level of significance was set at P < 0.05. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL).
**Results**

*Severe hepatic steatosis on treatment with CPT1 inhibitor TDGA*

To determine the acute effects of β-oxidation inhibition during fasting, *i.e.*, when the body strongly depends on fatty acid oxidation for its energy, we treated male C57BL/6J mice with the CPT1 inhibitor TDGA and fasted them for 12 h. From 3 h fasting onward, the blood glucose levels in TDGA-treated mice were statistically significantly lower than in the control mice (figure 6.1). Plasma NEFAs, TG, and free cholesterol levels were increased on TDGA treatment (table 6.1). Relative liver weight was increased by 18% on treatment, probably related to a marked increase in TG content (table 6.1). In contrast, liver glucose-6-phosphate levels were lower in the treated mice. Oil-Red-O staining for neutral lipids on frozen liver sections showed massive microvesicular lipid accumulation in periportal (zone 1) as well as in perivenous (zone 3) hepatocytes in mice receiving TDGA (figure 6.2). TDGA treatment lead to significant changes in the hepatic fatty acid composition (figure 6.3). Relative amounts of saturated fatty acids (SAFAs) and polyunsaturated fatty acids (PUFAs) decreased in favor of monounsaturated fatty acids (MUFAs).

*Insulin sensitivity of VLDL production is not affected by treatment with CPT1 inhibitor TDGA*

Figure 6.4 shows the plasma TG concentration *vs.* time curve after injection of Triton WR-1339 in control and TDGA-treated mice, with and without infusion of insulin. From these curves, the VLDL-TG production rates were calculated. TDGA treatment did not affect VLDL-TG production rate and insulin suppressed the production rate in control and treated mice by 43 and 54%, respectively. The concentrations of cholesterol, phospholipids, and TG in nascent VLDL particles isolated from plasma obtained at 90 min after Triton WR-1339 injection are summarized in table 6.2. Under basal conditions, the VLDL TG-to-phospholipid ratio tended to increase on TDGA treatment, indicative of the presence of larger particles (12). On insulin infusion, this ratio was decreased in both control and treated mice, suggesting that insulin-mediated suppression of VLDL-TG secretion was, at least partly, due to the secretion of smaller particles. Accordingly, direct measurement of particle sizes revealed that the mean diameter of the VLDL particles under basal conditions was increased on TDGA treatment (67 ± 5 vs. 59 ± 4 nm, *P* < 0.05). The diameters were smaller in both control and treated mice on insulin infusion (51 ± 3 and 53 ± 3 nm, control and treated, respectively). In accordance with these data, apoB100 and apoB48 contents in isolated VLDL fractions were increased on insulin infusion, but no significant difference was seen between control and TDGA treatment, as determined by Western blot analysis (figure 6.5). The fatty acid composition of the VLDL fraction was changed on treatment, resulting in changes similar to those seen for the hepatic fatty acids, *i.e.*, increased MUFAs and decreased SAFAs and PUFAs (figure 6.6). The hepatic expression of genes encoding enzymes involved in VLDL secretion was investigated with real-time RT-PCR (figure 6.7). Expression of Apob and Mttp, the latter encoding microsomal TG transfer protein, was slightly increased on TDGA treatment. Expression of lipoprotein lipase (*Lpl*) was slightly reduced in livers of TDGA-treated mice.
Table 6.1. Plasma and hepatic parameters after 12 h of fasting in mice treated with or without CPT1 inhibitor TDGA. Values are means ± SD; n = 6 mice. CPT1, carnitine palmytoyl transferase-1; TDGA, tetradecylglycidic acid. *P < 0.05, TDGA vs. control.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>TDGA</th>
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<tr>
<td>Plasma triglycerides (mM)</td>
<td>0.39 ± 0.16</td>
<td>0.60 ± 0.10 *</td>
</tr>
<tr>
<td>Plasma free cholesterol (mM)</td>
<td>0.61 ± 0.35</td>
<td>0.86 ± 0.36</td>
</tr>
<tr>
<td>Plasma cholesterylester (mM)</td>
<td>1.06 ± 0.54</td>
<td>1.07 ± 0.53</td>
</tr>
<tr>
<td>Plasma NEFA (mM)</td>
<td>0.68 ± 0.26</td>
<td>1.21 ± 0.41 *</td>
</tr>
<tr>
<td>Liver weight (% of bodyweight)</td>
<td>4.26 ± 0.66</td>
<td>5.03 ± 0.39 *</td>
</tr>
<tr>
<td>Liver proteins (mg/g liver)</td>
<td>228 ± 44</td>
<td>211 ± 39</td>
</tr>
<tr>
<td>Liver triglycerides (nmol/mg liver)</td>
<td>19.9 ± 8.3</td>
<td>112.4 ± 25.2 *</td>
</tr>
<tr>
<td>Liver cholesterol (nmol/mg liver)</td>
<td>9.91 ± 1.32</td>
<td>11.13 ± 2.92 *</td>
</tr>
<tr>
<td>Liver phospholipids (nmol/mg liver)</td>
<td>52.65 ± 11.50</td>
<td>49.81 ± 4.61</td>
</tr>
<tr>
<td>Liver glucose-6-phosphate (nmol/g liver)</td>
<td>50.6 ± 15.6</td>
<td>25.7 ± 2.6 *</td>
</tr>
</tbody>
</table>

Figure 6.1. Blood glucose levels during fasting period in mice treated with or without carnitine palmytoyl transferase-1 (CPT1)-inhibitor tetradecylglycidic acid (TDGA; 30 mg/kg body wt) (n = 6 mice; *P < 0.05, TDGA vs. control).
Hepatic insulin signaling is not affected on treatment with CPT1 inhibitor TDGA

To determine whether hepatic steatosis on TDGA treatment affected hepatic insulin signaling, we determined PI3-kinase activity and PKB-phosphorylation, both supposedly major steps in hepatic insulin signaling. Hereto, TDGA-treated and control mice were subjected to 30-min infusion with either saline or insulin. Both IRS1- and IRS2-associated insulin-induced increase of PI3-kinase activity did not differ between control and treated mice (figure 6.8). Moreover, insulin-induced phosphorylation of PKB, the key mediator of hepatic insulin signaling, was not affected by TDGA; insulin-induced PKB phosphorylation on Ser473 and Thr308 did not differ between control and TDGA-treated mice (figure 6.9).
Insulin sensitivity of VLDL production in fatty livers

Figure 6.4. Plasma triglyceride (TG) concentrations and VLDL-TG production rates of mice treated with or without CPT1-inhibitor TDGA, under basal and hyperinsulinemic conditions. Inset: VLDL-TG production rate in µmol·kg$^{-1}$·h$^{-1}$ calculated from the plasma TG vs. time curves (n = 6 mice; *P < 0.05, TDGA vs. control). Open bars, control mice; closed bars, TDGA-treated mice; open circles, control mice, basal conditions; closed circles, TDGA-treated mice, basal conditions; open squares, control mice, hyperinsulinemic conditions; closed squares, TDGA-treated mice, hyperinsulinemic conditions.

Figure 6.5. Representative apolipoprotein B (apoB) Western blots of VLDL fractions of mice treated with or without (Con) CPT1 inhibitor TDGA, under basal and hyperinsulinemic conditions.
In su lin sensitivity of hepatic glucose and lipid metabolism in animal models of hepatic steatosis

Figure 6.6. Relative fatty acid profile of VLDL fractions from mice treated with or without (control) CPT1 inhibitor TDGA (n = 6 mice; * P < 0.05, TDGA vs. control).

Table 6.2. Composition and size of nascent VLDL particles of mice treated with or without CPT1 inhibitor TDGA under basal and hyperinsulinemic conditions. Values are means ± SD; n = 6 mice; * P < 0.05 TDGA vs. control; † P < 0.05 hyperinsulinemic vs. basal.

<table>
<thead>
<tr>
<th></th>
<th>Basal Control</th>
<th>Basal TDGA</th>
<th>Hyperinsulinemic Control</th>
<th>Hyperinsulinemic TDGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides (%)</td>
<td>76.8 ± 1.6</td>
<td>79.1 ± 1.6 *</td>
<td>67.9 ± 1.6 †</td>
<td>66.8 ± 3.8 †</td>
</tr>
<tr>
<td>Phospholipids (%)</td>
<td>12.2 ± 0.7</td>
<td>11.7 ± 0.8</td>
<td>15.8 ± 0.9 †</td>
<td>16.2 ± 1.6 †</td>
</tr>
<tr>
<td>Cholesterol (%)</td>
<td>10.9 ± 0.9</td>
<td>9.2 ± 1.1 *</td>
<td>16.4 ± 1.5 †</td>
<td>17.0 ± 2.3 †</td>
</tr>
<tr>
<td>TG:PL ratio</td>
<td>6.3 ± 0.5</td>
<td>6.8 ± 0.6</td>
<td>4.3 ± 0.3 †</td>
<td>4.2 ± 0.7 †</td>
</tr>
<tr>
<td>Particle size (nm)</td>
<td>59.3 ± 3.6</td>
<td>66.6 ± 4.5 *</td>
<td>51.2 ± 2.5 †</td>
<td>53.2 ± 2.8 †</td>
</tr>
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Figure 6.7. Changes in hepatic gene expression patterns on TDGA treatment. Results were normalized to \( \beta \)-actin and \( 18S \) mRNA levels, data from control mice defined as 1. Apob, apolipoprotein B; Apobec1, apolipoprotein B editing complex-1; Apoe, apolipoprotein E; Mttp, microsomal triglyceride transfer protein, Lpl, lipoprotein lipase. N = 6 mice; * P < 0.05.
Figure 6.8. Hepatic phosphatidylinositol 3-kinase (PI3-kinase) activity in mice treated with or without CPT1-inhibitor TDGA on infusion of saline or insulin. Liver lysates were precipitated with antibodies against Insulin receptor substrate (IRS)1 (left) or IRS2 (right). The PI3-kinase activity in acquired pellets was determined by incorporation of 32P-ATP into PIP3. The amount of incorporated radioactivity was quantified using a phosphorimager. Hepatic PI3-kinase activity in control mice on saline infusion is set as value 100; n = 6 mice; # P < 0.05, insulin infusion vs. saline infusion.

Figure 6.9. Hepatic PKB-phosphorylation in mice treated with or without CPT1-inhibitor TDGA on infusion of saline or insulin. Liver lysates were applied to SDS-PAGE followed by Western blot analysis. Antibody staining for PKB phosphorylation at Ser473 (top) and Thr308 (bottom). Each lane represents an individual mouse that received infusion of saline or insulin.

Discussion

This study demonstrates that pharmacological inhibition of CPT1 by TDGA leads to severe hepatic steatosis without affecting insulin sensitivity of hepatic VLDL production. Moreover, the PI3-kinase insulin signaling cascade was not affected in the TDGA-induced fatty livers. Therefore, this study provides evidence that hepatic fat accumulation per se does not lead to insulin resistance in mice and underscores the fact that not all forms of hepatic steatosis are associated with insulin resistance.

In humans, various inherited β-oxidation disorders are associated with increased hepatic TG levels, hypoglycemia, and low plasma ketone body levels on fasting (33). The TDGA-treated mice showed marked hypoglycemia during fasting. From 3 h of fasting on, blood glucose levels were lower in TDGA-treated than controls and showed a steady state. Therefore, we performed all subsequent experiments within 9 to 12 h of fasting.
Two types of hepatic steatosis are usually discerned, i.e., macrovesicular and microvesicular steatosis. The latter type is particularly found in subjects with β-oxidation disorders (8,13); TDGA treatment was also associated with microvesicular hepatic steatosis in mice (figure 6.2). Despite the overabundance of fat in the liver and slightly increased hepatic $Apob$ and $Mttp$ gene expression (figure 6.7), basal VLDL-TG production rate was not affected on TDGA treatment (figure 6.4). Because this unaffected VLDL-TG production rate was accompanied by a 54% increase of plasma TG levels (table 6.1), clearance of TG-rich lipoproteins was, by definition, affected in TDGA-treated mice. Slightly reduced hepatic $Lpl$ expression (figure 6.7) might contribute to reduced VLDL lipolysis. Moreover, the increased NEFA levels on TDGA treatment (table 6.1) might also reduce the capacity of LPL to lipolyse VLDL-TG (30). The unaffected VLDL-TG production rate on TDGA treatment may, at first sight, seems surprising because VLDL production is one means for the liver to get rid of large amounts of TG that accumulate during fasting. A stimulatory effect on VLDL-TG production was found in mice with hepatic steatosis due to increased de novo lipogenesis on liver X receptor agonist treatment (16). On the other hand, $ob/ob$ mice show severe hepatic steatosis and increased de novo lipogenesis, but do not have increased VLDL-TG production under basal conditions (44). Furthermore, hepatic steatosis associated with inhibition of glucose-6-phosphatase activity was also without effect on VLDL production (4). These observations suggest that increased hepatic TG content per se does not stimulate hepatic VLDL production. Various factors, apart from the accumulation of TG, might contribute to changes in VLDL production. TGs are not the sole components of VLDL; hepatic cholesterol and phospholipid availability or synthesis might also influence the secretion and assembly of VLDL. In our TDGA-treated mice, hepatic cholesterol levels were slightly higher but phospholipid content was not affected. It has been suggested that de novo synthesis of cholesterol (19) and phospholipid (42), rather than their concentrations, are determinants of VLDL production. Although we have no direct measurements of these parameters, in a microarray experiment employing TDGA, we found significantly reduced hepatic gene expression of $Pemt$, encoding phosphatidylethanolamine N-methyltransferase, a key player in phospholipid synthesis (F. R. van der Leij, personal communication). Interestingly, PEMT is thought to play an important role in providing phospholipid needed for the surface of VLDL (28).

Another explanation for the unaffected VLDL-TG production might be duration of TG accumulation. It is reported that fatty acids taken up by the hepatocyte are not directly used for VLDL production (14) but are esterified and stored in a cytoplasmic pool. Utilization of TGs from this pool for VLDL assembly requires hydrolysis followed by reesterification. Thus an TG accumulation over a short period of time, as shown in this study, might not immediately lead to increased VLDL-TG production.

Hepatic steatosis is commonly associated with decreased insulin sensitivity (1,24,35). Hepatic insulin sensitivity was tested in a number of ways. First, we checked whether insulin would be able to effectively suppress the secretion of VLDL by the liver. Unexpectedly, insulin suppressed VLDL-TG production rate equally well in control and TDGA-treated mice (figure 6.4). This is in contrast to the situation in $ob/ob$ mice, a commonly used model of insulin resistance. Wiegman et al. (44) showed that insulin failed to effectively suppress VLDL-TG production in $ob/ob$ mice. Exactly how insulin suppresses VLDL production is not entirely clear. It has been suggested that insulin stimulates intracellular degradation of the major VLDL apolipoprotein apoB (7) through a mechanism involving PI3-kinase but not PKB (2,7,10). Therefore, we determined PI3-kinase activity and PKB phosphorylation in the steatotic livers of TDGA-treated mice. Indeed, Western blot analyses revealed that TDGA treatment did not affect insulin-induced hepatic PKB-phosphorylation at Ser473 nor at Thr308 (figure 6.9). Moreover, IRS1- nor IRS2-associated insulin-stimulated PI3-kinase
activities in the liver were affected on TDGA treatment (figure 6.8). Both results strongly suggest that hepatic insulin signaling is not affected in the rapidly developed fatty liver of TDGA-treated mice. From these data, and from figure 6.4, it cannot be concluded whether insulin-mediated suppression of VLDL production is independent from PKB, as suggested recently (2). In the study from Au et al. (2), however, it was concluded that PKB was not involved in insulin-mediated inhibition of apoB degradation. Other subclasses of PKB and/or PKC might be responsible for the effects of insulin on VLDL production. A recent report showed that PKC-Θ knockout mice are protected from fat-induced insulin resistance (21), suggesting that PKC-Θ is an important component in (skeletal muscle) insulin signaling.

An aspect that might be involved in the lack of association between hepatic steatosis and insulin resistance relates to the difference in the hepatic fatty acid profile between control and treated mice (figure 6.3). An increase in dietary MUFAs resulted in improved insulin sensitivity in healthy men and women (43), but had no effect on insulin secretion. Therefore, hepatic insulin sensitivity might be better preserved in fatty livers that contain relatively more MUFAs, as observed in TDGA-treated mice, than fatty livers with predominantly SAFA-containing TG.

In conclusion, CPT1 inhibition with TDGA led to the expected microvesicular hepatic steatosis in C57BL/6J mice. However, this was not associated with reduced hepatic insulin sensitivity because the hepatic insulin signaling cascade was not affected and the inhibitory effects of insulin on VLDL production in vivo were not blunted. Therefore, in combination with other data (4,16a,44), these observations support the notion that hepatocellular fat content per se is not causally related to induction of insulin resistance in mice.

References

In insulin sensitivity of VLDL production in fatty livers
