Chapter 4

Perturbed lipid metabolism in a mouse model of glycogen storage disease type Ia: differential contributions of de novo lipogenesis to liver steatosis in fed and fasted states

ABSTRACT

The present study aimed to examine the mechanisms leading to hepatic steatosis and hypertriglyceridemia in glycogen storage disease Ia (GSD Ia) using mice with a liver-specific glucose-6-phosphatase (G6PC) deficiency. In both fasted and fed conditions we examined 1) hepatic fatty acid synthesis using mass isotopomer distribution analysis (MIDA); 2) adipose tissue lipolysis; 3) hepatic and adipose tissue genes encoding for circulatory factors that are known to inhibit lipoprotein lipase (LPL).

Our results revealed differences in hepatic lipid metabolism between fasted and fed L-G6pc−/− mice. Hepatic triglycerides (TG) were increased both in fed and fasted L-G6pc−/− mice. In fed L-G6pc−/− mice accumulated hepatic TG were found with a concomitant increase in de novo fatty acid synthesis, while in fasted L-G6pc−/− mice increased chain elongation of existing fatty acids together with enhanced adipose tissue lipolysis were suggestive of increased hepatic fatty acid influx. The difference in origin of hepatic steatosis likely results from differences in glycaemia and its effects on the insulin to glucagon ratio.

In both fed and fasted L-G6pc−/− mice hypertriglyceridemia was found. Under both nutritional conditions L-G6pc−/− mice display altered hepatic expression of genes involved in VLDL-TG secretion and modulation of LPL activity, suggestive of enhanced VLDL-TG production and reduced VLDL-TG clearance.

In conclusion, the origin of hepatic steatosis in L-G6pc−/− mice is dependent on nutritional status. Since GSD I patients are usually kept in a semi-fed state to prevent hypoglycemic events, animal models of GSD I should for better comparison also be examined in a fed state.
INTRODUCTION

Glycogen storage disease I (GSD I) is an autosomal recessive inborn error caused by a deficiency of either the catalytic subunit (G6PC, GSD type Ia) or the translocase (G6PT, GSD type Ib) of the glucose-6-phosphatase (G6Pase) complex [80]. G6Pase catalyzes the last step in gluconeogenesis and glycogenolysis, converting newly formed G6P or G6P formed from glycogen stores to glucose. Upon fasting GSD I patients cannot maintain glycaemia since glucose producing organs (liver, small intestine, kidney) are unable to hydrolyze G6P to glucose and G6P is retained inside the cells. The clinical picture of fasting hypoglycemia is accompanied by among others high plasma lactate, triglyceride (TG) and uric acid concentrations. In the liver characteristics of the enzymatic defect are increased amounts of G6P, glycogen and TG (e.g. [81]).

To maintain blood glucose levels within a normal range patients have to adhere to a strict diet. The usual strategy comprises frequent meals in combination with uncooked cornstarch supplements, resulting in a slow release of glucose from the intestines into the circulation. Although hypoglycemic episodes are greatly reduced, and many secondary metabolic derangements are corrected by dietary treatment, comorbidities still occur frequently [82]. Of considerable concern are severe hepatic steatosis (e.g. [145]) and hyperlipidemia (e.g. [145, 146]).

Hepatic TG accumulation is the result of an imbalance between processes that add to the hepatic TG pool (hepatic fatty acid influx and de novo synthesis of fatty acids) and pathways that reduce this pool (TG catabolism and secretion in very low density lipoprotein, VLDL). In GSD Ia hepatic steatosis is generally thought to mainly result from increased hepatic de novo lipogenesis. In patients increased rates of hepatic de novo lipogenesis and choleseterogenesis were found [146, 147]. In rats, acute inhibition of G6PT with the chlorogenic derivative S4048 also led to increased hepatic de novo lipogenesis [85].

The mechanisms that lead to hypertriglyceridemia in GSD Ia have not been examined systematically. Plasma TG concentrations are determined by a balance between the synthesis and breakdown of VLDL and chylomicron particles. The main cause of hypertriglyceridemia in GSD I is thought to be decreased catabolism of VLDL. Several case reports show largely diminished lipoprotein lipase (LPL) and hepatic lipase (HL) activities [148, 149, 150, 151]. Other studies have shown delayed clearance and/or hypocatabolism of VLDL particles [146, 151] or TG [148]. An early study shows it is likely that a circulating factor inhibits LPL and HL in GSD I [150].
In human GSD I patients it is difficult to systematically examine the underlying causes of metabolic derangements. Also, there is a restricted possibility to examine tissue-specific mechanisms and interactions between organs in detail. Recently, several animal models became available that can be used to unravel molecular mechanisms underlying secondary metabolic derangements in GSD I [85, 87, 152].

Research in different conditional knockouts of $G6pc$ has shown that hepatic steatosis and hypertriglyceridemia in GSD I are driven by hepatocyte loss of $G6pc$ [153]. Therefore, we examined the effects of the absence of hepatic $G6pc$ on lipid metabolism in a liver-specific mouse model of GSD Ia (L-$G6pc^{-/-}$ mice, [86] under fed and fasted conditions. We show that in fasted L-$G6pc^{-/-}$ mice hepatic TG content is mainly determined by increased chain elongation of adipocyte-derived fatty acids, while in a fed state de novo lipogenesis has a predominant contribution to hepatic fatty acid synthesis. We also examined the possible causal role of circulating factors that are known to impact on LPL activity in hypertriglyceridemia in GSD I.

**EXPERIMENTAL PROCEDURES**

**Animals**

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Groningen. $G6pc^{lox/lox}$ and SACRED2-$G6pc^{lox/lox}$ mice [86] were housed in a light (lights on: 7:00 AM-7:00 PM) and temperature-controlled facility and fed a standard laboratory chow diet (RMH-B, Abdiets, Woerden, The Netherlands). Animals received i.p. injections of tamoxifen for five consecutive days to induce liver-specific deletion of the $G6pc$ gene. Ten days after the end of injections animals were sacrificed by heart puncture (8:00 AM) in either a fed or a 2-hour fasted (11:00 PM-8:00 AM) state. Tissues were quickly dissected, and stored at -80°C. Livers were freeze clamped immediately after dissection, unless otherwise specified. Blood was centrifuged (4000 rpm for 10 minutes at 4°C), and plasma was stored at -20°C.

**Liver and plasma biochemistry**

Commercially available ultrasensitive ELISA kits were used to determine plasma insulin (Chrystal Chem, Downers Grove, IL, USA) and glucagon (Alpco, Salem, NH, USA). Hepatic lipids were extracted according to Bligh and Dyer [99].
Commercially available kits were used to measure hepatic and plasma levels of TG (Roche, Mannheim, Germany) and plasma concentrations of total and free cholesterol and FFA (Diasys, Holzheim, Germany).

**Hepatic and white adipose tissue gene expression analysis**
RNA was extracted from livers and WAT using Tri Reagent (Sigma-Aldrich, St. Louis, MO, USA). cDNA obtained by reverse transcription was amplified using the appropriate primers and probes (Appendix A). All mRNA levels were calculated relative to the expression of 36b4 for liver and relative to 18S for WAT and normalized to expression levels in wildtype mice.

**Determination of de novo lipogenesis and chain elongation in vivo.**
In order to measure lipogenesis in the liver, mice received a sodium [1-13C]-acetate solution (241 mM; 99 atom %, Isotec/Sigma- Aldrich, St. Louis, MO, USA) as drinking water during the final 24 hours before sacrifice. Liver homogenates were prepared and fatty acids were measured using a Agilent 5975 series GC/MSD (Agilent Technologies, Santa Clara, CA). Normalized mass isotopomer distributions [104] were used in MIDA algorithms to calculate the acetyl-CoA precursor pool enrichment, fractional synthesis rate, and chain elongation rates, as described before [88]. Hepatic fatty acid composition was analyzed by gas chromatography after transmethylation [100].

**Mitochondrial oxygen consumption**
Mitochondria were isolated from liver by a differential centrifugation procedure, which was adapted from described previously [154]. The liver was quickly removed and placed into ice-cold isotonic (0.9 %) KCl solution. The tissue was cut into small pieces and homogenized in a glass Teflon homogenizer with mito buffer (10 mM Tris-HCl, 250 mM sucrose, pH 7.4 at 4°C). The homogenate was centrifuged at 800 xg for 10 min and the supernatant was centrifuged at 8600 xg for 12 min. The obtained pellet was washed in mito buffer and the suspension was centrifuged at 8600 xg for 12 min. The mitochondrial pellet was resuspended in 250 µL of mito buffer. Protein content was determined using a BCA protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA).

The O2 fluxes in isolated liver mitochondria were measured at 37°C in a two-channel high-resolution Oroboros oxygraph-2 k (Oroboros, Innsbruck, Austria). The assay medium (MiR05) contained 110 mM sucrose, 60 mM potassium lactobionate, 20 mM taurine, 20 mM HEPES, 0.5 mM EGTA, 10 mM KH2PO4, 3 mM
MgCl₂, and 1 mg/ml bovine serum albumin, at pH 7.1. The oxidizable substrates were: (i) 5 mM pyruvate plus 2 mM malate, or (ii) 25 µM palmitoyl-CoA plus 2 mM L-carnitine plus 2 mM malate. The maximal ADP-stimulated O₂ flux (state 3) was achieved by adding 1.5 U/mL hexokinase, 12.5 mM glucose and 1 mM ATP. The basal O₂ flux (state 4) was determined after blocking ADP phosphorylation with 1.25 µM carboxyatractyloside. Data acquisition and analysis were performed with DatLab software version 4.2 (Oroboros, Innsbruck, Austria).

**Ex vivo adipose tissue lipolysis**

Epididymal white adipose tissue (WAT) was removed and pieces of around 20 mg cut and kept on ice in Krebs buffer (12 mM HEPES, 4.9 mM KCl, 121 mM NaCl, 1.2 mM MgSO₄, 0.33 CaCl₂ and 0.1 % glucose, pH 7.4) until further processing. WAT pieces were transferred to Eppendorf tubes with 10x w/v KREBS with 3.5 % FFA free BSA prewarmed to 37°C. Samples were subsequently incubated at 37°C. Every hour for four hours one sample per animal was spun down at maximum speed and buffer was stored at -20°C until further analysis. Glycerol concentrations were measured according to manufacturer's protocol using a commercially available kit (Cayman Chemical, Ann Arbor, MI, USA).

**Immunoblotting**

Epididymal WAT was homogenized with a potter homogenizer in RIPA buffer supplemented with protease and phosphatase inhibitors (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 30 mM Na₃P₂O₇, 50 mM NaF, 1 mM PMSF, 2 mM Na₃VO₄, 12 µg/mL aprotinine and 1 % Triton X-100) and with complete protease inhibitor (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA). Tissue lysates were sonicated and cleared by centrifugation (15000 xg for 30 min at 4°C. Lysates were resolved by SDS-PAGE, and proteins were transferred to nitrocellulose membranes using semi-dry transfer (Trans-Blot Turbo Transfer System, Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked for 1 hour with 5 % BSA in TBS-T (20 mM Tris base, 137 mM NaCl, 0.1 % Tween-20, pH 7.5) and incubated overnight at 4°C in TBS-T with 5 % BSA containing primary antibody (1:1000) and for 1 hour with secondary antibody (1:1000). TBS-T was used as a washing buffer after antibody incubations. Proteins were detected by chemiluminescence using Pico substrate (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA). The following antibodies were used (Cell Signaling Technology, Danvers, MA, USA): HSL (#4107), phosphoHSL Ser660
Nutritional-state dependent changes in lipid metabolism in GSD Ia

(#4126), phopshoHSL Ser565 (#4137), phosphoHSL Ser563 (#4139), HRP-linked anti-rabbit IgG (#7074).

Plasma lipoprotein analysis
Plasma lipoproteins were separated by fast protein liquid chromatography gel filtration using a Superose 6 column (GE Healthcare, Uppsala, Sweden) as described [101]). TG content of the collected fractions was determined using a commercially available kit (Roche Diagnostics, Mannheim, Germany).

Statistics
Values in graphs are depicted as boxplots (middle line: median, box: 25th to 75th percentiles, whiskers 5th to 95th percentiles). Non-parametric statistical analysis (Mann Whitney U) was performed at an 0.05 level of probability. Data in tables are presented as mean and s.e.m. and parametric statistical analysis was performed (unpaired Student’s t-test). The null hypothesis was rejected at the 0.05 level of probability. Data points were considered outliers when their value differed from the group average by more than two times the standard deviation.

RESULTS
The origin of hepatic steatosis in L-6pc−/− mice depends on nutritional status
As shown previously [86, 155], the metabolic profile of L-6pc−/− mice resembled that of GSD I patients [82]. Fasted L-6pc−/− mice were hypoglycemic (Figure 4.1A) and showed a decreased plasma insulin to glucagon ratio (Figure 4.1B). Under both fed and fasted conditions hepatic TG (Figure 4.1C), liver weight and hepatic concentrations of glycogen and G6P (Table 4.1) were increased in L-6pc−/− mice.

In both fed and fasted L-6pc−/− mice hepatic expression of genes involved in de novo lipogenesis and fatty acid chain elongation were upregulated (Figure 4.2A and 4.2B). However, previous results from our lab have shown discrepancies between lipogenic gene expression and actual de novo lipogenic fluxes [130]. Therefore, we subsequently examined the metabolic origin of hepatic fatty acids using [1-13C]-acetate and MIDA, because this allows to distinguish between fatty acids that are formed either from de novo synthesis or by elongation of pre-existing fatty acids [88].
Figure 4.1: Biochemical parameters of L-G6pc−/− mice resemble those of GSD Ia patients. A) Blood glucose concentration. B) Plasma insulin to glucagon ratio. C) Hepatic TG. n=6-7 mice per group, *p<0.05

Under fed conditions isotope enrichment of the acetyl-CoA pool was lower in L-G6pc−/− mice than in wildtype littermates (Figure 4.2C), indicative of a larger dilution of [1-13C]-acetate. This indicates an enhanced turnover of acetyl-CoA in fed L-G6pc−/− mice, consistent with previous reports in GSD Ia patients [146] and a S4048 induced rat model for GSD Ib [85].

Using MIDA it is possible to distinguish between fatty acids that are formed either from de novo synthesis or by elongation of pre-existing fatty acids [88]. As expected, hepatic de novo fatty acid synthesis was higher in fed than in fasted wildtype (L-G6pc+/+) mice (Figure 4.2D). In fed L-G6pc−/− mice increased hepatic TG storage (Figure 4.1C) was accompanied by increased fractional de novo synthesis of stearate and oleate, while no changes in chain elongation were found (Figure 4.2D). Surprisingly, in fasted L-G6pc−/− mice no increase in fractional de novo synthesis of either palmitate, oleate or stearate was found (Figure 4.2E). On the contrary, under fasted conditions the contribution of hepatic oleate and stearate from elongation of pre-existing fatty acids was increased in L-G6pc−/− mice (Figure 4.2E), which was resembled by increased concentrations of stearate and oleate, but not palmitate (Table 4.2), in the livers of fasted L-G6pc−/− mice compared to their wildtype littermates. In both fed and fasted L-G6pc−/− mice, the most abundant hepatic fatty acid was oleate (Table 4.2), though oleate concentrations were three times higher in fasted than in fed L-G6pc−/− mice, reflecting the higher degree of TG accumulation in fasted versus fed L-G6pc−/− mice.

Metabolic adaptations to nutritional status in L-G6pc−/− mice

In order to examine what drives the difference in origin of hepatic fatty acids between fasted and fed L-G6pc−/− mice, we analyzed plasma metabolite con-
Figure 4.2: The origin of hepatic steatosis in L-G6pc−/− mice is dependent on nutritional status. A) and B) Relative mRNA fold induction of hepatic lipogenic genes in fed (A) and 9h fasted (B) mice. C) Acetyl-CoA pool enrichment. D) and E) Fractional de novo synthesis of palmitate (C16:0), stearate (C18:0) and oleate (C18:1) and stearate and oleate synthesized from chain elongation in fed (D) and 9h fasted (E) mice. n=6-7 mice per group, *p<0.05
Table 4.1: Liver and plasma parameters in fed and 9h fasted L-G6pc+/+ and L-G6pc−/− mice.

<table>
<thead>
<tr>
<th></th>
<th>Fed L-G6pc+/+</th>
<th>L-G6pc−/−</th>
<th>Fast L-G6pc+/+</th>
<th>L-G6pc−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.1 ± 0.1</td>
<td>1.9 ± 0.1***</td>
<td>1.5 ± 0.1</td>
<td>2.3 ± 0.1***</td>
</tr>
<tr>
<td>Glycogen (µmol/g)</td>
<td>123 ± 15</td>
<td>375 ± 34***</td>
<td>254 ± 43</td>
<td>350 ± 14</td>
</tr>
<tr>
<td>G6P (µmol/g protein)</td>
<td>4.3 ± 0.8</td>
<td>16.4 ± 2.3**</td>
<td>4.6 ± 1.0</td>
<td>16.8 ± 3.0**</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterylesters (mM)</td>
<td>1.3 ± 0.1</td>
<td>2.0 ± 0.1**</td>
<td>1.1 ± 0.1</td>
<td>2.0 ± 0.1***</td>
</tr>
<tr>
<td>Free cholesterol (mM)</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.1*</td>
<td>0.7 ± 0.0</td>
<td>1.3 ± 0.1***</td>
</tr>
</tbody>
</table>

Values are given as means ± s.e.m. for n=6-7 mice per group *p<0.05, **p<0.01, ***p<0.001 compared to wildtype littermates in the same nutritional status.

centrations. Plasma lactate concentration tended to be higher in both fed and fasted L-G6pc−/− mice (Figure 4.3A). Together with increased de novo lipogenesis (Figure 4.2D) and acetyl-CoA turnover (Figure 4.2C) this suggests that under fed conditions acetyl-CoA for lipid synthesis is mainly derived from glycolysis. Plasma FFA (Figure 4.3B) and β-hydroxybutyrate concentration (Figure 4.3C) were increased in L-G6pc−/− mice under both nutritional conditions and tended to be highest in fasted L-G6pc−/− mice. This suggests increased sequestering of hepatic acetyl-CoA derived from β-oxidation towards ketogenesis in fasted L-G6pc−/− mice.

We analyzed the function of hepatic mitochondria from fasted L-G6pc−/− mice by high-resolution respirometry. Maximal ADP stimulated mitochondrial oxygen consumption (state III) was similar for fasted L-G6pc+/+ and L-G6pc−/− mice when using pyruvate (Figure 4.3D) or palmitoyl-CoA (Figure 4.3E) as a substrate, indicating that hepatic capacity for oxidative metabolism of either glucose or fatty acids is not inherently altered in fasted L-G6pc−/− mice. These data suggest that the observed changes in hepatic lipogenesis between fed and fasted conditions are driven by an extra-mitochondrial mechanism. Since lipogenic gene expression is upregulated both in fed and fasted L-G6pc−/− mice, the differential phenotype may be driven by different enzyme expression or enzyme activity or otherwise it may be substrate driven.

Increased adipose tissue lipolysis in fasted L-G6pc−/− mice

To examine whether the differences in de novo lipogenic flux between fasted
and fed mice were substrate driven, we examined adipose tissue lipolysis. We hypothesized that in L-\(G6pc^{-/-}\) mice under fasting conditions an increased flux of adipose-derived FFA would provide the liver with fatty acids that are subsequently elongated to oleate and stearate. Both in fasted and fed L-\(G6pc^{-/-}\) mice circulating FFA concentrations were increased (Figure 4.3B), which has also been reported in GSD I patients [145, 156]. 

Ex vivo glycerol release was

Table 4.2: Hepatic concentrations of specific fatty acids (in µmol/g) in fed and 9h fasted L-\(G6pc^{+/+}\) and L-\(G6pc^{-/-}\) mice.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Fed (L-G6pc^{+/+})</th>
<th>Fed (L-G6pc^{-/-})</th>
<th>Fast (L-G6pc^{+/+})</th>
<th>Fast (L-G6pc^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>0.4 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.5</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>C16:1</td>
<td>1.5 ± 0.6</td>
<td>1.8 ± 0.6</td>
<td>9.3 ± 5.6</td>
<td>9.0 ± 3.2</td>
</tr>
<tr>
<td>C16:0</td>
<td>23.5 ± 6.1</td>
<td>23.6 ± 4.8</td>
<td>47.7 ± 13.8</td>
<td>45.1 ± 10.0</td>
</tr>
<tr>
<td>C18:3o6</td>
<td>0.2 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>1.5 ± 1.1</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>C18:2o6</td>
<td>18.1 ± 4.4</td>
<td>19.3 ± 2.7</td>
<td>41.3 ± 13.3</td>
<td>37.9 ± 6.9</td>
</tr>
<tr>
<td>C18:3o3</td>
<td>1.0 ± 0.4</td>
<td>1.4 ± 0.6</td>
<td>1.8 ± 0.6</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>C18:1o9</td>
<td>20.9 ± 11.6</td>
<td>35.6 ± 5.9*</td>
<td>53.5 ± 23.5</td>
<td>105.4 ± 17.8***</td>
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<td>C18:1o7</td>
<td>2.5 ± 0.8</td>
<td>3.2 ± 0.6</td>
<td>4.6 ± 1.7</td>
<td>8.8 ± 1.9**</td>
</tr>
<tr>
<td>C18:0</td>
<td>13.3 ± 3.3</td>
<td>15.7 ± 2.5</td>
<td>13.3 ± 1.8</td>
<td>17.9 ± 1.4***</td>
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<tr>
<td>C20:4o6</td>
<td>10.0 ± 2.2</td>
<td>9.0 ± 2.0</td>
<td>14.0 ± 1.7</td>
<td>12.4 ± 1.1</td>
</tr>
<tr>
<td>C20:5o3</td>
<td>0.7 ± 0.4</td>
<td>0.8 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
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<tr>
<td>C20:3o9</td>
<td>0.5 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>0.9 ± 0.2***</td>
</tr>
<tr>
<td>C20:3o6</td>
<td>1.1 ± 0.4</td>
<td>1.6 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>2.4 ± 0.3***</td>
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<tr>
<td>C20:2o6</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.0</td>
<td>0.4 ± 0.2</td>
<td>0.7 ± 0.1**</td>
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<td>C20:3o3</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.1 ± 0.1**</td>
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<td>C20:0</td>
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<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
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<tr>
<td>C22:5o6</td>
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<td>0.1 ± 0.0</td>
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<td>0.4 ± 0.1</td>
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<tr>
<td>C22:6o3</td>
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<td>C22:5o3</td>
<td>0.7 ± 0.3</td>
<td>0.6 ± 0.1</td>
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<td>0.6 ± 0.1</td>
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<td>C22:0</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1*</td>
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<tr>
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<tr>
<td>C24:0</td>
<td>0.3 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0***</td>
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Values are given as means ± s.e.m. for n=6-7 mice per group. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) compared to wildtype littermates in the same nutritional status.
Figure 4.3: Indications for substrate driven changes in lipid metabolism between fasted and fed L-\textit{G6pc}^{-/-} mice. Plasma A) lactate, B) FFA, C) \(\beta\)-hydroxybutyrate concentrations. D) and E) Maximal mitochondrial oxygen consumption in hepatic mitochondria from fasted mice using pyruvate (D) and palmitoyl-CoA (E) as substrates. n=6 mice per group, *\(p<0.05\)

considerably higher in adipose tissue from fasted than from fed mice, regardless of genotype (Figure 4.4A and 4.4B). Interestingly, adipose tissue from fasted \(\textit{L-G6pc}^{-/-}\) mice released more glycerol compared to fasted wildtype littermates (Figure 4.4A and 4.4B). No changes were found in adipose mRNA expression of lipolytic genes in either fed or fasted \(\textit{L-G6pc}^{-/-}\) mice (Appendix C). In adipose tissue of fasted, but not of fed, \(\textit{L-G6pc}^{-/-}\) mice increased phosphorylation of hormone sensitive lipase (HSL) on serine 563 was found together with a tendency for increased phosphorylation at serine 660 and no alterations in phosphorylation at serine 565 (Figure 4.4C and 4.4D and Appendix C). Adipose triglyceride lipase (ATGL) protein expression was not changed (data not shown). Interestingly, under fasting conditions a significant correlation (\(r^2=0.60, \ p = 0.002\)) was found between \textit{ex vivo} glycerol release from adipose tissue and protein expression of pHSL Ser563 (Figure 4.4E).

Several hormones are known to influence adipose tissue lipolysis. In the fed state insulin inhibits lipolysis, while under fasting conditions this inhibition is relieved and lipolysis is enhanced by increased concentrations of glucocorticoids, catecholamines [44, 157] and glucagon [158]. In fasted \(\textit{L-G6pc}^{-/-}\) mice
Figure 4.4: Increased adipose tissue lipolysis in fasted L-\(G6pc^{-/-}\) mice. A) \textit{Ex vivo} glycerol release from epididymal fat pads as a function of time and B) represented as concentration glycerol released per hour. C) and D) Quantification of western blot for adipose tissue of fasted (C) and fed (D) mice showing phosphorylated HSL at different sites relative to HSL and normalized against wildtype (L-\(G6pc^{+/+}\)), numbers represent average of 2-5 blots per protein of \(n=7\). E) Correlation between pHSL Ser563 and adipose \textit{ex vivo} glycerol release under fasted conditions. F) Relative hepatic mRNA fold induction of hepatokines involved in adipose tissue lipolysis. G) Plasma FGF21 expression measured with ELISA. \(n=5-7\) mice per group, \(*p<0.05\)
we found a decreased plasma insulin to glucagon ratio (Figure 4.1B), but no changes in plasma corticosterone and norepinephrine concentrations and even a slight decrease in epinephrine concentrations (data not shown).

More circulating factors are involved in adipocyte lipolysis, of which fibroblast growth factor 21 (FGF21) [65, 159] and angiopoietin-like proteins (ANGPTL) 3 and 4 [67, 160, 161] are synthesized and secreted by the liver in response to FFA-induced peroxisome proliferator activated receptor (PPAR)α [162, 163] and/or δ [164, 165, 166] activity. While Fgf21 expression was increased in the livers of fasted but not in fed L-G6pc−/− mice (Figure 4.4F and [167], circulating FGF21 concentrations were four-fold elevated in both the fed and fasted state, but reached especially high levels in fasted L-G6pc−/− mice (Figure 4.4G). FGF21 has been implicated to control hepatic ketogenesis during fasting [163]. In accordance with increased plasma FGF21 concentrations, β-hydroxybutyrate concentration was elevated both in fed as well as in fasted L-G6pc−/− mice (Figure 4.3C). Hepatic Angptl4 (Figure 4.4F), but not its mRNA expression in adipose tissue (data not shown) was induced in fasted and fed L-G6pc−/− mice.

Circulating factors that may inhibit LPL activity in L-G6pc−/− mice

Previous case reports and studies taken together suggest that decreased VLDL catabolism may contribute to hyperlipidemia in GSD Ia [146, 148, 151] and that a circulating factor inhibits activity of LPL and HL [148, 149, 150, 151]. Both fasted and fed L-G6pc−/− mice have hypertriglyceridemia (Figure 5.5A-5.5C) and hypercholesterolemia (Table 4.1). Hepatic Mttp expression is increased in both nutritional states, but its fold induction is higher in fasted L-G6pc−/− mice (Figure 5.5D).

The determining role of LPL in plasma TG clearance is well established. LPL catalyzes the hydrolysis of plasma TG in VLDL and chylomicrons into free fatty acids (FFA), which are subsequently further utilized or stored locally. Most physiological variation in LPL activity is regulated at the posttranscriptional level. Two distinct classes of extracellular proteins that are known to modulate LPL activity can be distinguished: apolipoproteins (namely apoC1, apoC2, apoC3, apoA5 and apoE) and angiopoietin-like proteins (ANGPTL3, 4 and 8) (reviewed in: [168]). In GSD Ia patients, elevated plasma concentrations of several of the mentioned apolipoproteins have been found [169, 170]). In the livers of fasted L-G6pc−/− mice ApoC2 (Figure 4.5E) and Angptl4 (Figure 4.4F) expression were upregulated, while Angptl3 expression was similar to wildtype littermates (Figure 4.4F). In fed L-G6pc−/− mice hepatic ApoC2,
ApoC3 (Figure 4.5E) and Angptl4 (Figure 4.4F) were induced compared to L-G6pc\(^{+/+}\) mice. In adipose tissue no differences in Lpl or Angptl4 expression were found (data not shown).

**DISCUSSION**

The present study aimed to elucidate the mechanisms leading to hepatic steatosis and hypertriglyceridemia using a liver-specific mouse model for GSD Ia. We found that the origin of hepatic TG is determined by nutritional status in L-G6pc\(^{-/-}\) mice. Fasted L-G6pc\(^{-/-}\) mice displayed elevated plasma FFA levels and increased glycerol release from WAT accompanied by enhanced hepatic fatty acid chain elongation. This indicates an increase in hepatic fatty acid uptake from the circulation. A lower insulin to glucagon ratio probably induced WAT lipolysis in fasted L-G6pc\(^{-/-}\) mice. Under fed conditions WAT lipolysis is not affected and *de novo* synthesis of hepatic fatty acids likely represents the prevailing cause of hepatic steatosis in L-G6pc\(^{-/-}\) mice. Both fed and fasted L-G6pc\(^{-/-}\) mice exhibited hypertriglyceridemia. LPL inhibition by an increase in plasma apoC3 content seems a likely mechanism leading to hypertriglyceridemia in L-G6pc\(^{-/-}\) mice.

Although the precise mechanisms leading to hepatic steatosis are not completely understood, it is caused by an imbalance between metabolic pathways delivering input to and output from the hepatic TG pool [11, 130, 171]. It is generally assumed that hepatic steatosis in GSD I results from an increase in hepatic fatty acid synthesis driven by a high glycolytic flux [85, 146, 147, 172]. Analysis of VLDL synthesis, glycerol turnover as a measure for adipose tissue lipolysis and *de novo* fatty acid synthesis in patients, indeed indicated that the leading cause of steatosis in GSD Ia is an increase in hepatic *de novo* lipogenesis [146]. An earlier study however reported enhanced hepatic FFA uptake and concomitant increases in adipose tissue lipolysis in GSD I patients [173]. It should be noted that these two studies were performed under different experimental conditions. The investigations by Bandsma *et al*. involved patients receiving continuous glucose infusion to prevent hypoglycemia. This resulted in a semi-fasted state which may explain the unaltered glycerol turnover rates found in patients as compared to healthy controls [146]. On the other hand, Havel *et al*. studied fasted GSD I patients [173]. Importantly, our current data in fed and fasted L-G6pc\(^{-/-}\) mice strongly indicate that a difference in nutritional status determines the contribution of different fluxes to hepatic TG in
Figure 4.5: Hypertriglyceridemia in L-\textit{G}6\textit{pc}^{-/-} mice. A) Plasma TG concentration. B) and C) Lipoprotein TG profiles in pooled (n=6) plasma samples from fed (B) and fasted (C) mice. D) Relative hepatic mRNA fold induction of genes involved in VLDL-TG secretion. E) Relative hepatic mRNA fold induction of genes involved in LPL inhibition. n=5-7 mice per group, *\(p<0.05\)
Nutritional-state dependent changes in lipid metabolism in GSD Ia

GSD I. Large differences in glycaemia and altered insulin to glucagon ratios seem to dictate these metabolic differences between nutritional states.

We found that \textit{ex vivo} lipolysis was enhanced in adipose tissue from fasted L-G6pc\textsuperscript{−/−} mice. Several circulating factors regulate TG hydrolysis in adipose tissue. Adipose tissue lipolysis is inhibited upon feeding in response to insulin in healthy subjects. Upon fasting, a reduction in circulating insulin concentrations and a concomitant increase in catecholamine and glucagon levels induce cyclic AMP (cAMP) and subsequent protein kinase A (PKA) dependent phosphorylation of hormone sensitive lipase (HSL), resulting in its activation (reviewed in: [44]). We show increased phosphorylation of HSL at serine 563 and a tendency for increase at serine 660 in fasted L-G6pc\textsuperscript{−/−} mice, but no differences at serine 565. PKA phosphorylates HSL at serine 563 and serine 660, which stimulates HSL activity [174]. In contrast, phosphorylation at serine 565 occurs via 5′ AMP-activated protein kinase (AMPK) and reduces HSL phosphorylation at Ser563 by PKA and inhibits HSL activity [175]. Interestingly, a recent report shows evidence for reduced hepatic AMPK signaling in a GSD Ia mouse model [176].

Glucocorticoids are also known to stimulate lipolysis by inducing HSL and adipocyte triglyceride lipase (ATGL) gene expression (reviewed in: [177]). GSD I has been associated with increased levels of cortisol and its metabolites [155, 178, 179]. However, under fasted conditions, circulating corticosterone concentrations were not elevated in L-G6pc\textsuperscript{−/−} mice as compared to wildtype controls, and expression levels of \textit{Atgl}, \textit{Hsl} or \textit{Plin} were unaltered in adipose tissue of these animals. Therefore we have no reason to believe that increased glucocorticoid signaling contributed to increased adipose tissue lipolysis in fasted L-G6pc\textsuperscript{−/−} mice.

On the other hand, and consistent with previous studies [86, 155], we observed a reduced plasma insulin to glucagon ratio in fasted L-G6pc\textsuperscript{−/−} mice. Multiple studies have shown that glucagon stimulates adipose tissue lipolysis (reviewed in: [44, 158]). \textit{Ex vivo} studies in human adipocytes have shown that glucagon increases human adipose tissue lipolysis through a rise in adipocyte intracellular cAMP levels [180]. The relevance of the lipolytic effects of glucagon has however been debated [158] because glucagon administration triggers insulin release \textit{in vivo} and thereby induces a counter-regulatory effect on adipose tissue lipolysis. Indeed, glucagon administration can acutely stimulate lipolysis in healthy men [181] and glucagon stimulates adipose tissue lipolysis in insulin resistant subjects and sheep indicating that glucagon exerts lipolytic effects when insulin action is impaired [181, 182, 183]. We therefore conclude
it is likely that the lower insulin to glucagon ratio in fasted \textit{L-G6pc\textsuperscript{−/−}} mice increased adipose tissue lipolysis via cAMP-PKA mediated activation of HSL. This in turn provided FFAs to the liver that were elongated, resulting in an increased contribution of chain elongation to hepatic TG in fasted \textit{L-G6pc\textsuperscript{−/−}} mice.

Also, ANGPTL4 is produced in the liver and the adipose tissue [184] and is involved in fasting-induced adipose tissue lipolysis through increases in intracellular cAMP and phosphorylation of HSL at serine 660 [67]. In the livers of both fed and fasted \textit{L-G6pc\textsuperscript{−/−}} mice we found an induction of \textit{Angptl4} mRNA expression, but no differences were found in adipose tissue. A role for ANGPTL4 in GSD Ia is however still to be determined, since we did not examine plasma concentrations and it is unclear whether this factor can work as an endocrine stimulator of adipocyte lipolysis [67].

Both fed and fasted \textit{L-G6pc\textsuperscript{−/−}} mice displayed increased FGF21 concentrations. Although reports of \textit{ex vivo} and \textit{in vitro} effects of FGF21 on adipocyte lipolysis are conflicting [66, 163, 185, 186], the phenotypes of FGF21 transgenic [163] and knockout mice [159] suggest that FGF21 promotes adipose tissue lipolysis and a recent study suggests that FGF21 stimulates lipolysis via catecholamines [187]. The role of FGF21 in adipose tissue lipolysis may furthermore be dependent on metabolic state, since FGF21 stimulates lipolysis in adipose tissue during feeding but inhibits it during fasting [159]. FGF21 is only induced in the late fasting response in humans [188, 189]. Whether FGF21 plays a role in metabolic derangements GSD Ia patients remains unknown. It is conceivable that metabolic control is of large influence on the FGF21 concentrations and effects in these patients.

Previous clinical studies strongly suggest that impaired VLDL catabolism is a commonly overlooked consequence of GSD Ia that likely contributes to hypertriglyceridemia [146, 148, 151]. Early studies proposed that LPL activity in GSD I patients is inhibited by a circulating factor [150, 169]. Increased circulating concentrations of apoC3 have been linked to hypertriglyceridemia in GSD I [149, 169], together with elevated apoC1, apoC2 [169, 190] and apoE levels [169, 170]. Our study shows that hepatic \textit{ApoC3} and \textit{Angptl4} mRNA expression were induced in \textit{L-G6pc\textsuperscript{−/−}} mice. Enhanced levels of apoC3, as well as increases in apoC1 and apoE can cause hypertriglyceridemia by post-translational inhibition of LPL activity and thereby inhibition of hydrolysis of TG-rich lipoproteins [168]. This is for instance illustrated by experiments in apoC1 [191] and apoC3 transgenic mice [192] and by the effects of apoE on LPL-mediated lipolysis of chylomicrons [193]. It has been proposed that apoC1 and apoC3 increase the
Figure 4.6: Schematic representation of hepatic lipid metabolism in fed (A) and fasted (B) L-G6pc−/− mice and putative factors affecting and/or driving metabolic pathways. See text for details.
inhibitory effect of ANGPTL4 on LPL activity by displacing LPL from TG-containing lipoproteins hence increasing LPL accessibility for ANGPTL4 [194]. Thus, elevated apoC1/3 concentrations in L-\(G\beta pc^{-/-}\) mice render LPL more susceptible for inactivation by ANGPTL4.

ApoC3 has also been implicated in assembly and secretion of VLDL [195] and point mutations in apoC3 lead to lowered ability to secrete VLDL [196, 197]. It is generally thought that increased hepatic lipogenesis in GSD I is, via enhanced VLDL-TG production and secretion, an important cause of hypertriglyceridemia in these patients. However, in fasted L-\(G\beta pc^{-/-}\) mice we show hypertriglyceridemia without increases in \textit{de novo} lipogenesis. Data on VLDL secretion rates in GSD I patients are also scarce and conflicting. Case reports have shown unchanged [149, 151], reduced or increased [146] VLDL production rates with increased VLDL TG content [151] or VLDL size [149]. In the S4048 induced rat model of GSD Ib hepatic VLDL-TG production rates were not affected [85]. Increased conversion of [1-\textsuperscript{14}C]-palmitate into TG in fasted GSD I patients could suggest increased VLDL-TG production and secretion after increased hepatic lipogenesis [173], but these effects may also be due to inhibited VLDL-TG catabolism. Insulin is known to inhibit VLDL secretion [198], therefore low fasting insulin concentrations may lead to an increase in VLDL-secretion during fasting in GSD Ia. More detailed assessments of VLDL-TG production rates should still be done in a larger number of patients and in animal models under differing nutritional conditions.

In summary, the present study demonstrates a marked difference in the origin of hepatic steatosis in a mouse model of GSD Ia depending on nutritional status (Figure 4.6A and 4.6B). In fed L-\(G\beta pc^{-/-}\) mice hepatic steatosis was accompanied by increased \textit{de novo} lipogenesis of fatty acids. In fasted L-\(G\beta pc^{-/-}\) mice hepatic TG is probably derived from elongation of fatty acids derived from enhanced WAT lipolysis, that presumably resulted from a decreased insulin to glucagon ratio and perhaps other circulating factors (ANGPTL4 and FGF21) that regulate cAMP-PKA phosphorylation of HSL. Hypertriglyceridemia may result from LPL inhibition by circulatory factors such as apoC3, apoE and ANGPTL4, though increases in VLDL-TG production cannot be excluded.

The frequent intake of uncooked cornstarch or gastric drip feeding aims to prevent fasting hypoglycemia and maintains GSD Ia patients in a continuous (semi)-fed state. As a consequence of adequate dietary management, the insulin to glucagon ratio remains within the fed range, preventing excessive adipose tissue lipolysis. Our findings stress the impact of nutritional state in the field of GSD I research. GSD Ia mouse models are commonly investigated in the fasted
state [86, 155, 167]. We propose that a systematic mechanistic investigation of GSD Ia in fed versus fasted states will provide clinically relevant insights into the contributions of perturbed lipid metabolism in patients with adequate versus poor metabolic control.

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