Interactions between carbohydrate and lipid metabolism in metabolic disorders
Bandsma, Robertus

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Enhanced glucose cycling and suppressed de novo synthesis of glucose-6-phosphate results in a net unchanged hepatic glucose output in \textit{ob/ob} mice

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\textit{Diabetologia, in press}
Abstract

Diabetes mellitus type 2 (DM2) is associated with insulin resistance and hyperglycemia. Leptin-deficient \textit{ob/ob} mice are hyperinsulinemic and hyperglycemic and provide an appropriate model for DM2. The mechanisms behind the hyperglycemia in \textit{ob/ob} mice has remained largely undefined so far. To evaluate the effects of chronic leptin deficiency on glucose metabolism, \textit{ob/ob} mice and lean littermates were fasted for 9 hours after which they were infused with [U-$^{13}$C]-glucose, [2-$^{13}$C]-glycerol, [1-$^{2}$H]-galactose and paracetamol for 6 h. Mass isotopomer distribution analysis was applied on blood glucose and urinary paracetamol-glucuronide. When expressed on the basis of body weight, endogenous glucose production (109 ± 23 vs. 152 ± 27 µmol/kg/min, obese vs. lean, \(P < 0.01\)) and \textit{de novo} synthesis of glucose-6-phosphate (122 ± 13 vs. 160 ± 6 µmol/kg/min, obese vs. lean, \(P < 0.001\)) were lower in \textit{ob/ob} mice than in lean littermates. In contrast, glucose cycling was greatly increased in obese mice (56 ± 13 vs. 26 ± 4 µmol/kg/min, obese vs. lean, \(P < 0.001\)). As a result, total hepatic glucose output remained unaffected (165 ± 31 vs. 178 ± 28 µmol/kg/min, obese vs. lean, NS). Metabolic clearance rate was significantly lower in obese mice (8 ± 2 vs. 18 ± 2 ml/kg/min, obese vs. lean, \(P < 0.001\)). Hepatic mRNA levels of genes encoding for glucokinase and pyruvate kinase were markedly increased in \textit{ob/ob} mice. Unaffected total hepatic glucose output in face of hyperinsulinemia reflects hepatic insulin resistance in \textit{ob/ob} mice, which is associated with markedly increased rates of glucose cycling. However, hyperglycemia in \textit{ob/ob} mice primary results from a decreased metabolic clearance rate of glucose.
Introduction

Hyperinsulinemia and fasting hyperglycemia are hallmarks of type 2 diabetes. Insulin resistance of peripheral organs, i.e., muscle and adipocytes, as well as of the liver may contribute to fasting hyperglycemia. Peripheral insulin-resistance reduces the ability of peripheral organs to clear glucose from the circulation. Hepatic insulin-resistance develops in two stages. During early stages in the development of type 2 diabetes, characterized by hyperinsulinemia and normoglycemia, hepatic glucose production is still normal under fasting conditions. However, hepatic glucose production remains inappropriately high during absorptive phases when insulin concentrations are elevated. At later stages in the development of type 2 diabetes in humans, hepatic glucose production is increased also under fasting conditions.1

Both gluconeogenesis and glycogenolysis may contribute to an elevated hepatic glucose production. Furthermore, data indicates that cycling of glucose, i.e., the process of sequential glucose phosphorylation by glucokinase (GK) and dephosphorylation by G6Pase, occurs at increased rates in humans with type 2 diabetes.2,3 Little is known about the quantitative role of glucose cycling in the increased hepatic glucose production in type 2 diabetes. Depending on the methodologies used for quantification of hepatic glucose fluxes, increased glucose cycling may affect the reported rates of gluconeogenesis and glycogenolysis.

Ob/ob mice suffer from severe obesity and diabetes due to leptin-deficiency and provide a model for type 2 diabetes. These mice display age-dependent hyperglycemia and hyperinsulinemia. Quantitative data on the perturbations of glucose metabolism in these mice in vivo are scarce. In vitro studies in perfused isolated livers of ob/ob mouse showed that glycogen turnover was increased.4 Lahtela et al. found greatly increased glucose cycling rates in hepatocytes isolated from 24-h fasted ob/ob mice.5

Novel methodologies using multiple stable isotopes in vivo now allow for determination of flux rates through the separate metabolic pathways involved in hepatic carbohydrate metabolism.6-8 In the current study, we used these methods to evaluate the quantitative role of gluconeogenesis, glycogenolysis, and glucose cycling in hyperglycemia in modestly fasted ob/ob mice.

Methods

Animals
Female ob/ob mice (n = 7) and lean littermates (n = 7) were purchased from Harlan (Zeist, The Netherlands) and were housed in a temperature-controlled (21°C) room on a 12-hr dark, 12-hr light cycle. Experimental procedures were approved by the Ethics Committee for Animal Experiments of the State University Groningen. Body weights were 22.7 ± 1.2 g for the lean mice and 49.7 ± 3.0 g for the ob/ob mice. Mice were equipped with a permanent...
catheter in the right atrium, via the right jugular vein as described previously. Mice were allowed to recover from surgery for at least 4 days.

Materials
The following isotopes were used: [2-13C]-glycerol (99% 13C APE), [1-2H]-galactose (98% 2H APE) (Isotec Inc., Miamisburg, OH, USA), [U-13C]-glucose (99% 13C APE) (Cambridge Isotope Laboratories, Inc., Andover, MA, USA). All chemicals used were reagent pro analysis grade. Bloodspots and urine were collected on Schleicher en Schuell No. 2992 filter paper (Schleicher en Schuell, ’s Hertogenbosch, The Netherlands). Infusates were freshly prepared and sterilized by the Hospital Pharmacy at the day before the experiment.

Animal experiments
Experiments were performed in awake, chronically-catheterized mice, essentially as described by Van Dijk et al. Mice were fasted for 9 h after which they were placed in metabolic cages to allow frequent collection of bloodspots and urine. Mice were infused with a sterile solution, containing [U-13C] glucose (13.9 µmol.ml\(^{-1}\)), [2-13C]glycerol (160 µmol.ml\(^{-1}\)), [1-2H]galactose (33 µmol.ml\(^{-1}\)) and paracetamol (1.0 mg.ml\(^{-1}\)) at a rate of 0.6 ml.hr\(^{-1}\). During the experiment, blood glucose was measured using EuroFlash™ test strips (LifeScan Benelux, Beerse, Belgium). Bloodspots were collected on filterpaper before the start of the infusion and hourly afterwards until 6h after the start of the infusion. Bloodspots were air-dried and stored at room temperature until analysis. Timed urine samples were collected at hourly intervals on filter paper strips. Strips were air-dried and stored at room temperature until analysis. At the end of the experiment animals were anesthetized with isofurane and a large blood sample was collected in heparin-containing tubes by heart puncture, centrifuged immediately and stored at –20 °C until analysis. The liver was quickly excised, weighed and frozen immediately in liquid N\(_2\).

Metabolite concentrations
Plasma was isolated from blood by centrifugation and liver tissue was homogenized. Plasma β-hydroxybutyrate, lactate, free fatty acid were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany and Wako Chemicals GmbH, Neuss, Germany). Plasma insulin levels were determined with a radioimmunoassay (RI-13K, Linco Research, St. Charles, MO). Total liver protein content was determined according to Lowry et al. Hepatic glycogen was determined after extraction with a 1 mol/l KOH solution by sonication. The extract was incubated for 30 minutes at 90 °C, cooled and brought to pH 4.5 by addition of 3 mol/l acetic acid. Precipitated protein was removed by centrifugation. Glycogen was converted to glucose by treating the samples with amyloglucosidase, followed by assay of glucose at pH 7.4 with ATP, NADP\(^+\), hexokinase and G6P dehydrogenase. Liver samples for the determination of G6P were treated by sonification in a 5% (w/v) HClO\(_3\) solution. Precipitated protein was removed by rapid centrifugation at 10.000 rpm for 1 min in a cold microcentrifuge and the supernatant
Table 1. List of sequences of primers and probes used in PCR measurements.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>GenBank no.</th>
</tr>
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<td>(\beta)-actin</td>
<td>Forward: ACC CAC ACT GTG CCC ATC TAC</td>
<td>NM007393</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCT CGG TCA GGA TCT TCA TGA</td>
<td></td>
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<tr>
<td></td>
<td>Probe: AGG GCT ATG CTC TCC CTC ACG CCA</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Forward: CGG CTA CCA CAT CCA AGG A</td>
<td>X00686</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCA ATT ACA GGG CCT CGA AA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: CGC GCA AAT TAC CCA CTC CCG A</td>
<td></td>
</tr>
<tr>
<td>G6ph</td>
<td>Forward: GAG GCC TTG TAG GAA GCA TTG</td>
<td>NM008061</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCA TCC CAG CCA TCA TGA GTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: CTC TGT ATG GGA ACC CTC GCC ACG</td>
<td></td>
</tr>
<tr>
<td>G6pt</td>
<td>Forward: GAG GCC TTG TTG GAA GCA TTG</td>
<td>NM008063</td>
</tr>
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<td></td>
<td>Reverse: CCA TCC CAG CCA TCA TGA GTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: CTC TGT ATG GGA ACC CTC GCC ACG</td>
<td></td>
</tr>
<tr>
<td>Gk</td>
<td>Forward: CCT GGG CTT CAC TTT CTT CTT</td>
<td>NM010292</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAC GAA GAC AAG ACC ATC ATG AAT</td>
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<tr>
<td></td>
<td>Probe: TGC CAT TCC CAT TCC ATG CCT</td>
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</tr>
<tr>
<td>Gp</td>
<td>Forward: GAA GGA GGC AAA CGG ATC AAG</td>
<td>NM133198</td>
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<tr>
<td></td>
<td>Reverse: TCA CGA TGT CCG AGT GGA TCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe: CCT CTG CAT CGT GGG CTG CCA</td>
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<tr>
<td>Gs</td>
<td>Forward: GCT CTC CAG ACG ATT CTT GCA</td>
<td>NM145572</td>
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<tr>
<td></td>
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<td></td>
<td>Probe: CTC TTA CGG GTT TTG TAA ACA GTC ACG CC</td>
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<tr>
<td>Pk</td>
<td>Forward: CGT TTG TGC CAC ACA GAT GCT</td>
<td>NM013631</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAT TGG CCA CAT CGC TTC TCT</td>
<td></td>
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<tr>
<td></td>
<td>Probe: AGC ATG ATC ACT AAG GCT CGA CCA ACT CGG</td>
<td></td>
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<tr>
<td>Pepck</td>
<td>Forward: GTG TCA TCC GCA AGC TGA AG</td>
<td>NM011044</td>
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<td>Reverse: CTT TCG ATC CTG GCC ACA TC</td>
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<tr>
<td></td>
<td>Probe: CAA CTG TTG GCT GCC TCT CAC TGA CCC</td>
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was neutralized to pH 7 by addition of small amounts of a mixture of 2 mol/l KOH and 0.3 mol/l MOPS. G6P was determined fluorimetrically with NADP⁺ and G6P dehydrogenase.

**Hepatic mRNA levels**

Total RNA was isolated from liver tissue using the Trizol method (Invitrogen, Paisley, United Kingdom). RNA was converted to cDNA with M-Mulv-RT (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol using random primers. cDNA levels of the genes of interest were measured by real-time PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). An...
amount of cDNA corresponding to 20 ng of total RNA was amplified using the qPCR core kit (Eurogentec, Seraing, Belgium) according to the manufacturer’s protocol using the appropriate forward and reverse primers (Invitrogen, Paisley, United Kingdom), and a template specific 3’-TAMRA, 5’-FAM labeled Double Dye Oligonucleotide probe (Eurogentec, Seraing, Belgium). Calibration curves were run on serial dilutions of pooled cDNA solutions as used in the assay. The data were processed using the ABI Sequence Detector v1.6.3 (System Applied Biosystems, Foster City, CA, USA). Quantified expression levels were within the linear part of the calibration curves. PCR results were normalized by S18-rRNA levels. The sequence of the primers and probes used in this study are listed in Table 1.

Measurement and Analysis of Mass Isotopomer Distribution by GC-MS
Analytical procedures for extraction of glucose and paracetamol-glucuronide (Par-GlcUA) from bloodspot and urine filterpaper strips, respectively, derivatization of the extracted compounds and GC-MS measurements of derivatives were essentially performed according to Van Dijk et al.8,10 The measured fractional isotopomer distribution by GCMS was corrected for the fractional distribution due to natural abundance of 13C, by multiple linear regression as described by Lee et al.6 to obtain the excess mole fraction of mass isotopomers due to incorporation and dilution of infused labelled compounds, i.e., [2-13C]-glycerol, [U-13C]-glucose and [1-2H]-galactose. This distribution was used in mass isotopomer distribution analysis (MIDA) algorithms of isotope incorporation and dilution according to Hellerstein et al.7 as described by Van Dijk et al.8,10

Statistical analysis
All values reported are mean ± SD. Levels of significance of difference of metabolite concentrations, gene expression and the values of the individual timepoints during isotope infusion experiments were determined using the non-parametric Mann Whitney test for unpaired data. Levels of significance of differences between the averages of the values of the fluxes at the individual timepoints between 3 and 6 h during the experiment were estimated using ANOVA with repeated measurements. Differences were considered significant at \( P < 0.05 \).

RESULTS

Body and liver weights of obese mice were more than double than those of lean mice (Table 2). Consequently, normalization of liver weight to body weight yielded no difference between obese and lean mice. Protein contents per gram of liver tissue were not significantly different between obese and lean mice. Hepatic glycogen content was mildly increased in the ob/ob mice, whereas glucose-6-phosphate (G6P) levels were not significantly different between ob/ob and lean mice. Plasma NEFA concentrations were almost two-fold higher in the ob/ob mice than in lean mice at the end of the experiment.
Plasma lactate and alanine concentrations were similar in the two groups but plasma β-hydroxybutyrate concentrations were elevated in ob/ob mice.

**Table 2.** Hepatic and plasma parameters in ob/ob mice and lean littermates (n = 7/group), * P < 0.05.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>lean</th>
<th>ob/ob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>23 ± 1</td>
<td>50 ± 3 *</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.1 ± 0.0</td>
<td>2.6 ± 0.4 *</td>
</tr>
<tr>
<td>Relative liver weight (% body weight)</td>
<td>4.7 ± 0.3</td>
<td>5.0 ± 0.8</td>
</tr>
<tr>
<td>Total liver protein (mg)</td>
<td>156 ± 20</td>
<td>325 ± 63 *</td>
</tr>
<tr>
<td>Liver protein content (mg protein/g liver weight)</td>
<td>144 ± 18</td>
<td>127 ± 18</td>
</tr>
<tr>
<td>G6P (nmol/g liver weight)</td>
<td>118 ± 56</td>
<td>153 ± 34</td>
</tr>
<tr>
<td>Glycogen (µmol glucose/g liver weight)</td>
<td>179 ± 16</td>
<td>207 ± 11 *</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEFA (mM)</td>
<td>0.5 ± 0.1</td>
<td>0.9 ± 0.2 *</td>
</tr>
<tr>
<td>3-Hydroxybutyrate (mM)</td>
<td>0.8 ± 0.4</td>
<td>3.1 ± 1.4 *</td>
</tr>
<tr>
<td>Alanine (µM)</td>
<td>133 ± 113</td>
<td>176 ± 33</td>
</tr>
<tr>
<td>Lactate (mM)</td>
<td>3.5 ± 1.0</td>
<td>3.5 ± 1.0</td>
</tr>
</tbody>
</table>

**Figure 1.** Plasma glucose concentrations during experiments in female lean (open circles) and ob/ob mice (closed circles). Each value represents the mean ± SD; n = 7, * P < 0.05 compared to lean animals.
The time course of blood glucose concentrations during experiments are shown in Figure 1. Obese mice were clearly hyperglycemic (8.8 ± 0.5 vs. 13.2 ± 1.9 mM, lean vs. obese, \( P < 0.05 \)). Glucose concentrations remained constant during the experiment in both groups. Furthermore, obese mice were clearly hyperinsulinemic. Insulin concentrations remained constant (45 ± 10 pM at \( t = 0 \) and 60 ± 45 pM at \( t = 6 \) h) during the experiment in lean mice but decreased from 900 ± 480 pM at \( t = 0 \) to 435 ± 270 pM at \( t = 6 \) h in \( ob/ob \) mice.

In Figure 2, endogenous glucose production (2A) and metabolic clearance rates of glucose (2B) are shown. At isotopic steady-state, i.e., between 3 and 6 h after the start of the infusion of labeled compounds, whole body and hepatic glucose metabolism was evaluated. As is clear from this Figure, endogenous glucose production was significantly

**Figure 2.** Endogenous glucose production (A) and metabolic clearance (B) during the infusion experiment in lean (open circles) and \( ob/ob \) (closed circles) mice. Each value represents the mean ± SD; \( n = 7 \). * \( P < 0.05 \) compared to lean animals.
decreased in \textit{ob/ob} mice (152 ± 27 vs. 109 ± 23 μmol.kg$^{-1}$.min$^{-1}$, lean vs. obese, $P < 0.001$) as was the metabolic clearance rate of glucose (18 ± 2 vs. 8 ± 2 ml.kg$^{-1}$.min$^{-1}$, lean vs. obese, $P < 0.001$). The rate of \textit{de novo} synthesis of G6P in obese mice was significantly decreased in comparison with lean control mice (160 ± 6 vs.122 ± 13 μmol.kg$^{-1}$.min$^{-1}$, lean vs. obese, $P < 0.001$, Fig 3A). Partitioning of newly synthesized G6P towards plasma glucose or glycogen was not affected in obese mice when compared to lean control mice (Fig 3B). In contrast, glucose cycling was greatly enhanced (Fig 4), \textit{i.e.}, 56 ± 13 μmol.kg$^{-1}$.min$^{-1}$ in obese mice and only 26 ± 4 μmol.kg$^{-1}$.min$^{-1}$ in lean control animals ($P < 0.001$).

\textbf{Figure 3}. The total rate of \textit{de novo} synthesis of G6P in lean (open circles) and \textit{ob/ob} (closed circles) mice (A) and the partitioning of the rates of \textit{de novo} synthesis of G6P to UDP-glucose (black), and to glucose (white) (B) during last 3 h of the infusion experiment in lean and \textit{ob/ob} mice. Each value represents the mean ± SD; $n = 7$. * $P < 0.05$ compared to lean animals.
As a consequence, total hepatic glucose production, i.e. the sum of endogenous glucose production and glucose cycling, was similar in obese and lean mice (178 ± 28 vs. 165 ± 31 µmol.kg⁻¹.min⁻¹, lean vs. obese, NS , Fig 4B).

Figure 4. Rate of cycling between glucose and G6P during the infusion experiment in lean (open circles) and ob/ob (closed circles) mice (A) and the contributions of endogenous glucose production (white) and glucose cycling (black) to the total endogenous glucose production in lean and ob/ob mice, during the last 3 h of the infusion experiment. Each value represents the mean ± SD; n = 7. * P < 0.05 compared to lean animals.
Figure 5. Separate fluxes through the relevant enzymic pathways in hepatic carbohydrate metabolism during the last 3 h of the infusion experiment in lean and ob/ob mice. Each value represents the mean ± SD; \( n = 7 \). * \( P < 0.05 \) compared to lean animals.
In Figure 5 the calculated mean values obtained at steady-state are shown for the individual fluxes through the various enzymic pathways of hepatic glucose metabolism. As anticipated based on the strong increase of glucose cycling, the calculated isotopic flux through GK was strongly increased in obese mice. The G6Pase flux, equivalent to the total hepatic glucose production, was unchanged. The flux through glycogen phosphorylase was not altered significantly whereas the glycogen synthase flux was significantly decreased in obese mice in comparison with lean littermates.

Expression of relevant genes in livers of lean and ob/ob mice are shown in Figure 6. Expression of the genes encoding Gk andPk were strongly up-regulated in ob/ob livers.

**Figure 6.** Gene expression of enzymes involved in glucose metabolism at the end of the infusion experiment in lean and ob/ob mice, n = 3 per group. Levels of cDNA were measured by real-time PCR as described in the Methods section. Data are expressed relative to 18S rRNA and the results of the lean animals are set equal to 1. *P < 0.05 compared to lean animals. Glut 2, glucose transporter 2; Gk, glucokinase; Pepck, phosphoenolpyruvate carboxykinase; Gp, glycogen phosphorylase; G6pt, glucose-6-phosphate translocase; G6ph, glucose-6-phosphate hydrolase; Pk, pyruvate kinase; Gs, glycogen synthase. Expression of genes were normalized by 18S-rRNA, since the level of β-actin mRNA in livers of obese mice was increased by ~30% when normalized by liver weight. In contrast, 18S-rRNA levels were similar in livers of obese and lean mice.
The mRNA levels of other key genes involved in carbohydrate metabolism, i.e. *Pepck*, *G6ph* and *G6pt* did not differ significantly between obese and lean mice. Expression levels of *Srebp-1c* and *Ppary*, transcription factors involved in control of hepatic glucose and fat metabolism, were significantly elevated in livers of *ob/ob* mice. As shown previously, mRNA levels of *Irs-1*, but particularly *Irs-2*, were strongly repressed in livers of obese mice in comparison with lean littermates.

**DISCUSSION**

The leptin-deficient *ob/ob* mouse is a commonly used mouse model of type 2 diabetes, but quantitative in vivo data on the disturbances that underly hyperglycemia in this model are sparse. In this study, we determined flux rates through various enzymic pathways, relevant in hepatic carbohydrate metabolism, in *ob/ob* and lean mice. When expressed on the basis of body weight, activity of hepatic glucose metabolism was, in general, supressed in obese mice in comparison with their lean littermates. One exception was glucose cycling. This flux was greatly increased in obese mice. Interestingly, the newly produced G6P was not preferentially directed towards plasma glucose in *ob/ob* mice but partitioned to glycogen stores to a similar extent as in lean mice. Furthermore, expression of genes of key enzymes involved in glucose metabolism was only different for *Gk* and *Pk*, indicating enhanced glycolysis in *ob/ob* mice.

Before discussing the results, some methodological issues have to be addressed. In this study, a multiple isotope infusion protocol according to Hellerstein et al. was used to calculate the relevant fluxes of glucose metabolism. The validity of the isotope model, with the application of glycoconjugates, and the MIDA approach has been substantiated in various studies although some controversy still remains. We have validated the application of MIDA in 9 h fasted C57Bl6/J mice in a separate study. In the current study, metabolic fluxes were compared between groups of mice with strongly different body compositions. Flux rates can be either normalized to body weight or to lean body mass. Lean body mass is slightly less in *ob/ob* mice in comparison with lean littermates while fat mass is disproportionally increased. Because of the following reasons, we have used the first approach for our calculations. Firstly, adipose tissue actively metabolises glucose. Therefore, it contributes to peripheral glucose clearance which is not accounted for when flux rates are normalized to lean body mass. Secondly, we noticed that relative liver weights i.e., normalized to body weight, in obese and lean mice did not differ significantly. Furthermore, similar protein contents were measured in livers of obese and lean mice. This indicates, to our opinion, that irrespective of the increased fat content of livers of *ob/ob* mice, the increase in liver weight is not associated with an increase in metabolically active cell mass. Based on these considerations we felt that, in this particular case, comparison between *ob/ob* mice and lean littermates should be done on the basis of flux rates normalized to body weight.
We found that the flux through G6Pase, reflecting total hepatic glucose output, was not affected in \textit{ob/ob} mice in comparison to lean mice. In the calculation of total endogenous glucose production the isotopic model considers 3 contributions \textit{i.e.}, (1) \textit{de novo} synthesis of G6P, partitioned to plasma glucose, (2) glycogen breakdown and (3) glucose recycling. The unaffected G6Pase flux was observed in the face of hyperinsulinemia and hyperglycemia. Probably as a consequence of hepatic insulin resistance, \textit{de novo} synthesis of G6P was diminished by only 30\% in \textit{ob/ob} mice when compared to lean littermates. Furthermore, it is important to note that autoregulation of hepatic glucose output, that is normally observed in the presence of high glucose concentrations in plasma, apparently malfunctions in \textit{ob/ob} mice because normal rates of endogenous glucose production were observed. In livers of \textit{ob/ob} mice a major contribution to the G6Pase flux could be assigned to glucose cycling, the process of concomitant glucose phosphorylation by GK and dephosphorylation by G6Pase. Glucose cycling was increased by a factor of \~2.5 in livers of \textit{ob/ob} mice. Lahtela \textit{et al.}\textsuperscript{5} also observed a high rate of glucose cycling in hepatocytes isolated of livers from fasted \textit{ob/ob} mice. Blood glucose concentrations in \textit{ob/ob} mice during the experiment were 13.2 \pm 1.9 M compared to 8.8 \pm 0.5 mM in lean littermates, an increase by a factor of 1.5, \textit{i.e.}, well below the relative increase in glucose recycling.

Glycogen metabolism in livers of \textit{ob/ob} mice did not differ much from that observed in lean littermates, with the exception of a relatively small decrease in the flux through GS. This represents another hallmark of hepatic insulin resistance in \textit{ob/ob} mice. The role of leptin in hepatic glycogen metabolism is still controversial. \textit{In vivo} infusion of leptin into fasted Wistar rats suppressed the contribution of glycogenolysis to hepatic glucose production.\textsuperscript{16} Hyperleptinemia in Wistar rats, brought about by recombinant adenovirus gene delivery, had a glycogen sparing effect.\textsuperscript{17}

Besides hepatic insulin resistance, peripheral organs were also found to be insulin resistant in \textit{ob/ob} mice. Metabolic clearance of plasma glucose was found to be decreased by a factor of \~2, at blood glucose concentrations that was almost double that in lean mice. This indicates that net glucose uptake by peripheral tissue was similar in \textit{ob/ob} and lean mice, irrespective of the elevated insulin concentrations in the first group. As a consequence, it can be concluded that hyperglycemia in \textit{ob/ob} mice is mainly related to peripheral insulin resistance. These considerations are in agreement with an earlier publication showing peripheral insulin resistance in \textit{ob/ob} mice by different means: uptake of 2-deoxyglucose was severely inhibited in isolated skeletal muscle of obese mice in comparison to muscle from lean mice.\textsuperscript{18}

As discussed, we observed “normal” rates of total glucose output and high rates of glucose cycling which could not be explained by hyperglycemia \textit{per se}. In accordance with these observations, “normal” mRNA levels of the genes encoding the gluconeogenic enzymes \textit{G6ph} and \textit{Pepck} were observed, while mRNA levels of \textit{Gk} were significantly increased in livers of obese mice when compared to lean littermates. Similarly, in an earlier publication GK activity was found to be increased in isolated livers of fasted \textit{ob/ob} mice.\textsuperscript{19} Concomitantly, we observed a significant increase in mRNA levels of \textit{Srebp-1c}
Hepatic glucose metabolism in ob/ob mice

and its target genes in liponeogenesis, i.e., Fas and Acc1 in livers of fasted ob/ob mice, in accordance with Wiegman et al. and Shimomura et al. In view of the observed hepatic insulin resistance in ob/ob mice, it is of interest to note that recently data became available indicating that hyperglycemia per se was able to induce increased expression of Srebp-1c and the glycolytic enzyme Pk in an insulin-independent way.

It is tempting to speculate that high rates of glucose cycling observed in livers of ob/ob mice reflect a consequence of an inappropriate co-localization of GK and G6Pase activities in hepatocytes. The liver exhibits a marked metabolic heterogeneity along the radius of the hepatic lobule. Activities of enzymes involved in hepatic glucose metabolism are differentially distributed along the acinar porta-central axis. During fasting and refeeding these gradients changes reciprocally. During fasting the gradients of PEPCK and G6Pase extent from the periportal into the perivenous zone while GK activity gradient is largely confined to the perivenously located hepatocytes only. During refeeding the opposite takes place. In this way, co-localization of concurrent enzymic reactions in hepatocytes is minimized. In livers of fasted ob/ob mice this mechanism appeared to be perturbed. Levels of mRNA of Gk remained high during fasting which, to our opinion, can be considered an indication for a persistent perivenous-periportal activity gradient of GK. On the other hand, mRNA levels of Pepck and G6ph in livers of fasting ob/ob mice were similar to those observed in lean littermates, pointing to a periportal-perivenous activity gradient of these enzymes in livers of ob/ob mice as normally observed in livers of fasting wild type mice. As a result considerable overlap occurs of GK and G6Pase activities in hepatocytes of ob/ob mice. The mechanism by which Gk mRNA is maintained at high levels during fasting in ob/ob mouse liver remains elusive, but is likely related to the constitutively high expression of SREBP-1c.

In humans with diabetes mellitus type 2, evidence for contributions of both induced gluconeogenesis and glycogenolysis to fasting hyperglycemia has been found. However, until now only very few studies have explicitly considered the quantitative role of glucose cycling in hepatic glucose production. There are indications to suggest that hepatic cycling of glucose is elevated in humans with type 2 diabetes. If glucose cycling is indeed a major contributor to the elevated G6Pase flux in type 2 diabetes, as we observed in ob/ob mice, this would lead to an overestimation of both gluconeogenic flux, measured by $^3$H$_2$O method and glycogenolytic flux, measured by $^{13}$C-MRS in these patients. Furthermore, in most studies with (often obese) diabetic subjects data was normalized to lean body mass instead of bodyweight, leading to inappropriately elevated values for gluconeogenic and glycogenolytic fluxes when compared to non-diabetic subjects.

In conclusion, this study demonstrates that in ob/ob mice de novo synthesis of glucose-6-phosphate was diminished while glucose cycling was increased, resulting in a "normal" total glucose output by the liver. However, these normal values were observed in face of hyperglycemia and hyperinsulinemia. This points to a co-existence of hepatic and peripheral insulin resistance with peripheral insulin resistance as the major cause of hyperglycemia.
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