In vivo kinetic studies in inborn errors of metabolism: expanding insights in (patho)physiology
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Chapter 3

A potential role for muscle in glucose homeostasis: *in vivo* kinetic studies in Glycogen Storage Disease type 1a and fructose-1,6-bisphosphatase deficiency

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Submitted
Abstract

**Objective:** to study the role of extra-hepatic tissue in glucose homeostasis during fasting.

**Study design:** endogenous glucose production (EGP), glycogenolysis (GGL) and gluconeogenesis (GNG) were quantified with stable isotopes in a patient with Glycogen Storage Disease type 1a (GSD-1a), and in a patient with fructose-1,6-bisphosphatase (FBPase) deficiency. The [6,6-2H2]glucose dilution method in combination with the deuterated water method was used during individualized fasting tests.

**Results:** both patients became hypoglycemic. The patient with GSD-1a after 2.5 hrs of fasting, with EGP 3.84 μmol/kg·min (30% of normal EGP after an overnight fast), GGL 3.09 μmol/kg·min and GNG 0.75 μmol/kg·min. The patient with FBPase deficiency after 14.5 hrs of fasting, with EGP 8.53 μmol/kg·min (62% of normal EGP after an overnight fast), GGL 6.89 μmol/kg·min GGL and GNG 1.64 μmol/kg·min.

**Conclusion:** EGP was severely hampered in both patients resulting in hypoglycemia. However, despite the defective hepatic and renal GNG in both disorders, and the defective hepatic GGL in GSD-1a, both patients were still able to produce glucose via both pathways. Since all necessary enzymes of these pathways have now been functionally detected in muscle, a contribution of muscle to EGP during fasting via both GGL as well as GNG is suggested.

**Abbreviations**

- **EGP** endogenous glucose production
- **FBPase** fructose-1,6-bisphosphatase
- **GGL** glycogenolysis
- **GNG** gluconeogenesis
- **GSD** glycogen storage disease
Introduction

Endogenous glucose production (EGP) during fasting is predominantly derived from hepatic gluconeogenesis (GNG) and glycogenolysis (GGL), with a minor contribution from renal GNG (1). Recently, a potential additional role for muscle in EGP has been suggested, based on the characterization of an isof orm of glucose-6-phosphatase, glucose-6-phosphatase-β (Glc-6-Pase-β) expressed in muscle and in other extra-hepatic tissues (2,3). Glc-6-Pase-β has been shown to have structural and functional properties in muscle comparable to the glucose-6-phosphatase-α expressed in liver, kidney and intestine (EC 3.1.3.9; Glc-6-Pase-α) (4). As patients with Glycogen Storage Disease 1a (GSD-1a; MIM #232200) are deficient for Glc-6-Pase-α resulting in a defective hepatic and renal GNG and GGL, Glc-6-Pase-β activity in muscle might explain the residual EGP previously observed in these patients (5-8).

In order to investigate the potential role of extra-hepatic and extra-renal tissue in glucose homeostasis during fasting in vivo, we performed whole body kinetic studies in a patient with GSD-1a and in a patient with fructose-1,6-bisphosphatase (FBPase) deficiency (MIM #229700), an inborn error of hepatic and renal GNG. For the first time, the differential contributions of GGL and GNG to EGP during fasting were quantified in these disorders, using the [6,6-2H2]glucose isotope dilution method combined with the deuterated water method (9;10).

Materials and Methods

Subjects

Patient 1 presented with severe hypoglycemia (plasma glucose 0.3 mmol/L) and hepatomegaly at the age of 4 months. GSD-1a was diagnosed on the demonstration of complete absence of glucose-6-phosphatase activity in a fresh liver biopsy. This diagnosis

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Height (m)</th>
<th>Weight (kg)</th>
<th>Inborn error of metabolism</th>
<th>Enzyme activity (normal range)</th>
<th>DNA analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>M</td>
<td>17.9</td>
<td>1.76 (-1 SD)</td>
<td>75.0 (+1.5 SD)</td>
<td>Glucose-6-phosphatase deficiency (GSD Ia)</td>
<td>0.0 (10 – 30) nmole/min·mg protein*</td>
<td>R170X</td>
</tr>
<tr>
<td>Patient 2</td>
<td>F</td>
<td>16.7</td>
<td>1.50 (-2 SD)</td>
<td>60.0 (+2 SD)</td>
<td>Fructose-1,6-bisphosphatase deficiency</td>
<td>&lt;0.1 (3 – 20) nmole/min·mg protein†</td>
<td>ND</td>
</tr>
</tbody>
</table>

‡ in leucocytes; Baker L et al, Lancet 1970; 296 (7662): 13-16 (ref 10)
ND = not determined
was later confirmed by mutation analysis revealing two mutations, known to completely abolish Glc-6-Pase-α activity (Table 1) (11). **Patient 2** was admitted at 11 months because of convulsions due to hypoglycaemia. She was diagnosed with FBPase deficiency by demonstrating undetectable enzyme activity in leucocytes (Table 1) (12). The *in vivo* stable isotope studies were approved by the Institutional Review Board. Both patients and their parents gave informed consent prior to the studies.

**Study protocol (Figure 1)**

Fasting tests were performed at the age of 17.9 and of 16.7 years respectively. Both patients were admitted one day before the test. An intravenous catheter was inserted into antecubital veins of both arms after topical application of lidocaine cream. One catheter was used for administration of \([6,6-\text{H}_2]\)glucose, the other for blood sampling. At baseline a blood sample was collected to determine background enrichment of deuterated water in plasma. Fasting was started at a time considered save, based on previous experience with fasting in the patients. Prior to fasting both patients consumed their regular evening meal. Patient 1 received nocturnal nasogastric drip feeding. This drip feeding was discontinued two hours prior to initiation of \([6,6-\text{H}_2]\)glucose infusion and substituted by an unlabeled glucose infusion at a rate of 5 mg/kg·min which was continued until the start of the \([6,6-\text{H}_2]\)glucose infusion. Both patients remained fasted throughout the test and maintained bed rest.

Twelve hrs prior to \([6,6-\text{H}_2]\)glucose infusion, both patients drank deuterium enriched water (>99% pure; Cambridge Isotope Laboratories, Cambridge, MA) at a dose of 5 g per kg body water divided in 5 doses within 120 minutes (13). The total amount of

**Figure 1a (patient 1)**

<table>
<thead>
<tr>
<th>glucose IV</th>
<th>5 mg/kg·min</th>
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<tbody>
<tr>
<td>([6,6-\text{H}_2])glucose IV</td>
<td>bolus (26.4 μmol/kg)</td>
</tr>
<tr>
<td>blood sampling</td>
<td>continuous (0.33 μmol/kg·min)</td>
</tr>
<tr>
<td>fasting time (h)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td></td>
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<td>1</td>
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<tr>
<td>2</td>
<td></td>
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<td>3</td>
<td></td>
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</tbody>
</table>

**Figure 1b (patient 2)**

<table>
<thead>
<tr>
<th>([6,6-\text{H}_2])glucose IV</th>
<th>bolus (26.4 μmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>blood sampling</td>
<td>continuous (0.33 μmol/kg·min)</td>
</tr>
<tr>
<td>fasting time (h)</td>
<td>10</td>
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<td></td>
<td>11</td>
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<td>15</td>
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</table>

**Figure 1.** Study protocols in patients 1 (GSD-1a) and 2 (FBPase deficiency).
body water (kg) was estimated as 60% of body weight (kg) (14). Thereafter, patients were only allowed to drink tap water enriched to 0.5% with deuterated water until the end of the test. At the start of the fasting test in patient 1, and after 10 hrs of fasting in patient 2, and after collection of a blood sample to determine background enrichment of [6,6-2H2]glucose in plasma, a primed continuous infusion of [6,6-2H2]glucose (>99% pure; Cambridge Isotope Laboratories, Cambridge, MA) was started (bolus: 26.4 μmol/kg; continuous infusion: 0.33 μmol/kg·min) in order to reach an estimated 2% plasma enrichment (15). Blood samples were drawn every 5 minutes at the beginning and end of the test when patients became hypoglycaemic, and every 30 minutes during the test (Figure 1). Samples were centrifuged at 3000 rpm for 10 min, after which plasma was collected and stored at -20°C. Blood samples for determination of fractional GNG were immediately deproteinized by adding an equal amount of 10% perchloric acid. These samples were centrifuged at 4000 rpm for 20 min, after which the supernatant was collected and stored at -20°C. Blood glucose levels were monitored every hour and more frequently when glucose levels dropped below 3.5 mmol/L. The test was terminated when clinical symptoms of hypoglycemia occurred, after which patients were immediately given carbohydrate rich drinks and a meal.

Analytical methods

**Plasma glucose concentration:** plasma glucose levels were analyzed with the hexokinase method on a Roche MODULAR P800 analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

**Hormones:** plasma insulin and cortisol concentrations were determined on an Immulite 2000 system (Diagnostic Products Corporation, LA, USA). Insulin was measured with a chemiluminescent immunometric assay, cortisol was measured with a chemiluminescent immuno assay. Glucagon was determined by RIA (Linco Research, St. Charles, MO, USA). Plasma free fatty acid (FFA) levels were measured by an enzymatic method (NEFAC; Wako Chemicals GmbH, Neuss, Germany).

**Plasma [6,6-2H2]glucose enrichment:** plasma glucose enrichments were determined as described previously (13). Briefly, plasma was deproteinized with methanol and evaporated to dryness. The extract was derivatized with hydroxylamine and acetic anhydride (16). The aldonitrile pentaacetate derivative of glucose was extracted into methylene chloride and evaporated to dryness. The extract was reconstituted in ethylacetate and injected into a gas chromatograph/mass spectrometer (HP 6890 series GC system and 5973 Mass Selective Detector, Agilent Technologies, Palo Alto, CA, USA). Separation was achieved on a J&W DB17 column (30 m x 0.25 mm, d 0.25 μm; J&W Scientific, Folsom, CA). Glucose ions were monitored at m/z 187, 188 and 189. The isotopic enrichment of glucose was determined by dividing the peak area of m/z 189 by the peak area of m/z 187, after correction for background enrichment of [6,6-2H2]glucose.

**Deuterium enrichment in glucose at position C5 and in plasma water:** glucose was converted to a hexamethylene tetra-amine (9;13). Hexamethylene tetra-amine was injected
into a gas chromatograph/mass spectrometer (HP 6890 series GC system and 5973 Mass Selective Detector, Agilent Technologies, Palo Alto, CA, USA). Separation was achieved on an AT-amine column (30 m x 0.25 mm, d, 0.25 μm; Alltech Associates Inc, Deerfield, IL, USA). Hexamethylene tetra-amine ions were monitored at $m/z$ 140 and 141. Deuterium enrichment in plasma was determined by a method adapted from Previs et al (17).

Calculations and statistical analysis

Rate of appearance of glucose: the rate of appearance of glucose in plasma ($R_a$ glucose), reflecting whole body endogenous glucose production (EGP) during fasting, was calculated with Steele’s non-steady-state equation (18). The fraction of the total extracellular glucose pool was assumed to be equal to the extracellular water compartment, which was between 20% and 25% of body weight in the subjects studied (14). Calculated rates of EGP in the patients were compared to rates of EGP after overnight fasting in healthy subjects as predicted by an age-dependent regression model (19).

Absolute gluconeogenesis and glycogenolysis: absolute GNG was calculated by multiplying $R_a$ glucose by the fractional GNG. Fractional GNG was calculated as follows (9):

$$\text{fracGNG(\%)} = \frac{\text{deuterium enrichment in glucose at C5}}{\text{deuterium enrichment in plasma water}} \times 100\%$$

Absolute GGL was calculated by subtracting absolute GNG from $R_a$ glucose.

Results

Plasma glucose, FFA and glucoregulatory hormones

Plasma glucose: in patient 1, after stopping exogenous glucose supplementation, plasma glucose decreased from 6.3 mmol/L to 1.1 mmol/L within 2.5 hrs (Figure 2). In patient 2 plasma glucose decreased from 3.7 mmol/L to 2.5 mmol/L from 12 to 14.5 hrs of fasting (Figure 3).

Plasma FFA: plasma FFA concentration was 1.64 mmol/L in patient 1 and 2.16 mmol/L in patient 2 at the end of the test. This may have been inaccurate in patient 1 as the hypertriglyceridemia could have interfered with the enzymatic assay.

Glucoregulatory hormones: At the end of the test in both patients plasma insulin levels were undetectable. Plasma glucagon was 190 ng/L in both patients and plasma cortisol was 666 nmol/L in patient 1 and 792 nmol/L in patient 2.

Glucose kinetics

Patient 1 (GSD-1a; Figure 2): after 2 hrs of fasting EGP was 5.09 μmol/kg·min, (normal after an overnight fast 13.23 μmol/kg·min (19)). The test was terminated at 2.6 hrs of
fasting (EGP was 3.84 μmol/kg·min). GGL decreased from 4.39 to 3.09 μmol/kg·min between 2 and 2.6 hrs of fasting, representing 86.2% to 80.5% of EGP, respectively. GNG was low but detectable: 0.60 and 0.78 μmol/kg·min (13.7% to 19.6% of EGP) at 2 to 2.6 hrs of fasting.

**Patient 2 (FBPase deficiency; Figure 3):** after 12 hrs of fasting EGP was 13.27 μmol/kg·min, corresponding with the predicted EGP after an overnight fast for this age (13.77 μmol/kg·min) (19). EGP decreased to 8.53 μmol/kg·min during the subsequent 2.5 hrs (14.5 hrs of fasting). At 12 hrs of fasting GGL was 10.44 μmol/kg·min (78.7% of EGP), decreasing to 6.88 μmol/kg·min (80.7% of EGP) during the subsequent 2.5 hrs of fasting. GNG was 2.83 μmol/kg·min (21.3% of EGP) at 12 hrs of fasting, decreasing to 1.65 μmol/kg·min (19.3% of EGP) during the subsequent 2.5 hrs.

**Figures 2 & 3** Glucose kinetics in relation to plasma glucose concentration and fasting duration in a patient with glucose-6-phosphatase deficiency (GSD-1a; Figure 2) and a patient with FBPase deficiency (Figure 3).

Plasma glucose concentrations are depicted in the left panels (Figures 2a and 3a). Endogenous glucose production (EGP; ○), glycogenolysis (GGL; △) and gluconeogenesis (GNG; ▽) are depicted in the right panels (Figures 2b and 3b).
Discussion

This paper reports for the first time the contribution of both GGL and GNG to EGP during fasting in a patient with Glc-6-Pase-α deficiency (GSD-1a) and in a patient with FBPase deficiency. Our data on glucose kinetics show a persistent EGP from both GGL and GNG in both patients, despite their undetectable enzyme activities. On the basis of these results, a potential role of muscle in glucose homeostasis via both GGL and GNG in vivo is suggested.

In patient 1, Glc-6-Pase-α activity was completely deficient, which was confirmed by mutation analysis (Table 1) (11). This fully excludes any contribution from either liver, kidney or small intestine to EGP. However, EGP in this patient still was 30% of the predicted EGP in healthy subjects of the same age after an overnight fast (19). This in line with previous studies showing residual EGP, even up to 60% of normal, in patients with GSD-1 (5-8).

Three different explanations for the presence of EGP in GSD-1 have been proposed. First, EGP by increased cycling through hepatic glycogen via the action of amylo-1,6-glucosidase. However, this has been ruled out (20). Second, EGP by lysosomal digestion of hepatic glycogen through α-1,4-glucosidase activity. This is highly unlikely (6;21) as α-1,4-glucosidase is not susceptible to substrate or hormonal regulation and EGP in patients with GSD-1 is influenced by exogenous glucose supplementation (6;7). The third proposed mechanism is EGP through muscular GGL and/or GNG. This is made plausible by the recent characterization of muscular Glc-6-Pase-β (3;4). The reported range in residual EGP between GSD-1a patients of the same age with completely abolished enzyme activity, reflected by the observed inter-individual differences in fasting tolerance in patients with GSD-1 (22-24), may be then explained by differences in muscle mass and/or muscular glycogen content.

GGL contributed more than 80% to the observed EGP in the patient with GSD-1a. In addition, we showed that GNG still contributed up to 19% to the EGP in this patient (Figure 2b). This contrasts with data from Kalderon et al who excluded GNG as a source for EGP in GSD-1, based on a lack of carbon recycling from [U-13C]glucose (21;22). Their method, however, only provides an indirect and non-quantitative assessment of GNG, whereas GNG determined with the deuterated water method, as used in our study, is a direct method to quantify GNG yielding accurate results (10). Possibly, in the experiments of Kalderon et al the 13C label from [U-13C]glucose was diluted beyond detection in the triose phosphate and oxaloacetate carbon pools (25). The observed GNG may also be of muscular origin. Apart from Glc-6-Pase-β a specific isoform of FBPase, another key enzyme in GNG, is functionally expressed in muscle (26;27). Based on the formerly presumed lack of glucose-6-phosphatase activity in muscle this muscle specific FBPase was previously considered not to be involved in GNG (28;29). Alternatively, the observed residual GNG in the patient with GSD-1a might also be explained by renal and/or intestinal GNG, as Glc-6-Pase-β is also expressed in kidney and small intestine (2). However, expression of
Glc-6-Pase-β is much lower in kidney and small intestine than in muscle (2), and muscle has by far the largest body reservoir of glycogen (4).

A potential role for muscle in GNG is further supported by the results of our study in the patient with a deficiency of the liver and kidney specific isoform of FBPase (EC 3.1.3.11) as we detected that GNG still contributed up to 20% to EGP in this patient (Figure 3). This might be explained by the activity of the muscle specific isoform of FBPase in combination with activity of Glc-6-Pase-β.

In conclusion, we have provided in vivo evidence for both GNG and GGL contributing to the EGP in GSD-1a. This, in combination with substantial residual GNG in FBPase deficiency, demonstrates an important role of extra-hepatic tissue in glucose homeostasis. A role of muscle in glucose homeostasis via both GGL and GNG is strongly suggested.

Acknowledgements

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