In vivo kinetic studies in inborn errors of metabolism: expanding insights in (patho)physiology
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Discrepancy between endogenous glucose production and glucose uptake causes hypoglycemia in HMG-CoA lyase deficiency

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Submitted
Abstract

Pathophysiology of hypoglycemia in HMG-CoA lyase (HL) deficiency remains unclear. We therefore studied the endogenous glucose production (EGP), peripheral glucose uptake (GU), glycogenolysis (GGL) and gluconeogenesis (GNG) during fasting in two siblings with HL deficiency using stable isotope techniques. Only the youngest patient (age 8.4 yrs) became symptomatic. After 21 hrs of fasting his plasma glucose was 3.1 mmol/L, plasma ketones were undetectable and plasma 3-hydroxyisovalerylcarnitine and 3-methylglutarylcarnitine were increased. During the test, EGP decreased from 21.72 to 10.89 μmol/kg-min, while GU decreased significantly less: from 21.91 to 14.61 μmol/kg-min. GGL decreased from 16.19 to 5.54 μmol/kg-min and GNG remained almost constant (5.53 to 5.35 μmol/kg-min). Patient 2 (age 15.1 yrs) maintained normoglycemia during 24 hrs of fasting. Plasma ketones were undetectable and plasma 3-hydroxyisovalerylcarnitine and 3-methylglutarylcarnitine remained constant. During the test his EGP and GU decreased concomitantly, both from 16.48 to 11.75 μmol/kg-min. GGL decreased from 10.01 to 6.65 μmol/kg-min and GNG from 6.47 to 5.10 μmol/kg-min. These data show that hypoglycemia in HL deficiency results from a mismatch between GU and EGP. This mismatch is due to inability to sufficiently reduce GU during fasting caused by the complete lack of ketones. This results in rapid depletion of glycogen and failure to sustain EGP. Inhibition of gluconeogenesis due to HMG-CoA accumulation appears not to play a role as GNG remained constant despite evidence of accumulating HMG-CoA.

Abbreviations

- **EGP**: endogenous glucose production
- **GGL**: glycogenolysis
- **GNG**: gluconeogenesis
- **GU**: peripheral glucose uptake
- **FFA**: free fatty acids
- **HL**: HMG-CoA lyase
- **HMG-CoA**: 3-hydroxy-3-methylglutaryl-CoA
- **KH**: ketotic hypoglycemia
- **mHS**: HMG-CoA synthase
- **R_a**: rate of appearance
- **R_d**: rate of disappearance
Introduction

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase deficiency (HL; OMIM #245050) is a rare autosomal recessive disorder of ketogenesis, resulting in episodes of non-ketotic hypoglycemia provoked by fasting. The hypoglycemia is considered to be caused by either an increased glucose demand exceeding endogenous glucose production (EGP) due to complete absence of ketone bodies as an alternate substrate, and/or to inhibition of gluconeogenesis caused by accumulated HMG-CoA (1). The differential contribution of these mechanisms to the observed fasting induced hypoglycemia has, however, never been studied.

We quantified the main fluxes of glucose metabolism during fasting in two siblings with HL deficiency in order to study the pathophysiology of hypoglycemia. Endogenous glucose production (EGP), peripheral glucose uptake (GU), glycogenolysis (GGL) and gluconeogenesis (GNG) were quantified with use of the [6,6-2H2]glucose isotope dilution method combined with the deuterated water method (2;3).

Materials and Methods

Subjects

Two patients with HL deficiency were included. HL deficiency was confirmed in both by enzymatic analysis (Table 1).

**Patient 1** is the youngest child in a family of nine children in which five died due to hypoglycemic incidents in HL deficiency. The diagnosis of HL deficiency was established in cord blood. He was started on frequent feedings and had an uncomplicated neonatal period. In his first years of life he had several minor metabolic derangements due to intercurrent infectious diseases without any neurological sequelae. Fasting is avoided and he receives carnitine supplementation.

**Patient 2** is the older brother of patient 1. Plasma glucose shortly after birth was 0.4 mmol/L. He was started on frequent feedings and carnitine supplementation. Enzyme analysis in cord blood confirmed HL deficiency. In his first years of life he had several hypoglycemic episodes during intercurrent infectious diseases. He has a

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<td>Patient 1</td>
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<td>Patient 2</td>
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*in leucocytes; Wanders et al, Clin Chim Act 1990; 189(3): 327-334
mild delay in psychomotor development. Fasting is avoided and he receives carnitine supplementation.

All studies were approved by the Institutional Review Board. The older patient and the parents gave informed consent prior to the studies.

Study protocol
Both patients were admitted one day before the fasting test. An intravenous catheter was inserted into the antecubital vein of both arms after topical application of lidocaine cream. One catheter was used for administration of [6,6-2H2]glucose, the other for blood sampling. A baseline 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 these patients. Prior to fasting both patients consumed their regular evening meal and maintained bed rest from that time on. After this evening meal they 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 (4). The total amount of body water (kg) in the subjects was estimated as 65% (patient 1) or 60% (patient 2) of body weight (kg) (5). Thereafter, they were only allowed to drink tap water enriched to 0.5% with deuterated water until the end of the test. At 8 a.m. the next day, 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 (patient 1: 26.4 μmol/kg bolus and 0.33 μmol/kg·min continuous infusion; patient 2: 17.6 μmol/kg bolus and 0.22 μmol/kg·min continuous infusion) in order to reach an estimated 2 % plasma enrichment (6). Thereafter, blood samples were drawn every 30 minutes and more frequently at the beginning and end of the test or when the patients became hypoglycemic. Total blood volume drawn did not exceed 5% of total estimated blood volume in the subjects. At several time points during the test urine was collected for organic acid analysis. Urine was stored at 4°C until analysis. Blood 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 gluconeogenesis 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 either when the blood glucose level was ≤ 2.5 mmol/L or when clinical symptoms of metabolic derangement occurred, or at the end of the afternoon on the day of the test. After cessation of the test, patients were immediately given carbohydrate rich drinks and a meal.

Analytical methods
Plasma glucose, FFA, ketone bodies, acylcarnitines and hormones: plasma glucose levels were analyzed with the hexokinase method on a Roche MODULAR P800 analyzer (Roche...
Plasma FFA concentration was measured by an enzymatic method (NEFAC; Wako Chemicals GmbH, Neuss, Germany). Plasma ketone bodies were determined with a fluorometric enzyme method according to Williamson and Corkey (7). Acylcarnitines were determined using electrospray tandem-mass spectrometry (8). Plasma insulin and cortisol concentrations were determined on an Immulite 2000 system (Diagnostic Products Corporation, LA, USA). Insulin was measured with a chemiluminescent immunoassay, cortisol was measured with a chemiluminescent immuno assay. Glucagon was determined by RIA (Linco Research, St. Charles, MO, USA). Norepinephrine and epinephrine were measured by an in-house HPLC method.

Plasma $[6,6-^{2}H_{2}]$glucose enrichment: plasma glucose enrichments were determined as described previously (4). Briefly, plasma was deproteinized with methanol and evaporated to dryness. The extract was derivatized with hydroxylamine and acetic anhydride (9). 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$_{f}$ 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 $[6,6-^{2}H_{2}]$glucose.

Deuterium enrichment in glucose at position C5 and in plasma water: glucose was converted to a hexamethylene tetra-amine (2;4). 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$_{f}$ 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 (10).

Calculations and statistical analysis

Rates of appearance and disappearance of glucose: the rates of appearance and disappearance of glucose in plasma (R$_a$ glucose and R$_d$ glucose), reflecting whole body endogenous glucose production (EGP) and whole body glucose uptake (GU) during fasting, were calculated with Steele’s non-steady-state equations (11). 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 (5). 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 (12).

Absolute gluconeogenesis and glycogenolysis: absolute gluconeogenesis (GNG) was calculated by multiplying R$_a$ glucose by the fractional gluconeogenesis. Fractional gluconeogenesis was calculated as follows (2):
\[ \text{fracGNG(\%) = } \frac{\text{deuterium enrichment in glucose at C5}}{\text{deuterium enrichment in plasma water}} \cdot 100\% \]

Absolute glycogenolysis (GGL) was calculated by subtracting absolute GNG from \( R_a \) glucose.

Results

Plasma parameters in relation to fasting duration

Plasma glucose, FFA, glucoregulatory hormones, acylcarnitines and ketone body concentrations in relation to fasting duration in both patients are reported in Table 2. Patient 1 developed symptoms of autonomic dysregulation and metabolic derangement after 21 hrs of fasting. At this time plasma glucose concentration was 3.1 mmol/L. Patient 2 maintained normoglycemia during 24 hrs of fasting. Activated lipolysis, fatty acid oxidation and leucine degradation in patient 1 are demonstrated by increased plasma FFA, acetylcarnitine, 3-hydroxyisovalerylcarnitine and 3-methylglutaryl-carnitine. In patient 2, plasma FFA increased to a lesser extent and acetylcarnitine, 3-hydroxyisovalerylcarnitine and 3-methylglutaryl-carnitine remained almost constant. No ketone bodies could be detected during fasting in both patients.

Glucose kinetics

Patient 1 (Figure 1): after 16 hrs of fasting EGP was 21.72 μmol/kg-min, which is normal for his age (12). A rapid decrease in EGP was observed after 19 hrs of fasting. At the

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<tr>
<td>Fasting duration</td>
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<td>Glucose (mmol/L)</td>
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<td>FFA (mmol/L)</td>
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<td>Acetoacetate (mmol/L)</td>
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<td>Acetylcarnitine (μmol/L)</td>
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<td>3-Hydroxyisovalerylcarnitine (μmol/L)</td>
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<td>3-Methylglutarylcaritine (μmol/L)</td>
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<td>Insulin (pmol/L)</td>
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<td>Glucagon (ng/L)</td>
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<td>Cortisol (nmol/L)</td>
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ND = not determined
end of the test EGP was 10.89 μmol/kg·min. Initially, GU equalled EGP (both 21.91 μmol/kg·min after 16 hrs of fasting). However, a dissociation between EGP and GU was observed from 19 hrs of fasting onwards, with EGP becoming increasingly lower than GU (Figure 1). GU at the end of test was 14.61 μmol/kg·min.

After 16 hrs of fasting GGL was 16.19 μmol/kg·min (74.5% of EGP). GGL initially decreased at constant rate, but showed a rapid decline at the end of the test to 5.54 μmol/kg·min at the end of the test (50.9% of EGP). GNG was 5.53 μmol/kg·min at 16 hrs of fasting (25.5% of EGP) and remained almost constant until the end of the test.

Patient 2 (Figure 2): after 16 hrs of fasting EGP was 16.48 μmol/kg·min, which is normal for his age (12). EGP decreased slowly during the test to 11.75 μmol/kg·min after 24 hrs of fasting. GU equaled EGP during the whole test.

GGL was 10.01 μmol/kg·min (60.7% of EGP) after 16 hrs of fasting. GGL decreased gradually during the test and was 6.65 μmol/kg·min after 24 hrs of fasting (56.6% of EGP). GNG was 6.47 μmol/kg·min after 16 hrs of fasting (25.5% of EGP) and 5.10 μmol/kg·min after 24 hrs of fasting (43.4% of EGP).

Figures 1&2. Glucose kinetics in relation to plasma glucose concentration and fasting duration in patients 1 (Figure 1) and 2 (Figure 2). Plasma glucose concentration is depicted in the ‘A’ panels in mmol/L and endogenous glucose production (EGP; ●), peripheral glucose uptake (GU; ○), glycogenolysis (GGL; △) and gluconeogenesis (GNG; ▽) are depicted in the ‘B’ panels in μmol/kg·min.
Discussion

We studied all major glucose fluxes during fasting in two patients with HMG-CoA lyase (HL) deficiency in vivo and demonstrated a discrepancy between EGP and GU, causing hypoglycemia. EGP is the main denominator of plasma glucose concentration in children (13) and adults (14). The rapid decrease in EGP observed after 19 hrs of fasting in patient 1 resulted in a decrease in plasma glucose concentration. This decrease in EGP was due to a decrease in GGL (Figure 1), suggesting glycogen depletion. This fits the absent glycemic response to glucagon administration at the time of hypoglycemia observed in a patient with HL deficiency (15). However, in healthy individuals, increasing plasma ketone concentrations reduce peripheral glucose utilization (16) by decreasing glucose oxidation via the inhibition of pyruvate dehydrogenase (EC 1.2.4.1) and phosphofructokinase (EC 2.7.1.11) (17). In HL deficiency ketone bodies are not synthesized. Therefore, limitation of peripheral glucose oxidation via inhibition of these enzymes will not occur. In addition, ketone bodies are not available as an alternate substrate source for energy production. Both mechanisms will result in sustained glucose utilization, leading to a more rapid depletion of glycogen. Indeed, the dissociation between GU and EGP observed in patient 1 after 19 hrs of fasting demonstrated that peripheral glucose demand could not be balanced by EGP, revealing the importance of ketone bodies for glycemic control in vivo.

In patient 2, glucose requirement per kg bodyweight was significantly lower as a result of his advanced age (6;12). As GU equalled EGP during the fasting period, EGP could be maintained at a sufficient rate to meet glucose demand (Figure 2).

It has been suggested that fasting hypoglycemia in HL may result from inhibition of gluconeogenesis by accumulating HMG-CoA, impairing gluconeogenesis through sequestration of CoA (1). The ensuing low intra-mitochondrial CoA levels may subsequently reduce the activity of the acetyl-CoA-dependent pyruvate carboxylase (EC 6.4.1.1), a key enzyme in gluconeogenesis (18). In addition, the increased acyl-CoA:CoA ratio might decrease the activity of other CoA-dependent enzymes involved in gluconeogenesis (19). However, we demonstrated that GNG was not inhibited and remained approximately constant during fasting, whereas HMG-CoA was indeed expected to accumulate, as illustrated by the continuous increase of 3-hydroxyisovaleryl carnitine and 3-methylglutaryl carnitine in plasma (Table 2).

In conclusion, we demonstrate that fasting hypoglycemia in HL is caused by the inability to reduce glucose utilization during fasting and not by the inhibition of gluconeogenesis as a result of HMG-CoA accumulation. Peripheral glucose utilization is maintained due to the absence of ketone bodies as an alternate substrate for energy production and due to the lack of the inhibitory effect of ketone bodies on enzymes in the glycolytic pathway. This results in more rapid depletion of glycogen and a failure to sustain an adequate EGP during fasting.
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References

