Studies on the role of glycosphingolipids in metabolism
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Chapter 4

Type I Gaucher Disease
A Glycosphingolipid Storage Disorder
Is Associated with Insulin Resistance

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Abstract

Objectives and background
Complex glycosphingolipids, in majority GM3, surround the insulin receptor in a special membrane compartment (raft) and modulate signaling through this receptor. Increased levels of GM3 in rafts impair insulin signaling, resulting in insulin resistance. Gaucher disease is a lysosomal storage disorder in which impaired breakdown of glucosylceramide leads to its accumulation in macrophages. Secondary to this defect GM3 concentrations, for which glucosylceramide is the precursor, in plasma and several cell types are elevated. We studied the influence of glycosphingolipid storage on whole body glucose and fat metabolism, by measuring insulin mediated (IMGU) and non-insulin mediated glucose uptake (NIMGU) and suppression of free fatty acids (FFA) by insulin.

Methods
We studied six Gaucher patients, either naive to treatment or with considerable remaining burden of disease and six matched healthy control subjects in the basal state, during an euglycemic and a hyperglycemic clamp with somatostatin measuring NIMGU and during an euglycemic hyperinsulinemic clamp measuring IMGU, using stable isotopes.

Results
NIMGU (both during eu- and hyperglycemia) did not differ between patients and control subjects. IMGU was lower in Gaucher patients, compared to controls. Suppression of lipolysis by insulin tended to be less effective in Gaucher patients.

Conclusions
Gaucher disease, a lysosomal glycosphingolipid storage disorder, is associated with (peripheral) insulin resistance, possibly through the influence of glycosphingolipids on insulin receptor functioning.
Introduction

In obesity induced insulin resistance, high plasma levels of free fatty acids (FFA) are causally linked to the impaired insulin signaling. One of the most abundant FFA, palmitate is a precursor for the sphingolipid ceramide. Ceramide is elevated in skeletal muscle of obese insulin resistant individuals and is negatively correlated with whole body insulin sensitivity. This effect is probably mediated by interference of cellular ceramide with insulin signal transduction at the level of Akt/PKB. In contrast, short term elevation of plasma FFA induces insulin resistance, but does not affect ceramide concentration. Conflicting data on increased levels of skeletal muscle ceramide in animal models of insulin resistance are reported. Ceramide is a precursor for more complex glycosphingolipids such as GM3 (5-acetyl-alpha-neuraminic acid(2-3)beta-D-galactopyranose(1-4)beta-D-glucopyranose(1-1)ceramide) which has been shown to negatively influence insulin sensitivity. Glycosphingolipids are complex molecules that are found in specialized membrane domains (rafts) where they influence signaling of receptors imbedded in these domains. The insulin receptor is one of the raft localized receptors. Its signaling is influenced by the glycosphingolipid composition of the raft. Especially the ganglioside GM3 present in these rafts appears to influence insulin receptor signaling, since GM3 synthase knockout mice display higher insulin sensitivity and are protected from high fat diet induced insulin resistance. Moreover, in 3T3-L1 adipocytes TNFα-induced insulin resistance is accompanied by increased GM3 in rafts, leading to exclusion of the insulin receptor from these rafts, resulting in impaired signaling. Depletion of GM3 restores insulin signaling in these cells. In mouse models of obesity-induced insulin resistance pharmacological inhibition of conversion of ceramide to glycosphingolipids improves insulin sensitivity.

Type I Gaucher disease is an autosomal recessive inherited glycosphingolipid storage disorder. It is caused by mutations in the gene encoding for the enzyme glucocerebrosidase, which degrades glucosylceramide into glucose and ceramide. This results in accumulation of glucosylceramide in lysosomes of macrophages which reside mainly in liver, spleen and bone marrow, leading to hepatosplenomegaly, cytopenia and skeletal complications. It can be successfully treated by Enzyme Replacement Therapy (ERT, Cerezyme, Genzyme, MA) in which a recombinant form of the deficient enzyme is administered intravenously, resulting in clearance of the storage material from Gaucher macrophages.
Plasma levels of glucosylceramide and GM3 are elevated in Gaucher disease. Though lysosomal storage only occurs in macrophages, there is evidence that the increased availability of glucosylceramide leads to an enhanced production of gangliosides. Increased synthesis of gangliosides, among them GM3, was found in cultured fibroblasts from Gaucher patients and increased levels of the ganglioside GM3 were noted in spleen and liver autopsy specimens from a small number of Gaucher patients. Whether ganglioside levels are also elevated in muscle and fat tissue of Gaucher patients is unknown.

Type I Gaucher disease is associated with abnormalities in whole body glucose metabolism. Earlier studies showed that this condition leads to higher endogenous glucose output, but similar plasma glucose concentrations. This may be explained by higher glucose uptake by large numbers of metabolically highly active macrophages. Glucose uptake via the glucose transporters GLUT 1 and GLUT 3 in macrophages is non-insulin mediated and seems to depend on the activation status of the macrophages since activation (by LPS, fMLP or PMA) leads to an increase in GLUT 1 and/or 3 expression.

We hypothesized that these earlier findings in Gaucher patients are caused by increased glucose utilization by activated macrophages. The presence of several kilograms of activated Gaucher cells could then be reflected in an increased non-insulin mediated glucose uptake. Furthermore, since glycosphingolipids may be involved in the induction of insulin resistance and the primary genetic defect in Gaucher disease leads to increased levels of these molecules, Gaucher patients constitute a human model to study the relationship between glucose metabolism and glycosphingolipids. We hypothesized that these patients are insulin resistant compared to matched controls.

**Patients and methods**

**Subjects**

Six type I Gaucher disease patients and six control subjects (all male) were studied. The control subjects were matched for age and BMI, were healthy except for overweight in some subjects and did not use any medication. Gaucher disease severity can be assessed using the Severity Scoring Index (SSI) which takes into account cytopenia, hepatosplenomegaly and bone complications. Using this score, type I Gaucher disease can be classified as mild.
(SSI 4-12), moderate (SSI 13-17) or severe (SSI >17). Since the introduction of ERT in 1991, severely affected, untreated patients are no longer available for study. We selected from our cohort those patients with the highest burden of disease based on SSI, excess liver and spleen volumes and chitotriosidase and CCL18 activity (both plasma markers for Gaucher cell burden). This resulted in the inclusion of three treatment naive patients and three severely affected patients that received ERT, but had suboptimal treatment response. All patients had stable disease (no recent infections or bone complications). They did not use any medication known to influence glucose metabolism and there was no significant co-morbidity. The study was approved by the Medical Ethical Committee of the Academic Medical Center in Amsterdam and written informed consent was obtained from all participants.

**Body composition**

Lean body mass (LBM) and total fat mass were determined using dual energy x-ray absorptiometry (model QDR 4500 W; Hologic, Waltham, MA).

**Glucose metabolism**

The study consisted of a basal measurement in the postabsorptive state and three clamps, an euglycaemic clamp during somatostatin infusion (blocking pancreatic insulin secretion, studying non-insulin mediated glucose uptake), a hyperglycemic clamp during somatostatin infusion and an euglycaemic clamp during somatostatin and insulin infusion (studying insulin mediated glucose uptake) (figure 1). Subjects consumed at least 250 g of carbohydrates during 3 days before the study and were asked to refrain from vigorous exercise. They were admitted to the metabolic research unit at 07.30h after fasting from 20.00h the previous day. They were allowed to drink water only. Subjects were studied in supine position. One catheter was inserted into an antecubital vein of the left arm to infuse stable isotopes, glucose, insulin and somatostatin and one catheter was inserted into a contralateral hand vein. The right hand was kept in a thermo-regulated (600°C) plexiglas box for sampling of arterialized venous blood.

At T = 0:00 h (08.00h), blood samples were drawn for determination of background enrichments and a primed (8.8 μmol/kg) continuous (0.11 μmol/kg*min) infusion of [6,6-2H2] glucose (>99% enriched; Cambridge Isotope Laboratories, Cambridge, MA) was started and continued until the end of the study. After an equilibration period of two hours, 3
blood samples were drawn for the measurement of glucose enrichments and 1 for glucose, glucoregulatory hormones, free fatty acids (FFA), glycolipids and chitotriosidase activity measurements. At T = 2:30 h (10.30h), a continuous infusion of somatostatin (UCB Pharma, Breda, the Netherlands) was started at a rate of 550 µg/h and continued until the end of the study. The plasma glucose concentration was determined every 5 minutes (glucose analyzer 2; Beckman, Palo Alto, CA) and euglycemia (5.0 mmol/L) was maintained by a variable infusion of a 10% (w/v) glucose solution. [6,6-2H2]glucose was added to the 10% glucose solution to achieve glucose enrichments of 1% to approximate the values for enrichment reached in plasma and thereby minimizing changes in isotopic enrichment due to changes in the infusion rate of exogenous glucose. After 145 minutes of somatostatin infusion, five plasma samples at 5 minutes-intervals were drawn for measurements of glucose enrichments and one to determine plasma levels of glucoregulatory hormones and FFA. At T=5:10h (13.10h) the hyperglycemic clamp started by increasing the 10% glucose infusion to reach a plasma glucose concentration of approximately 11 mmol/L. At T = 6:55 h (14.55h) 5 samples at 5
minutes-intervals were drawn for measurements of isotopic enrichment of plasma glucose and one for above mentioned parameters. At T=7:10h (15.10 h) the hyperinsulinemic euglycemic clamp started by infusing insulin (Actrapid 100 IU/ml; Novo Nordisk Farma bv, Zoeterwoude, The Netherlands) at a rate of 60 mU/m2 body surface area per min and a variable infusion of the enriched glucose 10% solution to maintain the plasma glucose concentration at 5 mmol/L. In Gaucher patients this insulin infusion rate resulted in remarkably variable plasma insulin concentrations (expected insulin concentration: approximately 600 pmol/L, measured insulin concentration in Gaucher patients: median 501, range 340-735 pmol/L). The insulin infusion rate in the matched control subjects were therefore adjusted to the insulin levels of the patients, to reach comparable plasma insulin levels and be able to compare the glucose disposal rates. At T=8:55 h (16.55h), five plasma samples were drawn at 5 minutes-intervals for measurements of isotopic enrichments of glucose and one for above mentioned parameters. Thereafter, the insulin infusion was stopped and subjects were offered a carbohydrate rich meal.

Gas chromatography-mass spectrometry
Plasma samples for glucose enrichment of [6,6-2H2]glucose were determined as described earlier\(^\text{25}\). Briefly, aldonitril pentaacetate derivative of glucose was injected into a gas chromatograph mass spectrometer system. Separation was achieved on a DB17 column (30 m x 0.25 mm, df 0.25 µm; J&W Scientific, Folsom, CA). Glucose concentrations were determined by using gas chromatography, using xylose as an internal standard. Glucose was monitored at mass-to-charge ratios of 187, 188, and 189. The enrichment of [6,6-2H2]glucose (TTR) was determined by dividing the peak area of m/z 189 by the peak area of m/z 187 and correcting for natural enrichments.

Measurements of hormones, cytokines, FFA and glycolipids
Plasma insulin and cortisol concentrations were measured on an Immulite 2000 system (DPC, Los Angeles, CA). Insulin was determined with a chemiluminiscent immunometric assay, detection limit 15 pmol/L. Cortisol was determined with a chemiluminiscent immunoassay detection limit 50 nmol/L. Glucagon was determined by RIA (Linco Research, St Charles, MO), detection limit 15 ng/L. Catecholamines were measured by an in-house high-performance liquid chromatography method, norepinephrine detection limit 0.05
nmol/L and epinephrine detection limit 0.05 nmol/L. Plasma FFAs were measured by an enzymatic method (NEFAC, Wako Chemicals, Neuss, Germany), detection limit 0.02 mmol/L. Ceramide and glucosylceramide in plasma were measured with a high performance liquid chromatography method as described previously. The limit of quantification was 2 pmol. GM3 was detected by analysis of the upper phase of the Folch extraction. The upper phase was desalted on a C18 Sep-Pak (Bakerbond) column as described by Kundu. Gangliosides were eluted in methanol and quantified after the digestion with ceramide glycanase (recombinant endoglycoceramidase II, Takara Bio Inc., Otsu, Shiga, Japan). Released oligosaccharides were labeled at their reducing end with the fluorescent compound anthranilic acid (2-aminobenzoic acid), prior to analysis using normal-phase high-performance liquid chromatography. Throughout the procedure monosialoganglioside-GM1 (Sigma, St Louis, Mo, USA) was used as an internal standard. The limit of quantification was 5 pmol.

**Calculations and statistics**

Endogenous glucose production (EGP) and peripheral glucose disposal (Rd) were calculated using the modified form of the Steele equations

\[
EGP = \frac{TTR_{tracer} \times I_{tracer}}{TTR_{plasma}}
\]

and

\[
Rd(t) = \frac{TTR_{tracer} \times I_{tracer}}{TTR_{plasma}(t)} + \frac{TTT_{exogenous} \times I_{exogenous}}{TTR_{plasma}(t)} - pV \times \frac{C_{org}(t)}{TTR_{plasma}} \frac{dTTR_{plasma}(t)}{dt} - pV \times \frac{dC_{org}(t)}{dt}
\]

in which TTR is tracer trace ratio, I is infusion (umol/kg.min), pV is plasma dilution volume (40 ml/kg), C is concentration (mmol/L) and dCong(t)/dt the change of tracee concentration in time (in mmol/L*min).

**Statistical analysis**

All data are presented as median and range [minimum-maximum]. A Wilcoxon signed rank test was performed to detect differences between Gaucher patients and control subjects. There was a large spread in BMI (20-29 kg/m²) in the studied patients. Body composition is known to determine the variability in insulin sensitivity by more than 20% and compari-
son therefore has to be made between patients and healthy control subjects with the same body composition by using paired analysis, pairs being based on BMI. A P-value of <0.05 was considered statistically significant. A P-value of <0.10 was considered indicative of a trend.

Results

Subject characteristics
As shown in table 1, there was no difference in anthropometric variables between patients and controls.

Basal state
FFA and cortisol levels did not differ between patients and control subjects. Plasma glucose levels tended to be lower in patients. Basal endogenous glucose production did not differ between patients and controls (table 1). There was a trend towards lower plasma ceramide concentrations in Gaucher patients compared to control subjects (median 6.4 [range 5.3-8.0] vs. 8.3 [range 7.2-15.4] µmol/L, p=0.075). Plasma glucosylceramide (median 13.0 [range 8.8-16.6] vs. 6.1 [range 4.3-8.5] µmol/L, p=0.028) and GM3 levels (median 7.6 [range 5.4-12.0] vs. 4.9 [range 4.0-7.0] nmol/L, p=0.027) were elevated in patients compared to controls (figure 2).

NIMGU during euglycemia
Plasma glucose levels did not differ between patients and controls. During somatostatin infusion, plasma insulin levels were completely suppressed. Non-insulin mediated glucose uptake during euglycemia was comparable between patients and control subjects (table 2).

NIMGU during hyperglycemia
Plasma glucose levels were comparable in Gaucher patients and control subjects. In two control subjects and one patient, the insulin levels were not completely suppressed by somatostatin, but this did not result in hyperinsulinemia and there was no significant difference in insulin concentrations between patients and controls. NIMGU at hyperglycemia did not
Table 1  Basal characteristics of Gaucher patients and healthy control subjects. Data are presented as median [minimum-maximum]. BMI=Body Mass Index, FFA=Free Fatty Acid, lbm= lean body mass, EGP= Endogenous Glucose Production.

<table>
<thead>
<tr>
<th></th>
<th>Gaucher patients (n=6)</th>
<th>Control subjects (n=6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>37 [25-49]</td>
<td>35 [26-41]</td>
<td>0.225</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 [20-29]</td>
<td>24 [19-30]</td>
<td>1.000</td>
</tr>
<tr>
<td>Fat mass (%)</td>
<td>24 [10-29]</td>
<td>19 [11-27]</td>
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</tr>
<tr>
<td>SSI</td>
<td>5 [3-14]</td>
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<td></td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.3 [4.8-5.5]</td>
<td>5.5 [4.9-5.8]</td>
<td>0.084</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>63 [40-97]</td>
<td>53 [19-87]</td>
<td>0.173</td>
</tr>
<tr>
<td>FFA (mmol/L)</td>
<td>0.36 [0.25-0.68]</td>
<td>0.36 [0.23-0.46]</td>
<td>0.345</td>
</tr>
<tr>
<td>Cortisol (nmol/L)</td>
<td>217 [152-365]</td>
<td>265 [64-361]</td>
<td>0.753</td>
</tr>
<tr>
<td>EGP (µmol/kg*min)</td>
<td>12.3 [8.5-15.0]</td>
<td>12.6 [10.7-14.7]</td>
<td>0.753</td>
</tr>
</tbody>
</table>

Figure 2  Basal study. Dot plots of ceramide (median 6.4 [range 5.3-8.0] vs. 8.3 [range 7.2-15.4] µmol/L) (A), glucosylceramide (median 13.0 [range 8.8-16.6] vs. 6.1 [range 4.3-8.5] µmol/L) (B) and GM3 (median 7.6 [range 5.4-12.0] vs. 4.9 [range 4.0-7.0] nmol/L) (C) plasma concentrations in Gaucher patients and control subjects. In all dot plots the bar represents the median.
Table 2  NIMGU during euglycemia, hyperglycemia and IMGU during euglycemia. Data are presented as median [minimum-maximum]. NIMGU= Non-Insulin Mediated Glucose Uptake, IMGU= Insulin Mediated Glucose Uptake, Rd= rate of disappearance.

differ between Gaucher patients and control subjects (table 2).

**IMGU during euglycemia**
In one of the Gaucher patients, we did not manage to achieve euglycemia within the time limit of the clamp. The duration of the exposure to insulin before reaching euglycemia in this patient was 210 minutes compared to approximately 125 minutes in the matched control subject and all other patients and control subjects. Since the effect of insulin on glucose uptake is enhanced by longer insulin exposure, resulting in a final Rd of 37.2 µmol/kg*min in the patient, we decided to exclude this pair from the results. All following results from the hyperinsulinemic clamp were obtained in 5 matching pairs.

Plasma glucose levels were not statistically different between groups. Plasma insulin concentrations were comparable between groups. Insulin-mediated glucose uptake was significantly lower in the Gaucher patients compared to the control subjects (table 2 and figure 3). Endogenous glucose production was suppressed by insulin to comparable levels in Gaucher patients and controls (2.8 µmol/kg*min [0.22-4.7] vs. 1.3 µmol/kg*min [1.2-5.4], p=0.893). Insulin-mediated suppression of lipolysis, as reflected by the plasma FFA concentrations, showed a trend towards less suppression in the patients compared to controls (0.03 mmol/L [0.02-0.08] vs. 0.02 mmol/L [0.00-0.04], p=0.066).
Glycosphingolipid metabolism influences insulin sensitivity\textsuperscript{6,13}. Since the primary genetic defect in Gaucher disease results in elevated glycosphingolipid concentrations, it constitutes a human model for the study of the relationship between altered glycosphingolipid levels and glucose metabolism. Therefore, we studied glucose metabolism in patients with Gaucher disease in a case-control setting.

We could not reproduce the earlier observation of an increased basal hepatic glucose output in Gaucher patients\textsuperscript{20}. This discrepancy can be explained by a difference in study population. In comparison to the earlier study\textsuperscript{20}, our patients had less severe disease, measured by SSI (median 5 [range 3-14] in the current study versus 7 [5-16] in the previous study), excess liver (median 277 ml [0-1237] versus 1401 ml [711-3514]) and excess spleen volume (median 1130 ml [993-3650] versus 2452 ml [894-3178]). This selection bias was inevitable, since introduction of ERT has led to prompt treatment of patients with severe Gaucher disease to prevent complications.

In an earlier study, low dose ERT did not lead to a decrease in EGP after six months\textsuperscript{31}, but it is likely that long term, higher dosed treatment may have influenced the outcome of the EGP in the current study. Accumulation of glucosylceramide within macrophages results in activation of these cells and hypothetically in increased glucose utilization. Macrophages do not express the glucose transporter GLUT 4 and their glucose uptake is therefore entirely

\begin{figure}[h]
\centering
\includegraphics[width=0.4\textwidth]{figure3.png}
\caption{Hyperinsulinemic clamp. Dot plot of insulin mediated glucose uptake as measured by hyperinsulinemic euglycaemic clamp. Each matched pair (Gaucher patient and control subject) is depicted by a different symbol.}
\end{figure}
non-insulin mediated. In-vitro studies showed that activation of macrophages (by phagocytosis or other stimuli) leads to an increased expression of the GLUT 1 and/or GLUT 3 protein resulting in an increased glucose uptake\textsuperscript{21,22,33,34}. Therefore, we postulated that the presence of a large amount of activated macrophages in liver, spleen and bone marrow would lead to an increase in whole body non-insulin mediated glucose uptake (NIMGU) in Gaucher patients. This might compensate for an assumed lower glucose uptake in skeletal muscle due to insulin resistance induced by accumulation of glycolipids. However, measurements of NIMGU during euglycemia and hyperglycemia did not show an increase in NIMGU in Gaucher patients with moderate disease severity compared to controls. Whether NIMGU is elevated in more severely affected Gaucher disease patients, with a higher burden of activated macrophages, remains to be established.

We found lower peripheral insulin sensitivity in our patients compared to matched control subjects. The difference in insulin-mediated peripheral glucose uptake might be explained by increased glycosphingolipid (GM3) levels in Gaucher patients since lowering of glycolipid levels with an experimental glucosylceramide synthase inhibitor results in improved insulin sensitivity in several animal models of insulin resistance\textsuperscript{6}. However, whether elevated plasma levels of GM3 and glucosylceramide as found in our patients, also lead to increased levels of GM3 within the lipid rafts of myocytes and adipocytes, which account for the majority of insulin-mediated glucose uptake, is not known. In a mouse model of Niemann Pick type C disease (a lysosomal glycosphingolipid and cholesterol storage disorder), altered lipid composition of the rafts surrounding the insulin receptor was shown to be associated with insulin resistance in hepatocytes\textsuperscript{35}. Alternatively, the difference in peripheral insulin sensitivity might hypothetically be explained by differences in systemic inflammation in our patients reflected by higher plasma TNF-α levels, which may result in disturbed insulin signaling in adipocytes and myocytes. However, this possibility is less likely, as several earlier studies in type I Gaucher disease patients have failed to show significantly elevated TNF-α levels\textsuperscript{36,37}.

In conclusion, patients with type I Gaucher disease display peripheral insulin resistance compared to matched control subjects. Since GM3 has shown to be involved in insulin resistance, this may be caused by accumulation of GM3 in lipid rafts of myocytes and adipocytes, resulting in disturbed insulin signaling. The exact mechanism remains to be clarified.
References


29. Steele R Influences of glucose loading and of injected insulin on hepatic glucose output. Ann NY Acad Sci


