Interactions between hepatic glucose and fat metabolism in animal models of insulin resistance
Wiegman, Cornelis Harm

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High-fat feeding in rats abolishes the ability of insulin to suppress *de novo* glucose-6-phosphate synthesis

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* CH Wiegman and TH van Dijk contributed equally to this study

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ABSTRACT

A Western type high-fat diet predisposes to type 2 diabetes. We studied the effects of dietary fat content on hepatic glucose metabolism in Wistar rats fed a low-fat (LF) or a high-fat (HF) diet for 3 weeks. Pathways of glucose metabolism were quantified using [U-\textsuperscript{13}C]-glucose, [2-\textsuperscript{13}C]-glycerol and [1-\textsuperscript{2}H]-galactose, applying mass isotopomer distribution analysis on urinary paracetamol-glucuronide and plasma glucose, during postprandial (basal) and during euglycemic (5.3±0.5 mM), hyperinsulinemic (829±12 pM) clamp conditions. Enzyme activities and mRNA levels of glucose-6-phosphatase hydrolase (\textit{G6ph}) and glucokinase (\textit{Gk}) were measured. HF feeding did not affect basal plasma glucose concentration, although basal insulin concentrations were 2-fold increased. HF feeding decreased glucose disposal under clamp conditions (159±2 vs. 186±2 µmol/kg/min, respectively, p<0.05). Insulin suppressed endogenous glucose production in LF rats only by suppressing \textit{de novo} synthesis of G6P (39±2 vs. 24±1 µmol/kg/min, basal vs. clamp, p<0.05), whereas no effect of insulin was observed in HF rats (38±3 vs. 35±1 µmol/kg/min, basal vs. clamp, NS). The enzyme activity of G6Pase was not affected, while GK activity was decreased by HF feeding, associated with a 31% reduction in G6P content under basal conditions. Counterintuitively, mRNA levels of \textit{G6ph} were decreased, whereas those of \textit{Gk} were increased. In conclusion, HF feeding in rats strongly affects G6P metabolism and induces hepatic insulin resistance particularly with the respect to \textit{de novo} synthesis of G6P.
INTRODUCTION

A Western type diet, high in energy and fat content, is associated with insulin resistance and related disorders like obesity and type 2 diabetes. The exact etiology of insulin resistance is not clear. Genetic predisposition and environmental factors, including quality and quantity of dietary fat, both contribute to development of an inability to adequately dispose plasma glucose at normal plasma insulin levels.

During insulin resistance, insulin is unable to appropriately suppress hepatic glucose production. Together with a decreased plasma glucose clearance by liver and peripheral tissues, this will ultimately result in hyperglycemia. An increased contribution of gluconeogenesis (GNG) to total glucose production and a normal glycogen flux has been reported in type 2 diabetes patients (1). Several other studies, however, did not confirm the existence of enhanced GNG in diabetes (2,3). Various methodological reasons may explain the variability of the reported results, relating to the isotopic methods applied as well as to the subjects studied. The measured rate of partitioning of newly synthesized glucose-6-phosphate (G6P) into glucose and liver glycogen might vary, depending on the particular experimental design and thereby uncouple changes in de novo synthesis of G6P from changes in GNG. This partitioning depends on the relative enzyme activities of glucose-6-phosphatase (G6Pase) and glucokinase (GK) on the one hand and glycogen synthase (GS) and glycogen phosphorylase (GP) on the other hand.

The ratio of enzyme activities of hepatic glucose-6-phosphatase over glucokinase (G6Pase/GK-ratio) is increased in type 2 diabetes (4,5). During insulin resistance, insulin might not be able to reduce this ratio, as it normally does. Both an increased G6Pase activity and/or a decreased GK activity result in a net increase in endogenous glucose production (4). Overexpression of the G6Pase catalytic subunit in rat liver in vivo leads to a mild diabetic phenotype with hyperinsulinemia, hyperglycemia, glucose intolerance and a decreased hepatic glycogen content (6). In contrast, inhibition of the G6Pase catalytic subunit leads to the reversed condition; hypoinsulinemia, hypoglycemia, decreased hepatic glucose production and increased hepatic G6P glycogen contents (7). Adenovirus-mediated overexpression of GK in diet-induced diabetic mice normalized plasma glucose and insulin levels (8). In addition, GK regulatory protein (GKRP) knockout mice show decreased hepatic GK activity and protein levels and are hyperglycemic and hyperinsulinemic (9).

Increased de novo synthesis of G6P might take place since activity and gene expression levels of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in the de novo synthesis of G6P, is increased in models of diabetes (10-13). Overexpression of PEPCK in mice leads to increased fasting plasma glucose levels, decreased glycogen synthesis and an altered response during a glucose tolerance test (14).

High-fat feeding in rats results in hepatic and muscle insulin resistance. The time course and severity of insulin resistance depends on the dietary fatty acid composition, the amount of fat used and the duration of high-fat feeding (15,16). We used this animal model of insulin resistance to study the effects of high-fat feeding on hepatic carbohydrate metabolism. Specifically, we addressed the following questions: 1) Does diet-induced insulin resistance decrease total plasma glucose turnover, 2) is insulin able to decrease de novo G6P synthesis in diet-induced insulin resistance, 3) are the enzyme activities of G6Pase and GK affected by diet-induced insulin resistance and 4) are hepatic expression levels of genes involved in glucose metabolism affected by high-fat feeding? Glucose metabolism was studied by [U-13C]-glucose, [2,13C]-glycerol and [1-2H]-galactose, applying mass isotopomer distribution analysis on urinary paracetamol-glucuronide and plasma glucose during basal and hyperinsulinemic euglycemic clamping. Metabolic fluxes were compared to enzyme activities of G6Pase and GK and hepatic gene expression levels of key enzymes of glucose metabolism.
MATERIALS AND METHODS

Diets. Low- and high-fat semi-purified diets were purchased from Hope Farms (Woerden, The Netherlands). Total fat and carbohydrate content was 8 wt% and 60 wt%, respectively, in the low-fat diet (4148.01), whereas the high-fat diet (4148.02) contained 25 wt% fat and 25 wt% carbohydrate. The high-fat diet contained more palmitate (C16:0, 29.13 g/kg vs. 91.12 g/kg) and oleate (C18:1, 32.08 g/kg vs. 100.24 g/kg) compared to the low-fat diet. Other nutritional components were comparable between the two diets. The protein content was slightly lower in the low-fat diet, but due to a higher daily intake (20 ± 1 vs. 17 ± 1 g/day in low-fat and high-fat diet, respectively, mean ± SEM), total protein intake was similar between the groups.

Animals. Male Wistar (WU) rats (n=24) weighing between 300-350 gram (Harlan Laboratories, Zeist, The Netherlands) were fed either the high-fat diet (n=12) or low-fat diet (n=12). After two weeks on either diet, rats (n=6) of each diet-group were equipped with two permanent catheters in the right jugular vein to allow infusion and blood collection in unrestrained conditions (17). After surgery, rats recovered for a period of 7 days in individual cages in a light- (8 am-8 pm), and temperature- (20 ºC) controlled room with the appropriate diet and water available at libitum. Food intake returned to preoperative levels within two days. Experimental procedures were approved by the Ethical Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen.

In vivo experiments. To study the effects of high-fat feeding on hepatic carbohydrate fluxes at basal and hyperinsulinemic euglycemic conditions two sets of experiments were carried out. After a fasting period of 9 hours rats were placed individually in metabolic cages. To study hepatic carbohydrate fluxes under basal conditions rats received an intravenous infusion containing paracetamol (0.44±0.01 mg/kg/min, total dose 213±3 mg/kg), [U-13C6]-glucose (0.99±0.02 μmol/kg/min), [2-13C]-glycerol (9.6±0.2 μmol/kg/min) and [1-1H]-galactose (4.9±0.1 μmol/kg/min). The total infusion time was 8 hours and the infusion rate was 3 ml/hr.

To study hepatic carbohydrate fluxes during hyperinsulinemic euglycemic conditions rats underwent a comparable infusion with the addition of insulin (5.5 nmol/kg/min; Novo Nordisk, Bagsvaerd, Denmark) and somatostatin (1.56 μg/kg/min; UCB, Breda, The Netherlands). The [2-13C]-glycerol infusion was increased to 13.9±0.2 μmol/kg/min to compensate for the expected additional dilution by infused glucose for the mass isotopomers of glucose. The solution containing the hormones and isotopes was infused at a rate of 1.5 ml/hr. A second infusion was used to maintain normal blood glucose concentrations containing 1.67 M (30% w/v) D-glucose (Merck, Darmstadt, Germany), the rate of which varied between 1.1 and 1.6 ml/hr. All solutions were freshly prepared in saline containing 1.5% bovine serum albumin (BSA, Sigma, St. Louis, MO). Blood glucose concentrations were determined every 15 minutes with a GlucoTouch-glucose analyzer (LifeScan, Beerse, Belgium). In both studies blood samples were collected by tail bleeding. A drop of blood was collected on paper (bloodspots) before and during the infusion at 4, 5, 6, 7 and 8 hours after the start of the experiment. Bloodspots were stored at room temperature until analysis. Urine samples were also collected at hourly intervals and were stored at -20 ºC until analysis. At the end of the experiment, rats were anesthetized with pentobarbital; a large blood sample was taken by heart puncture and liver was excised and immediately frozen in parts in liquid nitrogen.

Metabolite isolation and derivatisation procedures. Isolation of glucose from bloodspots - On the day of analysis, a disk (6 mm in diameter) was punched out of a bloodspot. The disk
was transferred to an Eppendorf reaction vial and wetted with 40 µl water. Finally 400 µl ethanol was added to the disk and the solution was shaken for 45 minutes and then centrifuged. The supernatant was split into two fractions.

**Isolation of glucose from plasma** - On the day of analysis, 40 µl of plasma was deproteinized by adding 400 µl ice-cold ethanol. The mixture was placed on ice for 30 minutes and then centrifuged. The supernatant was split into two fractions.

**Isolation of Par-GlcUA from urine** - Paracetamol-GlcUA was isolated from urine samples by HPLC as described previously (7). The fraction containing Par-GlcUA was split into two fractions.

**Derivatisation** – Derivatisation was done as previously described (7). One fraction of either plasma or bloodspot was used to derivatise glucose to its pentaacetate derivative by reaction with acetic anhydride in pyridine. The second fraction was used to convert glucose into its aldonitril pentaacetate derivative by reaction with hydroxylamin in pyridine followed by reaction with acetic anhydride in pyridine. One fraction of urine extract containing paracetamol-GlcUA was converted into its diethyl-tetraTMS derivative by reaction with ethanol/acyetylchloride mixture followed by reaction with BSTFA/pyridine/TCMS. The other fraction was converted into the saccharic acid diethyl-tetraacetate derivative by reaction of Par-glcUA with sodium nitrite in nitric acid to saccharic acid which was subsequently converted into its diethyl-tetraacetate derivative by reaction with a mixture of ethanol/acyetylchloride. After reactions all derivatives were dried under nitrogen and after drying dissolved in ethylacetate prior to injection.

**GC-MS procedures.** All samples were analyzed by gas chromatography-mass spectrometry. Derivatives were separated with a HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, US) using an AT-5MS 30 m x 0.25 mm ID (0.25 µm film thickness) capillary column (Alltech, Breda, the Netherlands). Derivatives were measured by chemical ionization with methane. The ions monitored for glucose pentaacetate, aldonitril pentaacetate and saccharic acid diethyl-tetraacetate were m/z 331-337, m/z 328-334 and m/z 375-381, respectively, corresponding to the m<sub>0</sub>-m<sub>6</sub> mass isotopomer. Paracetamol-GlcUA ethyl-tetra-TMS was measured by electron impact at m/z 331-337 ions, representing the m<sub>0</sub>-m<sub>6</sub> mass isotopomer. The accuracy of the measurement was checked by injection a standard sample after each set of samples from one individual. The series were rejected when the reproducibility was less then 2% for m<sub>1</sub> and m<sub>2</sub> and less then 1% for m<sub>0</sub>. To check the range of reproducible analysis, dilution series were routinely made.

**Calculations.** Blood glucose concentrations and glucose infusion rates during the hyperinsulinemic euglycemic experiment were calculated as mean values from minus 0.5 hour to plus 0.5 hour around the sample point for the calculation of the hepatic carbohydrate fluxes. The metabolic fluxes at steady state were calculated according to Hellerstein et al. (18) described by van Dijk et al. (7). The isotopic model allows calculation of the whole body glucose appearance and disappearance, endogenous glucose production, the GNG, the de novo synthesis of G6P and the fluxes through GK and G6Pase. The model does not allow the calculation of the rate of hepatic glycolysis, so that the flux through GK represents only a minimal estimate of that flux, i.e. the flux of plasma glucose into UDP-glucose.

**Total infusion rate and correction of the mole percent enrichment of the infusate.** During the hyperinsulinemic euglycemic clamp, naturally labelled glucose was infused in addition to [U-<sup>13</sup>C] labelled glucose. To account for the additional dilution of infused [U-<sup>13</sup>C]-glucose in the calculations of the isotopic fluxes the isotopic enrichment of the combined glucose infusion was calculated as follows:
MPE(glc;m_6,total)_infuse = MPE(glc;m_6)_infuse \times \text{infusion(glc;m_6)} / \text{infusion(glc;total)} \quad [\text{Eq.1}]

\text{with}

\text{infusion(glc;total)} = \text{infusion(glc;m_6)} + \text{infusion(glc;m_n)} \quad [\text{Eq.2}]

in which MPE(glc;m_6)_infuse is the mole percent of enrichment of [U-^{13}C]-glucose in the infusate of [U-^{13}C]-glucose, infusion(glc;total) is the total infusion rate of glucose, infusion(glc;m_6) is the infusion rate of uniformly labelled [U-^{13}C]-glucose, and infusion(glc;m_n) is the infusion rate of naturally labelled glucose.

Rates of appearance and disposal. In steady state the whole body rate of appearance of glucose in plasma (Ra(glc;whole body)) equals the total rate of glucose disposal (R_d(glc;whole body)) and was calculated by isotope dilution as follows:

Ra(glc;whole body) = R_d(glc;whole body) = MPE(glc;m_6,total)_infuse / (MPE(glc;m_6)_plasma \times \text{infusion(glc;tot)} \quad [\text{Eq.3}]

in which MPE(glc;m_6)_plasma is the mole percent enrichments of [U-^{13}C]-glucose in plasma.

Endogenous productions, recycling, and exchange rates. The rates of endogenous production of plasma glucose (Ra(glc;endo)) and UDPglc (Ra(UDPglc)), were calculated as follows:

Ra(glc;endo) = (MPE(glc;m_6,total)_infuse / (MPE(glc;m_6)_plasma – 1) \times \text{infusion(glc;total)} \quad [\text{Eq.4}]

and

Ra(UDPglc) = (MPE(gal;m_1)_infuse / (MPE(pGlcUA;m_1)_urine – 1) \times \text{infusion(gal;m_1)} \quad [\text{Eq.5}]

The contribution of recycling should be added to these rates of endogenous production to obtain the total rates of appearance (18,19). For the calculation two exchange factors are introduced: the fractional contribution of plasma glucose to UDP-glucose formation (c(glc)),

\text{c(glc)} = \text{MPE(pGlcUA;m_6)_urine} / \text{MPE(glc;m_6)_plasma} \quad [\text{Eq.6}]

in which MPE(pGlcUA;m_6)_urine and MPE(glc;m_6)_plasma are the mole percent enrichments of urinary p-GlcUA and plasma glucose, respectively, during an infusion of [U-^{13}C]-glucose and the fractional contribution of UDP-glucose to plasma glucose formation (c(UDPglc)),

\text{c(UDPglc)} = \text{MPE(glc;m_1)_plasma} / \text{MPE(pGlcUA;m_1)_urine} \quad [\text{Eq.7}]

in which MPE(glc;m_1)_plasma and MPE(pGlcUA;m_1)_urine are the mole percent enrichments of urinary p-GlcUA and plasma glucose, respectively, during an infusion of [1-^2H]-galactose.

Recycling of glucose (r(glc)) and UDP-glucose (r(UDPglc)) were calculated as follows,

r(glc) = (c(glc) / (1 – c(glc))) \times \text{Ra(glc;endo)} \quad [\text{Eq.8}]

which is also a measure of glucose/glc-6-P cycling, and

r(UDPglc) = (c(UDPglc) / (1 – c(UDPglc))) \times \text{Ra(UDPglc)} \quad [\text{Eq.9}]

in which MPE(pGlcUA;m_1)_urine and MPE(glc;m_1)_plasma are the mole percent enrichments of urinary p-GlcUA and plasma glucose, respectively, during an infusion of [1-^2H]-galactose.
Total rates of endogenous glucose production (totalRa(glc;endo)) and endogenous UDP-glucose production (total Ra(UDPglc)) were calculated according to,

\[
\text{total Ra(glc;endo)} = \text{Ra(glc;endo)} + r(\text{glc}) \quad \text{[Eq.10]}
\]

and

\[
\text{total Ra(UDPglc)} = \text{Ra(UDPglc)} + r(\text{UDPglc}) \quad \text{[Eq.11]}
\]

Rate of gluconeogenesis. The fractional gluconeogenic flux into both plasma glucose (f(glc)) and hepatic UDP-glucose (f(UDPglc)), measured in urinary p-GlcUA were calculated by mass isotopomer distribution analysis as described in detail elsewhere (20,21). The absolute gluconeogenic fluxes into plasma glucose (GNG(glc)) and into UDP-glucose (GNG(UDPglc)) was calculated according to:

\[
\text{GNG(glc)} = f(\text{glc}) \times (\text{totalRa(glc;endo)} + \text{infusion(glc;total)}) \quad \text{[Eq.12]}
\]

and

\[
\text{GNG(UDPglc)} = f(\text{UDPglc}) \times (\text{totalRa(UDPglc)} + \text{infusion(gal;m1)}) \quad \text{[Eq.13]}
\]

The total gluconeogenic flux (GNG\text{total}), which is equivalent to the de novo synthesis of G6P, is the sum of both GNG(glc) and GNG(UDPglc) corrected for the exchange of label between plasma glucose and hepatic UDP-glucose pools,

\[
\text{GNG\text{total}} = \text{GNG(glc)} \times (1 - c(\text{UDPglc})) + \text{GNG(UDPglc)} \times (1 - c(\text{glc})) \quad \text{[Eq.14]}
\]

Individual Fluxes through Enzymes. The individual fluxes through the enzymes GK and G6Pase were calculated according to the equation,

\[
\text{GK} = \text{glc(UDPglc)} + r(\text{glc}) \quad \text{[Eq.15]}
\]

which is a minimal estimate of the flux through GK since only two contributions to the total flux through GK are considered, i.e. the flux of plasma glucose into UDP-glucose and glucose/Glc-6-P cycling. The contribution of glycolysis is not assessable in this model.

\[
\text{G6Pase} = \text{total Ra(glc;endo)} \quad \text{[Eq.16]}
\]

Metabolic assays. Plasma glucose concentration was determined with a GlucoTouch-glucose analyzer (LifeScan, Beerse, Belgium). Plasma insulin was determined by a radioimmunoassay (RIA) RI-13K (Linco Research, Inc., St. Charles, MO). To determine hepatic glycogen and G6P levels, liver homogenates were diluted with 4 vol of ice-cold 0.9 % NaCl with 10 mM MOPS (pH 7.4) and centrifuged. After removal of the clear supernatant the pellets were dissolved in 0.1 M KOH and heated for 40 minutes at 85 °C. The solution was acidified to pH 4.5 with acetic acid (3 M) and centrifuged to remove proteins. To 100 µl of the supernatant 0.14 U amyloglucosidase was added and the mixture was incubated for 2 hr at 40 °C. The glucose formed was measured fluorometrically. Background glucose was measured in identically-treated samples, without addition of amyloglucosidase (22).
Enzyme activities. Glucokinase (GK) and glucose-6-phosphatase (G6Pase) enzyme activities were determined as described by Davidson et al. (23) and Stryker et al. (24), respectively.

Hepatic gene expression. Hepatic mRNA levels were quantified by real time PCR analysis during basal conditions. Total RNA was isolated from approximately 30 mg of liver tissue using Trizol-method (GIBCO, Paisley, United Kingdom) followed by the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the protocols provided by the manufacturer. Isolated total RNA was converted to single stranded cDNA by a reverse transcription procedure with M-Mulv-RT (Boehringer Mannheim, Mannheim, Germany) according to manufacturer’s protocol. cDNA levels were measured by real-time PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). For the PCR amplification studies, an amount of cDNA corresponding to 10 ng of total RNA was amplified using the qPCR core kit (Eurogentec, Seraing, Belgium) essentially according to manufacturer’s protocol and optimized for amplification of the particular gene using the appropriate forward and reverse primers (GIBCO, Paisley, UK) and a template specific 3'-TAMRA, 5'-6-FAM labeled Double Dye Oligonucleotide probe (Eurogentec, Seraing, Belgium). In the same experiments, calibration curves were run on serial dilutions of a 8x concentrated cDNA solution as used in the assay, resulting in a series containing 8x, 4x, 2x, 1x, 0.5x, 0.125x, 0.062x, and 0.031x of the cDNA present in the assay incubation. Both assay and calibration incubations were done simultaneously. During the amplification, the breakdown of the probe releases the fluorescent 6-FAM-dye, resulting in an increase in fluorescence. The fluorescence data obtained were processed using the software program ABI Sequence Detector v1.6.3 (System Applied Biosystems, Foster City, CA). All quantified expression levels were within the linear part of the calibration curves and calculated using these curves. The primer sequences used are listed in Table 1.

Table 1. List of primers and probes used.

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Statistics. All data described are mean ± standard error of mean (SEM) unless stated otherwise. Statistical significance was considered when p<0.05 using ONE WAY ANOVA with repeated measurements analysis.
RESULTS

High-fat diet fed rats maintain normal plasma glucose concentrations albeit at increased plasma insulin concentrations. After three weeks, body weights were similar between rats fed the low-fat diet and the high-fat diet (324.6±1.6 vs. 316.6±4.4 gr, respectively). Postprandial plasma insulin levels were more than 2 times higher in rats fed the high-fat diet (351±65 pM, n=6) compared to rats fed the low-fat diet (145±16 pM, n=6). However, plasma glucose concentrations were not different between both groups, indicating that more insulin was needed to maintain normal plasma glucose levels (Table 2). Rats fed the high-fat diet developed hepatic steatosis. Hepatic triglyceride and cholesteryl ester contents were increased ~2-fold and ~3-fold, respectively (Table 2). Free cholesterol and phospholipid contents were not different between rats fed the low-fat and high-fat diet. Hepatic glycogen and G6P concentrations were significantly decreased in rats fed the high-fat diet (Table 3).

Table 2. Basal plasma parameters and hepatic lipid composition after 3 weeks on diet, values are mean±SEM, n=6/group, * p<0.05, ONEWAY ANOVA with repeated measurements.

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<th>Plasma parameters</th>
<th>LF diet</th>
<th>HF diet</th>
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<tr>
<td>Glucose (mM)</td>
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<td>5.1±0.2</td>
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<tr>
<td>Insulin (pM)</td>
<td>145±16</td>
<td>351±65*</td>
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<td>Triglycerides (mM)</td>
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<td>Cholesterol (mM)</td>
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<td>Free fatty acids (mM)</td>
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<tbody>
<tr>
<td>Triglycerides (nmol/mg protein)</td>
<td>127±22</td>
<td>234±35*</td>
</tr>
<tr>
<td>Total cholesterol (nmol/mg protein)</td>
<td>55±5</td>
<td>75±9</td>
</tr>
<tr>
<td>Free cholesterol (nmol/mg protein)</td>
<td>48±2</td>
<td>47±6</td>
</tr>
<tr>
<td>Cholesteryl esters (nmol/mg protein)</td>
<td>9±3</td>
<td>30±6*</td>
</tr>
<tr>
<td>Phospholipids (nmol/mg protein)</td>
<td>231±3</td>
<td>234±7</td>
</tr>
</tbody>
</table>

Table 3. Hepatic glucose-6-phosphate and glycogen levels and enzyme activities of glucose-6-phosphatase and glucokinase under basal conditions after a 9 hr fast in rats fed a low-fat or high-fat diet for a period of 3 weeks, values are mean±SEM, n=6/group, *p<0.05, ONEWAY ANOVA with repeated measurements.

<table>
<thead>
<tr>
<th>Liver parameters</th>
<th>LF diet</th>
<th>HF diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate (μmol/g liver)</td>
<td>0.59±0.03</td>
<td>0.41±0.02*</td>
</tr>
<tr>
<td>Glycogen (μmol/g liver)</td>
<td>288±10</td>
<td>171±6*</td>
</tr>
<tr>
<td>G6Pase (nmol/min/mg protein)</td>
<td>142.4±9.06</td>
<td>129.7±6.84</td>
</tr>
<tr>
<td>Glucokinase (nmol/min/mg protein)</td>
<td>23.4±1.21</td>
<td>12.8±0.32*</td>
</tr>
<tr>
<td>G6Pase-GK (nmol/min/mg protein)</td>
<td>119.0±9.63</td>
<td>116.9±6.97</td>
</tr>
<tr>
<td>G6Pase/GK ratio</td>
<td>6.2±0.65</td>
<td>10.2±0.70*</td>
</tr>
</tbody>
</table>
High-fat feeding renders insulin less effective in stimulating whole body glucose disposal. Plasma insulin levels were clamped at high-physiologic concentrations of 825±143 and 833±10 pM in rats fed the low-fat and high-fat diet, respectively (Figure 1A). Plasma glucose concentrations were held constant by adjusting the unlabeled glucose infusion rate to maintain euglycemic plasma concentrations (5.1±0.1 vs. 5.7±0.5 mM, LF vs. HF, respectively, Figure 1C). Total glucose infusion rate, calculated according [Eq.2] (infusion(glc;total)), was significantly lower in rats fed the high-fat diet compared to rats fed the low-fat diet, 111.3±0.4 and 147.3±0.5 µmol/kg/min, respectively, indicating a decreased ability to disposed glucose (Figure 1B). High-fat feeding resulted in a decreased whole body glucose disposal calculated according to [Eq.3] (62.2±2.4 vs. 52.5±4.3 µmol/kg/min, LF vs. HF, respectively). During hyperinsulinemia, plasma glucose disposal increased in both groups but the ability to dispose glucose was significantly decreased in rats fed the high-fat diet (Figure 2A). Furthermore, plasma glucose disposal rate was shifted to the right in rats fed the high-fat diet. The endogenous glucose production (Figure 2B) was similar between the two groups at basal conditions and was decreased during hyperinsulinemia in rats fed the low-fat diet but not in rats fed the high-fat diet.

![Figure 1](image1.png)

**Figure 1.** Plasma insulin levels (A), total glucose infusion rate (GIR) (B) and plasma glucose levels (C) during hyperinsulinemic euglycemic Clamping in rats fed the low-fat diet (open squares) and rats fed the high-fat diet (closed squares), values are mean±SEM, n=3/group, * p<0.05 Difference between groups, † p<0.05 different from basal, ONE WAY ANOVA with repeated measurements.
High-fat feeding ablates the insulin sensitivity of de novo G6P synthesis. De novo G6P synthesis (Figure 2C) was not different at basal conditions between rats fed the low-fat or the high-fat diet. However, during hyperinsulinemia a clear discrepancy was observed between the groups. In rats fed the low-fat diet, de novo G6P synthesis decreased significantly in comparison to basal conditions, i.e. from 39±2 to 24±1 µmol/kg/min. However, in rats fed the high-fat diet de novo G6P synthesis did not decrease to any extent, i.e. from 38±3 to 35±1 µmol/kg/min. The isotopic G6Pase/GK-flux ratio, G6Pase flux and GK flux were not affected by high-fat feeding. Insulin decreased the isotopic G6Pase/GK-flux ratio in both groups but this effect was less pronounced in rats fed the high-fat diet (Figure 3A). Insulin decreased the isotopic G6Pase flux in rats fed the low-fat diet only (Figure 3B) and increased the isotopic GK flux in both groups of rats (Figure 3C).

**Figure 2.** Plasma glucose disposal (A), endogenous plasma glucose production (B) and de novo G6P synthesis (C) in rats fed the low-fat diet (white circles) and the high-fat diet (black circles) during basal (low insulin) and hyperinsulinemia, *p<0.05 diet effect, † p<0.05 insulin effect, ONE WAY ANOVA with repeated measurements.
High-fat feeding decreases GK enzyme activity resulting in an increased G6Pase/GK activity ratio. Hepatic GK enzyme activity was significantly decreased by 45% in rats fed the high-fat diet compared to rats fed the low-fat diet (Table 3), while hepatic G6Pase enzyme activities were not different between the groups (Table 3). This resulted in an increased hepatic G6Pase/GK activity ratio in rats fed the high-fat diet compared to rats fed the low-fat diet which was associated with a lower hepatic G6P concentration in rats fed the high-fat diet (Table 3).

**Figure 3.** Hepatic G6Pase/GK-flux ratio (A), isotopic G6Pase-flux (B), isotopic GK-flux (C) in rats fed the low-fat diet (white circles) and the high-fat diet (black circles) during basal (low insulin) and hyperinsulinemia, *p<0.05* diet effect, † *p<0.05* insulin effect, ONE WAY ANOVA with repeated measurements.
**Hepatic gene expression is altered by high-fat feeding.** To gain insight into the background of altered hepatic glucose metabolism hepatic gene expression levels of relevant genes involved in hepatic glucose metabolism were studied. Hepatic expression of the gene encoding the sterol element binding protein-1c (Srebp-1c), the master switch of hepatic fat and glucose metabolism, was not affected by high-fat feeding (Figure 4). Hepatic expression levels of some SREBP-1c target genes were clearly affected in rats fed the high-fat diet; glucokinase (Gk) was increased and phosphoenolpyruvate carboxykinase (Pepck) was decreased in rats fed the high-fat diet. Genes known to be controlled by G6P, i.e. glucose-6-phosphatase hydrolase (G6ph) and pyruvate kinase (Pk) were decreased in rats fed the high-fat diet except for Gp expression level, which was not affected by high-fat feeding.

![Figure 4](image-url)
DISCUSSION

High-fat feeding for a period of three weeks induced an insulin resistant state in rats, with normal plasma glucose and elevated plasma insulin concentrations. High-fat diet-induced metabolic changes in whole body and hepatic glucose metabolism were more clearly visible during hyperinsulinemic clamp conditions; whole body glucose disposal, \textit{de novo} G6P synthesis and endogenous plasma glucose production were less sensitive to the actions of insulin. At basal condition (9 hr fast), the enzyme activity-ratio of glucose-6-phosphatase and glucokinase (G6Pase/GK ratio) was increased in rats fed the high-fat diet indicating a net increase in glucose cycling towards glucose output. In spite of hepatic insulin resistance, \textit{Srebp-1c} gene expression levels were not affected but two of its putative target genes, i.e. \textit{Gk} and \textit{Pepck} were clearly affected. Metabolic changes induced by high-fat feeding were associated with decreased hepatic G6P and glycogen content and decreased expression levels of genes putatively controlled by intracellular glucose metabolites.

At basal condition, whole body glucose disposal was decreased but endogenous glucose production and \textit{de novo} G6P synthesis were not affected by high-fat feeding. This indicates that the compensatory increase in plasma insulin concentration was sufficient to maintain normal, basal glucose metabolism. During hyperinsulinemic euglycemic clamping, the sensitivity of whole body glucose disposal for insulin was shifted towards higher plasma insulin concentrations in rats fed the high-fat diet (Figure 2), indicating that the insulin resistance resulted from a post-receptor defect. Furthermore, endogenous glucose production decreased upon increasing insulin concentration in rats fed the low-fat diet but not in rats fed the high-fat diet. This was due to the inability of insulin to suppress \textit{de novo} G6P synthesis, which represents the first part of the gluconeogenesis (GNG). High-fat feeding was without effect on the isotopic fluxes to and from glycogen neither under basal and hyperinsulinemic euglycemic clamp conditions (unpublished observation).

Although the hepatic isotopic fluxes through G6Pase and GK were not different at basal condition, the G6Pase/GK activity ratio was increased due to a decreased hepatic GK activity in rats fed the high-fat diet in comparison with rats fed the low-fat diet. This discrepancy between GK isotopic flux and GK enzyme activity in rats fed the high-fat diet, likely results from the underestimation of the isotopic GK flux. In the isotopic model it is not possible to estimate the contribution of hepatic glycolysis to the isotopic flux through GK. The isotopic flux, which is estimated in this model, is the transfer of plasma glucose to UDPglc only. Therefore, changes in hepatic glycolytic flux due to changed enzyme activity of GK and resulting in decreased glycolysis are not detected by this isotopic model.

Changes in hepatic GK enzyme activity did not match the changes noted in hepatic \textit{Gk} gene expression. Whereas GK enzyme activity decreased upon high fat feeding \textit{Gk} mRNA increased. \textit{G6ph} mRNA decreased upon high-fat feeding while its activity remained unaffected. This discrepancy, particularly for GK, has also been observed in another rat model of diabetes type 2, the Zucker Diabetic Fatty (ZDF) rat. In primary hepatocytes isolated from ZDF rats enzyme activity of GK is about 20\% of the activity in hepatocytes from lean littermates (25). In another study it was shown that \textit{Gk} mRNA levels remained high irrespective fasting or feeding (26). In addition, liver type \textit{Pk} mRNA, enzyme activity and rate of glycolysis were depressed in hepatocytes from ZDF rats when compared to hepatocytes from lean littermates (25). A similar decrease in \textit{Pk} mRNA levels was observed in this study upon high fat feeding, supporting the suggestion that the isotopic flux through GK might have been decreased in parallel with GK enzyme activity when the isotopic flux through glycolysis could have been estimated as well.

When the changes in mRNA levels are considered in more detail it appeared that genes depending on glucose metabolism such as \textit{G6ph} and \textit{Pk} are down-regulated in line with
the decreased hepatic G6P concentration observed in high-fat fed rats. When compared to ZDF rats, increasing the expression of GK by adenovirus-mediated Gk gene transfection of isolated hepatocytes increased mRNA levels of Pk, its activity and rate of glycolysis, together with an increase in intracellular concentration of G6P. The effects of high fat feeding on Gk mRNA levels are more difficult to understand, since mRNA levels of Srebp-1c, the transcription factor regulating Gk gene transcription, were not affected by high-fat feeding. However, it is likely that other factors are also involved in regulating Gk gene expression. Recent studies in SREBP-1c knock out mice show that the change in Gk mRNA levels responded normal in these knock-out mice upon the transition from fasting to feeding (27).

We have previously shown that high-fat feeding in rats resulted in insulin resistance due to decreased hepatic insulin signaling. Phosphorylation of the hepatic insulin receptor, insulin receptor substrates (IRS) and protein kinase B (PKB) were decreased in rats fed the high-fat diet, indicating that in this model the defect was localized in the phosphatidylinositol 3-kinase (PI3K) pathway. Insulin increases glycogen storage and decreases gluconeogenesis and glucose output via the PI3K pathway in part by inactivation of glycogen synthase-3 (GSK-3) (28). This protein dephosphorylates and, thereby, activates of glycogen synthase (GS) (29). In the high-fat diet-induced insulin resistance, this pathway appears to be impaired leading to decreased glycogen storage and increased de novo G6P synthesis, in parallel with the effects of decreased intracellular concentration of G6P on glycogen metabolism and gene expression.

In conclusion, high-fat feeding results in insulin resistance and subsequently in an inability of insulin to modulate hepatic glucose metabolism. Insulin is not able to effectively suppress de novo G6P synthesis and glucose production and to increase plasma glucose disposal. Other studies have shown that gene expression levels and activities of key enzymes in these metabolic pathways are affected by insulin resistance. We show here that high-fat diet induced insulin resistance affects the metabolic fluxes through these pathways, resulting in the phenotype, which resembles the early stages in the development of diabetes.

ACKNOWLEDGEMENTS

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REFERENCES

High-fat feeding affects glucose metabolism


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