Regulators of hepatic glucose and glycogen metabolism
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Free fatty acids (FFA) are involved in the regulation of intrahepatic fluxes of gluconeogenesis and glycogen metabolism during short-term starvation in healthy volunteers


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Abstract

The influence of free fatty acids (FFA) on intrahepatic fluxes of gluconeogenesis, glycogenolysis and glycogen synthesis was investigated between 16 and 22 hours of fasting in 6 male volunteers. Each subject was studied twice, once with and once without acipimox, an inhibitor of lipolysis, and fluxes were measured by infusion of $[2^{-13}C_1]$glycerol, $[1^{-2}H_1]$galactose and $[U^{-13}C_6]$glucose. Acipimox almost completely suppressed plasma FFA levels within 1 hour, whereas FFA slowly increased in the control study. Glucose production was unchanged with acipimox but decreased by 19% in the control study. Total gluconeogenesis increased by 17% in the control study due to an increase in its direct pathway to plasma, whereas total and direct gluconeogenesis showed a transient decrease with acipimox. Indirect gluconeogenesis (via UDP-glucose and glycogen) was similar and constant in both studies. Acipimox prevented the decrease in total glycogenolysis and breakdown of hepatic glycogen stock found in the control study (decrease resp. 32% and 34%). Glycogen synthesis (retained for stock), and glycogen synthesis flux (sum of synthesis retained for stock and synthesis followed by subsequent breakdown) were constant and similar in the acipimox and the control study.

We conclude that during short-term starvation (less than 24 hours) 1) FFA stimulate the direct pathway of gluconeogenesis, 2) FFA inhibit the breakdown of glycogen stock, 3) FFA do not affect glycogen synthesis (or synthesis flux).

During short-term starvation intrahepatic fluxes are directed towards a relative sparing of liver glycogen stock, and FFA is an important regulator of these fluxes.
**Introduction**

Plasma levels of free fatty acids (FFA) play a crucial role in the pathophysiology of insulin sensitivity and the pathogenesis of type 2 diabetes mellitus. The influence of FFA on peripheral (muscle) and central (liver) glucose and glycogen metabolism is beyond doubt, but its exact physiological role is still not completely elucidated (1). Insulin resistance has been convincingly related to FFA, even at physiological FFA concentrations (50-800 µmol/l) (2).

FFA influence the regulation of glucose production in healthy subjects. Bergman et al. have described FFA as a “metabolic messenger” (3). Recent studies suggest that the primary route by which insulin maintains control over glucose production may be indirect and mediated by FFA (1,4). This strong and unique correlation of FFA with suppression of glucose production has led to the assumption that FFA is not just a metabolic substrate but the extrahepatic “second signal” in the control of insulin action on glucose production (1).

Administration of FFA stimulates glucose production, but the effect of suppression of plasma FFA is less clear; FFA suppression has been described to suppress, stimulate, or have no effect on glucose production (5-7). Different explanations can be found: firstly, in vivo, autoregulation between gluconeogenesis and glycogenolysis may prevent major changes in overall glucose production (8-11). Secondly, findings appear to depend on the study design. Under basal conditions, Chen et al., Stingl et al., Wang et al. and Fery et al. found that FFA inhibit glucose production through suppression of glycogenolysis, despite stimulation of gluconeogenesis (7,10,12-14). According to Boden et al., increasing FFA concentration under basal conditions does not alter glucose production, mainly due to concomitant stimulation of insulin secretion (5). Pancreatic clamp studies by the groups of Bergman and Boden show that suppression of FFA inhibits glucose production, and that re-infusion of FFA restores glucose production (5,6). After 4 days of fasting FFA-suppression was reported to induce an increase in glucose production and gluconeogenesis (15).

During short-term starvation (i.e. less than 24 hours), plasma glucose originates from gluconeogenesis and glycogen breakdown. Gluconeogenesis can be directed towards plasma directly (direct gluconeogenesis), or indirectly, via UDP-glucose and glycogen (indirect gluconeogenesis). Liver glycogen synthesis has two
sources: plasma glucose and indirect gluconeogenesis. Newly synthesized glycogen can be retained in the liver (for stock) or it can be subsequently degraded after synthesis. Total glycogen synthesis flux is the sum of retained glycogen synthesis and synthesis followed by subsequent breakdown. Total glycogenolysis is therefore the sum of the breakdown of pre-existing liver glycogen (stock) and breakdown of newly synthesized glycogen.

Between 16 and 24 hrs of fasting, the rate of appearance (Ra) of glucose decreases 20% in healthy volunteers (16,17). It is assumed that the decrease in Ra glucose is due to a decrease in glycogenolysis, driven by a decreasing glycogen content in the liver (18,19). During fasting, lipolysis is enhanced and FFA levels in plasma rise (12). During fasting, glucose metabolism adapts in a way compatible with effects previously ascribed to FFA; the influence of FFA on overall gluconeogenesis and glycogenolysis has been described before. However, there are no data so far on the influence of FFA on intrahepatic fluxes through the above-mentioned pathways of gluconeogenesis, glycogenolysis, and glycogen synthesis.

The aim of this study was to investigate changes in intrahepatic fluxes of glycogenolysis, gluconeogenesis and glycogen synthesis during short-term starvation in healthy male volunteers. The role of FFA in the regulation of these fluxes during short-term starvation was investigated using acipimox, an inhibitor of lipolysis.

**Subjects and methods**

**Subjects**: six healthy lean male volunteers (age: 32 ± 4 yr, weight: 68 ± 2 kg, height: 1.70 ± 0.02 m, body mass index: 22.6 ± 0.74 kg/m²) were included in a cross-over saline controlled study after obtaining written informed consent. None had experienced any febrile disease in the month prior to the study or used medication. For 3 days prior to the study, all volunteers consumed a weight-maintaining diet containing at least 250 grams of carbohydrates. The study was approved by the Research Committee and the Medical Ethical Committee of the Academic Medical Center in Amsterdam.

**Study protocol**: each subject was studied twice, receiving acipimox on one occasion and saline (0.65%) on the other (control study). Both study protocols were separated by at least 4 weeks. The subjects were fasted from 6 PM on the day prior
to the study until the end of the study. During both studies the subjects were confined to bed. At 6.45 AM, a catheter was placed into an antecubital vein for infusion of stable isotope tracers. Another catheter was inserted retrogradely into a contralateral hand vein kept in a thermoregulated (65°C) plexiglas box for sampling of arterialized venous blood. In both studies saline was infused as NaCl 0.65% at a rate of 50 ml/h to keep the catheters patent. At 7 AM (t= -3h) blood was sampled for determination of background enrichment of the tracers [2-\textsuperscript{13}C\textsubscript{1}]glycerol, [1-\textsuperscript{2}H\textsubscript{1}]galactose and [U-\textsuperscript{13}C\textsubscript{6}]glucose. Then, a primed continuous infusion of [2-\textsuperscript{13}C\textsubscript{1}]glycerol (9 mg/kg, 0.15 mg/kg/min), [1-\textsuperscript{2}H\textsubscript{1}]galactose (4.8 mg/kg, 0.08 mg/kg/min) and [U-\textsuperscript{13}C\textsubscript{6}]glucose (1.2 mg/kg, 0.02 mg/kg/min) was started and continued until the end of the study at 4 PM (t=6h). Acetaminophen (500mg) was given orally at 7 AM, 10 AM, 12 AM and 2 PM (t= -3h, 0h, 2h, 4h). In the intervention study, acipimox was ingested orally at t=0h (250mg), at t=2h (250mg), and at t=4h (500mg).

At t= -20min, -15 min, -10 min and t=0h, blood samples were drawn for determination of steady state isotopic enrichment. At t=0h, blood samples for baseline values of hormones and FFA were drawn.

Every 30 min between t=0h and the end of the study at 4 PM (t=6h), blood samples were drawn for measurement of isotopic enrichment of glucose by [U-\textsuperscript{13}C\textsubscript{6}]glucose to determine glucose turnover. Isotopic enrichment of glucose and Acetaminophen-glucuronate (GlcUA) by [2-\textsuperscript{13}C\textsubscript{1}]glycerol, [1-\textsuperscript{2}H\textsubscript{1}]galactose and [U-\textsuperscript{13}C\textsubscript{6}]glucose was measured every 2 hours between t=0h and the end of the study at 4 PM (t=6h).

Blood samples for measurement of glucoregulatory hormones were drawn every 30 min, except between t=2h and t=4h when they were drawn every hour. Blood samples for measurement of FFA were measured every hour in the control study, and every 30 min in the acipimox study. The total blood volume withdrawn was 200 ml.

**Chemicals, isotopes, and mass spectrometric analyses:** [2-\textsuperscript{13}C\textsubscript{1}]glycerol, [1-\textsuperscript{2}H\textsubscript{1}]galactose and [U-\textsuperscript{13}C\textsubscript{6}]glucose were purchased from Cambridge Isotopes (ARC laboratories B.V., Amsterdam, the Netherlands). Isotopes were >98% pure and >99% enriched. All chemicals were analytical grade. Isolation of metabolites, preparation for mass spectrometry, and mass spectrometric analyses were done as previously described by Hellerstein et al. (20). The gas chromatography column used was a 60 m x 0.25 mm, x 0.25 μm DB17 capillary column (J & W Scientific,
Chapter 9

Folsom, CA) on an Hewlett-Packard 5890 Series II gas chromatograph coupled to an HP 5989 A model mass spectrometer (Hewlett Packard, Palo Alto, CA).

**Calculations of metabolic fluxes:** calculations were done with mass isotopomer distribution analysis (MIDA) and the “secreted glucuronate technique” as previously described by Hellerstein et al.; [2-13C1]glycerol, [1-2H1]galactose and [U-13C6]glucose were infused as tracers (20-22). MIDA is a technique for measuring biosynthesis and turnover of polymers *in vivo*. By comparison of statistical distributions predicted from the binomial or multinomial expansion to the pattern of excess isotopomer frequencies observed in the polymer, the enrichment of the biosynthetic precursor subunits (or pool, p) for newly synthesized polymers is calculated (21). The pathway by which glucose and gluconeogenic fluxes were converted to glycogen were traced by use of acetaminophen as a non-invasive xenobiotic probe of intrahepatic UDP-glucose. Glucuronate-acetaminophen (GlcUA) was sampled in urine and used to determine UDP-glucose enrichment. The technique uses GlcUA for sampling the intrahepatic UDP-glucose pool, MIDA for measuring fractional contributions from gluconeogenesis to plasma glucose and hepatic UDP-glucose, the intrahepatic dilution technique for measuring the rate of appearance (R\text{a}) or turnover of hepatic UDP-glucose, and techniques for estimating hepatic cycles, including glycogen synthesis-breakdown and glucose phosphorylation-dephosphorylation (20,22).

**Pathways of glucose metabolism** (see fig 1): during short-term starvation (less than 24 hrs) under basal conditions, plasma glucose originates from gluconeogenesis and glycogen breakdown. Gluconeogenic flux can be directed towards plasma directly (direct gluconeogenesis), or indirectly by passing through UDP-glucose, glycogen, and reaching plasma after glycogen breakdown (indirect gluconeogenesis). Plasma glucose from glycogen (total glycogenolysis) originates from pre-existing liver glycogen stock, and the above-mentioned indirect gluconeogenesis passing through the glycogen-pool. Liver glycogen can be synthesized from plasma glucose and indirect gluconeogenic flux. Newly synthesized glycogen, i.e. glycogen synthesized in the course of the study, can be either retained in the liver (retained glycogen synthesis) or be degraded again rapidly (synthesis followed by breakdown). Total glycogen synthesis flux is calculated as the sum of glucose appearance from UDP-glucose (synthesis
followed by breakdown) and retained glycogen synthesis (originating from both plasma glucose and indirect gluconeogenesis). Total glycogenolysis is calculated as the sum of breakdown of pre-existing glycogen stock and breakdown of newly synthesized glycogen. The glycogen balance can be calculated as retained glycogen synthesis minus total glycogen breakdown.

*Fig 1. Intrahepatic pathways of glucose metabolism, glucuronate, and stable isotopes.* Plasma glucose (Glc); Glucose production (GP); glycogenolysis (GL); total gluconeogenesis (GNG), direct and indirect gluconeogenesis (dirGNG, indirGNG); total glygogen synthesis flux (FluxGS) and retained glycogen synthesis (GS).
Assays: all measurements in each individual subject were performed in the same run, and all samples were tested in duplicate. Glucose concentrations were measured on a Beckman glucose oxidizer (Beckman, Palo Alto, CA).

Plasma insulin concentration was determined by RIA (Insulin RIA 100, Pharmacia Diagnostic AB, Uppsala, Sweden; intra-assay coefficient of variation (CV) 3-5%, interassay CV 6-9%, detection limit 12 pmol/l (2mU/l). C-peptide was measured by RIA-coat c-peptide, Byk-Sangtec Diagnostica GmbH & Co. KG, Dietzenbach, Germany, intra-assay CV 4-6%, interassay CV 6-8%, detection limit 0.05 nmol/l. Glucagon was determined by RIA (Linco Research, St Charles, MO, USA, intra-assay CV 3-5%, interassay CV 9-13%, detection limit 15 ng/L. Cortisol was measured by enzyme-immunoassay on an Immulite analyzer (DPC, Los Angeles, CA, USA), intra-assay CV 2-4%, interassay CV 3-7%, detection limit 50 nmol/l.

Catecholamines were measured by an in-house HPLC method, norepinephrine: interassay CV 6-8%, intra-assay CV 7-10%, detection limit 0.05 nmol/l, and epinephrine: interassay CV 6-8%, intra-assay CV 7-12%, detection limit 0.05 nmol/l.

Plasma free fatty acids (FFA) were measured by an enzymatic method (NEFAC: Wako Chemicals GmbH Neuss, Germany), intra-assay CV 2-4%, interassay CV 3-6%, detection limit 0.02 mmol/l. Blood lactate was determined by enzymatic method (Boehringer Mannheim, Mannheim, Germany) on a Cobas Bio Centrifugal Analyzer.

Calculations and statistics: because plasma glucose concentrations and tracer/tracer ratios remained constant during each sampling phase of the study, calculations for steady state kinetics were applied, adapted for the use of stable isotopes (24,25). Results are given as mean ± s.e.m.

The overall differences in the parameters between the two groups were tested adjusted for the baseline value using analysis of repeated measures by means of the proc mixed procedure of the SAS software package (version 6.12, SAS institute, Cary, North Carolina, USA). For each parameter and for each group the regression coefficient was obtained from the same model in which time was entered as a continuous variable. For FFA, the difference between the studies at t=1h was tested using a t-test; in the acipimox study only, the initial decline in FFA levels between t=0h and t=1h was tested using a t-test. For glycogen balance results between time points t=0h and t=6h within the studies were also compared using a t-test. Statistical significance was set at p<0.05.
Results

Control study (results expressed in mg/kg/min unless indicated) (also see table 1). Plasma FFA increased 35% between t=1h and the end of the study (p=0.001) (fig 2). Between t=0h and t=6h plasma glucose concentration decreased 13% (p=0.0001) and glucose production decreased 19% (p=0.0009). Total gluconeogenesis increased 17% (p=0.02), due to a 14% increase in direct gluconeogenesis (p=0.003) whereas indirect gluconeogenesis did not change. Total glycogenolysis decreased 32% (p=0.0001), due to a 34% decrease in the rate of breakdown of liver glycogen stock (p=0.004) and a 24% decrease in the UDP-glucose-glycogen flux (p=0.004). Total glycogen synthesis flux did not change and neither did retained glycogen synthesis. Due to the decrease in glycogen breakdown, glycogen balance became less negative (p=0.04 (t-test)).

![Graph showing FFA concentration over time.](image)

Fig 2. Plasma FFA concentration. Control study (○) and acipimox study (●). Acipimox was given after t=0 (16h of fasting). FFA was lower with acipimox (p=0.003).

Acipimox study (results expressed in mg/kg/min unless indicated) (also see table 1) Plasma FFA decreased 75% within 1 hr (p=0.0001, t-test) and remained suppressed until t=6h (fig2). Between t=0h and t=6h plasma glucose concentration decreased 13% (p=0.001). Glucose production did not decrease with acipimox. Total
gluconeogenesis showed a transient decrease from 0.75±0.08 (t=0h) to 0.66±0.11 (t=4h) (p=0.02) and returned to baseline at t=6h; direct gluconeogenesis also showed a transient decrease from 0.59±0.08 (t=0h) to 0.45±0.06 (t=4h) (p=0.02) and returned to baseline at t=6h. Indirect gluconeogenesis did not change. Total glycogenolysis did not change, despite a 32% decrease in the UDP-glucose-glycogen flux (p=0.02), and glycogen breakdown from glycogen stock was unchanged. Total glycogen synthesis flux did not change and neither did retained glycogen synthesis. Glycogen balance was unchanged due to persistent glycogen breakdown.

Table 1. Intrahepatic fluxes of glucose metabolism after 16 and 22 hours of fasting in the presence (control) and absence (acipimox) of FFA.

<table>
<thead>
<tr>
<th>flux</th>
<th>Control mg/kg/min</th>
<th>acipimox mg/kg/min</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16h fast</td>
<td>22h fast</td>
<td>p-value</td>
</tr>
<tr>
<td>glucose concentration</td>
<td>5.28±0.13</td>
<td>4.57±0.25</td>
<td>0.0001</td>
</tr>
<tr>
<td>glucose production</td>
<td>2.88±0.14</td>
<td>2.32±0.12</td>
<td>0.0009</td>
</tr>
<tr>
<td>total gluconeogenesis</td>
<td>0.81±0.04</td>
<td>0.93±0.07</td>
<td>0.02</td>
</tr>
<tr>
<td>direct gluconeogenesis</td>
<td>0.64±0.07</td>
<td>0.73±0.06</td>
<td>0.003</td>
</tr>
<tr>
<td>indirect gluconeogenesis</td>
<td>0.17±0.03</td>
<td>0.21±0.02</td>
<td>n.s.</td>
</tr>
<tr>
<td>total glycogenolysis</td>
<td>2.08±0.11</td>
<td>1.41±0.17</td>
<td>0.0001</td>
</tr>
<tr>
<td>glycogenolysis from stock</td>
<td>1.69±0.12</td>
<td>1.12±0.17</td>
<td>0.004</td>
</tr>
<tr>
<td>glycogenolysis from UDP-Glc</td>
<td>0.39±0.03</td>
<td>0.30±0.02</td>
<td>0.004</td>
</tr>
<tr>
<td>glycogen synthesis flux</td>
<td>0.57±0.04</td>
<td>0.51±0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>retained glycogen synthesis</td>
<td>0.19±0.04</td>
<td>0.22±0.03</td>
<td>n.s.</td>
</tr>
<tr>
<td>glycogen balance</td>
<td>-1.89±0.12</td>
<td>-1.19±0.16</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Non-significant : n.s.

Acipimox vs control study
Plasma FFA were significantly lower in the acipimox study than in the control study from t=1h onwards (p=0.003, t-test) (fig 2). Plasma glucose concentrations
were not different. Glucose production was higher with acipimox than in the control study (p=0.0009) (fig 3). Total and direct gluconeogenesis with acipimox were lower than in the control study (p=0.003) (fig 4a and 4b); indirect gluconeogenesis was not different between the acipimox or the control study (fig 4c). Breakdown of glycogen stock was higher with acipimox than in the control study (p=0.0005) (fig 4f); with a similar UDP-glucose to glycogen flux this resulted in a higher rate of total glycogenolysis with acipimox than in the control study (p=0.0003) (fig 4d). Total glycogen synthesis flux and retained glycogen synthesis were not different in the control and the acipimox study. Glycogen balance was more negative with acipimox than in the control study (p=0.003).

Fig 3. Ra of appearance of glucose. Control study (○) and acipimox study (●). Acipimox was given after t=0 (16h of fasting). Glucose production was higher with acipimox (p=0.0009).
Fig 4. Pathways of gluconeogenesis and glycogenolysis. Control study (○) and acipimox study (●). With acipimox, total and direct gluconeogenesis showed a transient decrease (p=0.02). Total and direct gluconeogenesis with acipimox were lower than in the control study (p=0.003). Breakdown of glycogen stock was higher with acipimox (p=0.0005) and so was total glycogenolysis (p=0.0003).
Counterregulatory hormones (see fig 5):
Both insulin (fig 5a) and C-peptide decreased more in the acipimox than in the control study (p=0.0001) (insulin: acipimox: from 35±4 to 18±2 pmol/l (p=0.0001); control: from 38±6 to 29±6 pmol/l (p=0.04), and C-peptide: acipimox: from 367±64 to 120±35 pmol/l (p=0.0001); control: from 373±73 to 232±45 pmol/l (p=0.01)). Glucagon (fig 5b) did not change in the control study (from 54±6 to 54±5 ng/l) but rose in the last 30 min of the acipimox study (from 61±11 to 84±13 ng/l, p=0.001). Plasma cortisol levels were not significantly different between both studies (control: from 400±67 to 217±27 nmol/l; acipimox: from 302±26 to 302±46 nmol/l). Epinephrine (fig 5c) did not change in the control study (from 0.16±0.04 to 0.25±0.03 ng/l) but rose in the last hour of the acipimox study (from 0.16±0.04 to 0.77±0.22 nmol/l, p=0.0001) (fig 6c); Norepinephrine did not change in either of the studies (control: from 0.82±0.19 to 1.01±0.15 nmol/l and acipimox: from 1.13±0.16 to 1.03±0.25 nmol/l).

**Fig 5. Glucoregulatory hormones.** Control study (○) and acipimox study (●). Insulin was lower with acipimox (p=0.0001), glucagon (p=0.01) and epinephrine rose with acipimox (p=0.02).
Chapter 9

**Fig 6. Sites of action of physiological FFA concentrations on intrahepatic fluxes of glucose metabolism:** stimulation (↑); inhibition (↓); not affected (=).

**Discussion**

This study describes in detail the pathways of hepatic glucose and glycogen metabolism in the presence and absence of physiological FFA concentrations. The results indicate that FFA play an important role in the decrease of glucose production during short-term starvation, by limiting glycogen breakdown. Without the physiological rise in FFA, glycogen breakdown maintained glucose production at the expense of liver glycogen stock. Another important finding is the unchanged rate of glycogen synthesis during short-term starvation, albeit not regulated by FFA. These findings suggest that the changes of intrahepatic fluxes during short-term starvation are aimed at a relative sparing of glycogen content, only partly regulated by FFA.

In the control study, plasma FFA increased from t=1hr till the end of the study. Glucose production decreased, in large part due to a decrease in glycogenolysis from pre-existing stock. Gluconeogenesis increased due to an increased flux through the direct pathway to plasma. The gluconeogenic flux through the indirect
FFA and intrahepatic pathways of glucose

(UDP-glucose to glycogen) pathway remained unaffected. The increase in gluconeogenesis was insufficient to prevent the decrease in glucose production. Neither the flux through glycogen synthesis nor retained glycogen synthesis decreased during short-term starvation. Due to the decrease in glycogen breakdown, the glycogen balance became less negative over time. Acipimox suppressed plasma FFA almost completely, indicating that lipolysis was effectively inhibited. Simultaneously with the decrease in FFA, fluxes through different pathways changed. Glucose production ceased to decrease and remained constant thereafter due to sustained glycogen breakdown from glycogen stock. Without FFA, gluconeogenesis did not increase; the direct gluconeogenic pathway showed an initial decrease, but regained baseline rates between 20 and 22 hours of fasting. Indirect gluconeogenesis was unaffected by the absence of FFA. Total glycogen synthesis (flux) stayed constant, unaffected by the absence of FFA.

Technique
There is no golden standard available for the measurement of gluconeogenesis and fluxes through UDP-glucose and glycogen in vivo in humans. All available (isotopic) techniques come with assumptions and technical imperfections. The technique we used to measure intrahepatic carbohydrate metabolic fluxes was developed by Hellerstein et al. and combines MIDA, the "secreted glucuronate technique", and the standard isotope dilution technique (20-22,25,26). The glucuronate technique and MIDA rely on the validity of a number of biochemical key conditions. The technical backgrounds and the basis for calculations have been extensively discussed by Hellerstein et al., including the assumptions and imperfections (20-22,27).

The most important requirements for the validity of the "secreted glucuronate technique" and MIDA are that 1) galactose is exclusively taken up by the liver and that UDP-glucose enrichment from labeled galactose reflects the enrichment in the glucose-6-phosphate pool, that 2) in vivo, under conditions of fasting, the flux from glucose-1-phosphate to UDP-glucose is functionally irreversible (implicating that all glucose originating from UDP-glucose passed the glycogen pool), and that 3) [2-13C1]glycerol labels the gluconeogenic precursor pool uniformly, i.e. enrichment of the precursor must be the same in all cells that synthesize glucose.

Firstly, galactose must be exclusively taken up by the liver to use dilution of labeled galactose in GlcUA for measurement of intrahepatic UDP-glucose turnover.
in vivo, GlcUA samples have indeed been shown to measure a common UDP-glucose/glucose-6-phosphate pool (27-29). In mammals over 90% of galactose is utilized by the liver, and there is no evidence that labeled galactose taken up by the liver can be assimilated by any other route than via UDP-glucose (27). When infused in high doses in rats, galactose may be excreted in urine. This is not the case with the tracer amounts we infused. To the extent that extrahepatic tissues may take up galactose, the Ra UDP-glucose will be overestimated (27).

Secondly, the (ir)reversibility of the glucose-1-phosphate to UDP-glucose reaction (the UDP-glucose pyrophosphorylase reaction) has been questioned by Landau (27). Biochemically speaking the reaction is indeed reversible. However, in vivo and under conditions of fasting, when cytosolic AMP and PPi concentration are very low, the reaction will be thermodynamically and kinetically irreversible (27). During ingestion of high lactose loads in infants, galactose can form glucose-1-phosphate directly, i.e. without passing glycogen, after entering the UDP-hexose pool (30). In the absence of such a substrate load however, as in our study, the importance of this reaction pathway is unknown. With the tracer amounts of galactose enriching the hepatic UDP-glucose by only 1-2%, this alternative pathway would seem to be negligible (27).

Irreversibility of the glucose-1-phosphate to UDP-glucose reaction implies that UDP-glucose must pass the glycogen pool to reach plasma as glucose: if so, then the flux through UDP-glucose equals glycogen synthesis flux. This extrapolation of the Ra UDP-glucose to glycogen synthesis has been questioned (27). In rats, Hellerstein et al. and Rother et al. have shown that the Ra UDP-glucose correlates closely with glycogen deposition (29,31). The fractional recovery (R) of labeled UDP-glucose (i.e. from administered [1-\(^2\)H\(_1\)]galactose) in plasma glucose is measured as the exit of galactose label from the liver into plasma glucose. Vice versa, the fraction of galactose label that is not recovered (1-R) provides a measure of the retention of label in liver glycogen. Hellerstein et al. found that a majority (65-70%) of labeled carbon flux into UDP-glucose did not return into plasma glucose (i.e. was retained in the liver) during a 4-6 hr infusion of [1-\(^2\)H\(_1\)]galactose: retained label could be mobilized from the liver by administration of glucagon (20). In accordance with Hellerstein we found that 60 to 65% (control) and 55 to 70% (acipimox) of galactose label was retained in the liver between 16 and 22 hrs of fasting. This retained hepatic UDP-glucose flux provides a minimal estimate for glycogen synthesis, and this flux can originate from either plasma glucose or
indirect gluconeogenesis (discernable by its labeling from \([U-{}^{13}\text{C}_6]\)glucose or \([2-{}^{13}\text{C}_1]\)glycerol\) (20,27). Would a proportion of the UDP-glucose flux bypass the glycogen pool, or escape by hydrolysis of UDP-glucose, then the assumption that all UDP-glucose-plasma glucose flux is glycogen synthesis flux would no longer be valid. However, as Hellerstein pointed out, since 60-70\% of the label is retained in the liver (1-R) in fasting humans, the magnitude of this escape route (i.e. a proportion of the 30-40\% (R)) is not likely to be substantial. Nevertheless, as the retained UDP-glucose flux represents an estimate for glycogen synthesis, caution is in fact necessary in equating Ra UDP-glucose to glycogen synthesis (27).

Thirdly, an important assumption of MIDA is that the triose phosphate pool is uniformly \(^{13}\text{C}\)-enriched by infusion of \([2-{}^{13}\text{C}_1]\)glycerol. If not, then the fractional contribution of gluconeogenesis to glucose production is underestimated. Metabolic zonation or a decrease in the transhepatic concentration and enrichment of \([2-{}^{13}\text{C}_1]\)glycerol may cause a gradient in enrichment across the liver, especially at low infusion rates (32)(33). Landau et al. hypothesized a progressive decrease in the labeling of triose phosphates from the perportal to the perivenous region of the liver lobules (34). The influence of zonation on MIDA with \([2-{}^{13}\text{C}_1]\)glycerol is much debated and findings are contradictory. Hellerstein et al. and Neese et al. tested uniform labeling in the liver, by comparing the enrichment in plasma glucose and in GlcUA after \([2-{}^{13}\text{C}_1]\)glycerol and acetaminophen, the two end products of newly formed hexoses from the triose pool, after 24 hrs of fasting. They found that the enrichments in plasma glucose and GlcUA were the same, providing support for an isotopic equilibrium (or good mix) within the triose-phosphate and glucose-6-phosphate pools (26,31). Their findings were supported by independent groups of Peroni et al. and Schwenk et al. (28,35). Landau et al. however found that the isotopomer distributions in glucose and glucuronic acid from urinary acetaminophen on administration of \([2-{}^{13}\text{C}_1]\)glycerol were not compatible with gluconeogenesis from a single pool of triose phosphates (34). Non-uniform labeling has also been related to the tracer amount of glycerol infused by Previs et al. and to heterogeneity between hepatocytes (33). Due to contradictions and the impossibility to sample the triose phosphate pool directly in living humans, we cannot rule out that metabolic zonation may have influenced our results. With the available isotopic techniques, no golden standard exists for true absolute rates of gluconeogenesis. Nonetheless, comparing the rates of gluconeogenesis between the control and the acipimox study using the same
technique remains valid, as it seems unlikely that zonation with tracer amounts of \([2-^{13}C_1]\)glycerol would be different with or without acipimox.

It has been pointed out that the tracer amount of \([2-^{13}C_1]\)glycerol may serve as a substrate and increase gluconeogenesis (36). Our group however showed, in agreement with others, that the amount of glycerol used as a tracer in this study did not affect fractional contribution of gluconeogenesis to glucose production or glucose production itself with the \(^2\text{H}_2\text{O-}\) method (37).

Finally, the role of the kidney in this model has been discussed by Hellerstein et al. (20). The kidney can convert glycerol into glucose, and therefore plasma gluconeogenesis in our study is measured regardless of the tissue source. Neese et al. calculated that as long as extra-hepatic gluconeogenesis remained less than 20% of total gluconeogenesis and the precursor pool enrichment in the two tissues was similar (ratio less than 2:1), the effect of the contribution of the kidney on the calculations of gluconeogenesis would cause a difference less than a 5% (20, 26). The kidneys contribute to the glucuronidation of acetaminophen, but only to around 5%. As to the sampling of hepatic UDP-glucose enrichment in urine, urinary GlcUA enrichment can therefore be considered to represent mainly hepatic UDP-glucose enrichment (20).

**Hormones**

Acipimox caused hormonal changes that may have influenced our results; most of these hormonal changes would primarily affect glycogenolysis (38). Insulin and C-peptide levels decreased more with acipimox than in the control study; glucagon and epinephrine rose in the last hour of our acipimox study whereas they did not change in the control study. Fery et al., Wang et al. and Chen et al. described similar hormonal changes with both acipimox and nicotinic acid as the anti-lipolytic drug; glucagon however did not rise with nicotinic acid (7, 12, 14).

Although statistically significantly different from basal, the hormonal changes found in our and the above-mentioned studies are close to physiological levels, unlike in studies designed to investigate the effect of these hormones on (hepatic) glucose metabolism (39-41). Basal insulin and glucagon exert an important effect on hepatic glucose output, as described by Cherrington et al. Complete absence of insulin increases glucose output through glycogenolysis (38). In our study however, insulin was not absent but low, and insulin- and C-peptide levels decreased further with acipimox. Inhibition of basal insulin secretion rates with
nicotinic acid administration has been described before in healthy volunteers; the inhibition of insulin secretion was due to the absence of FFA and not to nicotinic acid (42). However, insulin levels just below basal levels cannot be held responsible for the higher glucose production and glycogenolysis rates found with acipimox (7,14,16).

Glucagon is a strong and immediate stimulator of glycogenolysis and gluconeogenesis; an increase of 10 ng/L has been reported to stimulate glucose output by 0.3 mg/kg/min, due to an effect of glycogenolysis (38,39). Although we cannot rule out a possible effect of glucagon on our results, the rise in glucagon occurred at the very end of the acipimox study whereas sustained higher rate of glycogenolysis where found from the moment FFA levels started to decrease. We therefore believe it is unlikely that the rise of glucagon explains our results. Epinephrine started to rise 2 hours before the end of the acipimox study. The threshold for an effect of epinephrine on glycaemia (resulting from mainly glycogenolysis and glucose clearance) has been described by others to lie between 0.55 and 1.1 nmol/l (43-45). That level was reached only in the last hour of our acipimox study. Because the rate of glycogenolysis began to rise long before the rise in epinephrine and simultaneously with the suppression of FFA, we believe that FFA themselves were responsible for the changes in the intrahepatic fluxes, irrespective of the influence of acipimox on epinephrine and glucagon secretion.

Glucose production
Although extensively studied, reports on the effect of suppression of FFA by inhibitors of lipolysis on glucose output differ depending on the study design. Our results on the inhibitory effect of FFA on basal glucose production in the postabsorptive state are in accordance with studies by Boden et al., Wang et al., Chen et al. Stingl et al. and Fery et al. (7,9-14).

Gluconeogenesis
In this study, gluconeogenesis increased during short-term fasting in the control study. In the acipimox study gluconeogenesis decreased initially during the first 4 hours but returned to baseline despite persistent suppression of FFA. Physiological FFA concentrations are known to stimulate GNG and antilipolysis is known to blunt this effect, as others have described in vivo in the basal state with a variety of different isotopic techniques (7,10,12-14,46). This study confirms the available
Chapter 9

evidence that FFA stimulate GNG. However, a novel finding in our study is that this effect of FFA on gluconeogenesis is transient. Although we cannot rule out an effect of glucagon and epinephrine on gluconeogenesis in the last 2 hours of the acipimox study, we note that gluconeogenesis returned to baseline before the rise in glucagon and epinephrine (38).

Gluconeogenic flux can be differentiated into a direct and an indirect pathway. Hellerstein et al. compared 11 hrs and 60 hrs of fasting under basal conditions using the same technique as we used in our study. In their study they showed that during short-term fasting, the direct pathway was quantitatively the most important component of total gluconeogenic flux; the indirect pathway was unaffected by fasting (20). Our results are in accordance with these findings. The description of the effect of FFA on the two pathways of gluconeogenesis is however novel. The present study showed that, simultaneously with the decrease in plasma FFA, (direct) gluconeogenesis fell due to a decrease in the contribution of the direct pathway, and then rose back to baseline values between 20 and 22 hours. The mechanism behind the transient decrease in gluconeogenesis could be that acipimox reduced FFA availability for oxidation; the intrahepatic energy depletion which results may stimulate proteolysis, releasing amino acids as an alternative source for gluconeogenesis, which in turn may stimulate gluconeogenesis. The indirect pathway was unaffected by fasting, and by (suppression of) physiological FFA concentrations.

Glycogenolysis

Our finding that during short-term fasting glucose turnover decreased due to a decrease in glycogenolysis has been shown by others (12,47,48). Our results showed that during fasting, the breakdown of liver glycogen stock was restrained. These findings are supported by two other groups. Hellerstein et al., using the same isotopic technique, compared 11 hrs to 60 hrs of fasting under basal conditions and also found that glycogen depletion from stock was restricted (14,20). In addition, Stingl et al., using $^{13}$C NMR spectroscopy, found that hepatic glycogen concentration declined during fasting, and that FFA restrict liver glycogen breakdown, in accordance with our results (13).

The distinction between the effects of FFA on both glycogenolytic fluxes, i.e. glycogen degradation from stock and from newly synthesized glycogen (the UDP-glucose to glycogen flux) has not been studied before. FFA restricted glycogen
breakdown from liver stock and thereby contributed to a relatively sparing of liver glycogen content. The degradation of newly formed glycogen however also contributed to a substantial (20%) and constant part of total glycogenolytic flux, and suppression of FFA by acipimox did not influence this pathway. A possible explanation for the persistence of glycogenolysis in the absence of FFA could be that in the acute absence of FFA, FFA oxidation decreases, leading to a shortage of ATP in the cell. The concomitant increase in the intracellular AMP concentration that will presumably follow, may act as an activator of glycogen phosphorylase b, inducing glycogen breakdown (49).

**Glycogen synthesis**

This study shows that between 16 and 22 hrs of fasting glycogen synthesis remains unchanged, confirming earlier data by Hellerstein et al. (20). As far as we know the influence of FFA on these pathways of liver glycogen synthesis has not been described before in humans *in vivo*. We showed that suppression of physiological FFA levels did not influence glycogen synthesis (flux) during short-term fasting; the gluconeogenic pathway and the plasma glucose flux contributed equally to (retained) glycogen synthesis, both in the presence and absence of FFA. The same was true for the total UDP-glucose to glucose flux, i.e. glycogen synthesis followed by breakdown, that did not decrease significantly, with or without FFA.

We know of only one study in which changes in net glycogen content in relation to FFA were measured with $^{13}$C magnetic resonance spectroscopy in healthy humans, during fasting and during lipid infusion; lipid infusion prevented the decrease in liver glycogen content found during fasting alone. This study however measured changes in net glycogen content, and not glycogen synthesis and breakdown separately (13).

**Conclusion**

This study shows that physiological concentrations of FFA exert important changes in intrahepatic fluxes of glucose metabolism in healthy fasting humans. The decrease in glucose production (i.e. glycogenolysis) normally seen during fasting is blunted in the absence of FFA. We confirm that FFA stimulate gluconeogenesis during short-term starvation, and that the direct gluconeogenic pathway is quantitatively the most important. Indirect gluconeogenic flux remains constant,
irrespective of physiological FFA levels. FFA counteract liver glycogen stock depletion by inhibiting glycogen breakdown. Glycogen synthesis is unaffected by FFA. This suggests that FFA do not regulate glycogen synthesis in the postabsorptive state. Plasma glucose and indirect gluconeogenic flux contribute substantially to glycogen synthesis during fasting.

During short-term starvation, intrahepatic fluxes are directed towards a relative sparing of liver glycogen stock. These data indicate that FFA are an important regulator of intrahepatic glucose fluxes during short-term (<24 hrs) starvation.

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