Regulators of hepatic glucose and glycogen metabolism

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CHAPTER 7

Glycogenolysis during short-term fasting in malaria and healthy subjects: the potential regulatory role of glycogen content on glycogen breakdown.

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Metabolism, Clinical and Experimental, submitted
Abstract

During short-term starvation (<24hrs) glucose production decreases 10-20% due to a decrease in glycogenolysis. In the fed state it has been suggested that glycogen regulates its rate of breakdown in order to limit glycogen accumulation. Whether in the fasted state a similar mechanism exists to preserve glycogen content is not known. In malaria, the rate of glycogen breakdown after an overnight fast is considerably lower than in healthy subjects, which may reflect a lower liver glycogen content. If glycogen content regulates its rate of breakdown during fasting, we postulate that the rate of glycogenolysis should decrease faster in patients with malaria than in healthy subjects. We studied 6 Vietnamese non-severe falciparum malaria patients between 16 and 22 hrs of fasting, and compared them to healthy Vietnamese controls. We measured glucose production with [6,6-2H2]-glucose, and glycogenolysis was calculated after measuring gluconeogenesis with the 2H2O-method.

Glucose production at baseline (16 hrs of fasting) was 15% higher in malaria patients than in controls. Glycogenolysis was much lower in malaria (2.3+/−0.37 μmol/kg/min versus 8.4 +/- 0.93 μmol/kg/min), while plasma glucose concentration was not different. Between 16 and 22 hrs of fasting, glucose concentration decreased similarly in patients and controls (malaria: 5.6±0.19 to 5.2±0.21 mmol/l (p=0.01), control: 5.1±0.13 to 4.8±0.15 mmol/l (p=0.03)). The absolute decrease in glycogenolysis was slower in malaria patients than in controls (p=0.001), whereas the relative decrease in glycogenolysis from baseline was not different.

We conclude that during fasting, the relative decrease in glycogenolysis is independent of the absolute rate of glycogenolysis. Breakdown of glycogen is not slowed down by a lower absolute glycogenolysis rate in malaria. The regulation of glycogenolysis seems not preferentially dictated by glycogen content but rather driven by the necessity to guarantee glucose output and maintain euglycaemia.
Introduction

During fasting, endogenous glucose production is the sum of glycogenolysis and gluconeogenesis. Data in rats indicate that glycogen synthase $a$ is fully active in the liver of fasted animals and that the absence of net glycogen accumulation is due to continuous glycogenolysis by phosphorylase $a$. Magnusson et al. showed that the relative turnover rate of glycogen, measured by $^{13}$C nuclear magnetic resonance spectroscopy (NMR) in healthy humans was significantly higher in the fed than in the fasted state, and they suggested that glycogen may regulate its rate of breakdown, preventing accumulation of liver glycogen. This suggestion is supported by studies by Magnusson et al. and Petersen et al. who showed that respectively type 2 diabetes patients and patients with liver cirrhosis synthesized less glycogen after a meal, and had a slower rate of glycogenolysis than matched healthy subjects.

In healthy subjects, glucose production decreases linearly by 10 - 20% between 16 and 22 hrs of fasting. This decrease is due to a decrease in glycogenolysis, as gluconeogenesis, expressed in absolute values, remains constant during the first 24 hrs of starvation. Concomitantly with the decrease in glycogenolysis, human hepatic glycogen content decreases linearly in the first 22 hrs of fasting. These data support a link between glycogen content and glycogen breakdown, as was previously explicitly suggested by Magnusson et al.

If a lower rate of glycogenolysis reflects a lower liver glycogen content, one would expect a faster decline in the rate of glycogenolysis in subjects with a lower initial rate of glycogenolysis, in order to restrain further loss of glycogen content. Malaria is a disease in which glycogenolysis is known to be considerably lower than in the healthy state after a similar overnight fast.

To test whether the lower rate of glycogenolysis in malaria would decrease faster than in healthy controls during fasting, we measured glucose production, gluconeogenesis and calculated glycogenolysis, in Vietnamese patients with non-severe falciparum malaria, and in healthy Vietnamese subjects during a 16 hr to 22 hr fast. We expected glucose production and glycogenolysis to decrease faster in time in malaria patients than in the healthy subjects.
Subjects and Methods

Subjects: twelve Vietnamese subjects were included in the study after written informed consent. Six malaria patients diagnosed with non-severe falciparum malaria were included, and six healthy volunteers (controls). Their characteristics are shown in Table 1. Patients were recruited immediately after laboratory confirmation of the clinical diagnosis and exclusion of quinine use by a quinine dipstick.15

Table 1. anthropometric and biochemical data, comparing baseline values (t=0h, 16 hrs of fasting) of six Vietnamese non-severe falciparum malaria patients, and six healthy Vietnamese controls.

<table>
<thead>
<tr>
<th>at baseline</th>
<th>malaria</th>
<th>p value</th>
<th>healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td>sex</td>
<td>1F:5M</td>
<td>n.s.</td>
<td>1F:5M</td>
</tr>
<tr>
<td>age, yr</td>
<td>26.3±4.2</td>
<td>n.s.</td>
<td>25.8±4</td>
</tr>
<tr>
<td>weight, kg</td>
<td>51.5±1.8</td>
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<td>length, m</td>
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<td>BMI, kg/m²</td>
<td>1.59±0.02</td>
<td>n.s.</td>
<td>1.64±0.02</td>
</tr>
<tr>
<td>parasitaemia</td>
<td>5.91±4 2185</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>creatinine μmol/L</td>
<td>116±7</td>
<td>n.s.</td>
<td>98±7</td>
</tr>
<tr>
<td>total bilirubin μmol/L</td>
<td>2143</td>
<td>n.s.</td>
<td>18±1</td>
</tr>
<tr>
<td>direct bilirubin μmol/L</td>
<td>6.8±1.5</td>
<td>n.s.</td>
<td>4.6±0.2</td>
</tr>
<tr>
<td>ASAT U/L</td>
<td>63±16</td>
<td>n.s.</td>
<td>31±3</td>
</tr>
<tr>
<td>ALAT U/L</td>
<td>60±16</td>
<td>n.s.</td>
<td>31±5</td>
</tr>
<tr>
<td>Hb mmol/L</td>
<td>8±0.73</td>
<td>n.s.</td>
<td>9±0.34</td>
</tr>
<tr>
<td>lactate, mmol/L</td>
<td>0.92±0.16</td>
<td>n.s.</td>
<td>0.78±0.13</td>
</tr>
</tbody>
</table>

Patients were admitted to Bao Loc Provincial Hospital, Lam Dong Province, Vietnam. Exclusion criteria were complicated malaria according to WHO-criteria, age under 16 years, treatment with quinine (quinine stimulates insulin secretion), concomitant infectious disease, pregnancy, malnutrition, diabetes mellitus or a family history of diabetes mellitus, and anorexia leading to a diminished food intake in the three days before the study.16,17 Each patient was treated with
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Both the patients and the controls had consumed a weight-maintaining diet containing at least 250 grams of carbohydrate in the three days preceding the study. The study protocol was approved by the institutional review board of Cho Ray Hospital, Ho Chi Minh City, under whose jurisdiction research in the Lam Dong Provincial Hospital is performed and by the institutional Medical Ethical Committee and Research Committee of the Academic Medical Center in Amsterdam, the Netherlands.

Study design: the patients participated in the study on the day after admission. On the evening before the study, patients and volunteers consumed a standard dinner at 6 p.m. (t= -16h), after which they were fasted until completion of the study (t= +6h). Water was ingested ad libitum. During the study, patients and volunteers were confined to bed. At 4 a.m. (t= -6h) a urine sample was obtained for determination of background enrichment with $^{2}$H$_2$O in urine, and blood was drawn for measurement of background (natural) $^2$H-enrichment in glucose. Patients and controls then ingested 5 doses of 1g/kg body water of $^2$H$_2$O each, with 30 minutes interval (total dose of 5g/kg body water). Body water was calculated as 60% of body weight in males and 50% body weight in females. From that moment onwards, all ingested water and all intravenously infused saline was 0.5% enriched with $^2$H$_2$O.

At t= -2hr, an i.v. cannula was inserted into the left and right forearm, one for isotope infusion and the other for drawing blood samples. Catheters were kept patent by a slow isotonic saline drip. After obtaining a baseline blood sample for determination of background (natural) isotope abundance of [6,6-$^2$H$_2$]-glucose, a primed (17.6 μmol/kg), continuous (0.22 μmol/kg/min) infusion of [6,6-$^2$H$_2$]-glucose (99%, Isotec Inc., Miamisburg OH, USA), dissolved in sterile isotonic saline and sterilized by passage of the solution through a millipore filter (size 0.2 μm; Minisart, Sartorius AG, Göttingen, Germany) was administered by a motordriven calibrated syringe pump (Perfusor Secura FT, Braun, Melsungen AG, Germany). At t= -15, t= -10, and t= -5 min, blood samples were collected for determination of plasma glucose concentration and [6,6-$^2$H$_2$]-glucose enrichment to confirm isotopic steady state.

At t=0h (10 a.m. and 16hrs of fasting), a baseline urine sample was obtained for determination of $^2$H-enrichment, and blood was drawn for determination of baseline $^2$H-enrichment in blood glucose. Baseline samples for routine
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haematological and biochemical tests, plasma concentration of lactate, insulin, glucagon, cortisol, epinephrine and norepinephrine were also obtained. Between t=0h and t=6h (22hrs of fasting, end of the study), blood samples for measurement of plasma glucose concentration and [6,6-\(^2\)H\(_2\)]-glucose enrichment were obtained every 30 min. Blood samples for determination of \(^2\)H-enrichment in blood glucose were drawn, and urine samples for \(^2\)H enrichment in body water were obtained at t=2h, t=4h and t=6h. After t=0h, blood samples for measurement of glucoregulatory hormones (insulin, cortisol, glucagon and catecholamines) were also measured at t= 4h, and t=6h. The total amount of blood taken was 100 ml. All samples were and collected in pre-chilled tubes, kept on ice and centrifuged promptly. A sample for lactate was collected into a fluoride tube and deproteinized immediately with PCA 1M. Samples for insulin and routine biochemical tests were collected in heparinized tubes, samples for haematological tests were collected in tubes with EDTA-buffer, whole blood was added to trasylool for glucagon determination, and to reduced glutathion-EGTA buffer for determination of epinephrine and norepinephrine. Aliquots of separated plasma were stored below -20°C before assay and were transported from Vietnam to the Netherlands on dry ice before assay.

Assays: all measurements in each individual subject were performed in the same run, and all samples were tested in duplicate, except the deuterium enrichment at carbon 5 of glucose. The plasma glucose concentration and [6,6-\(^2\)H\(_2\)]-glucose enrichment in plasma were measured using a method adapted from Reinauer et al.\(^b\). The aldonitri l penta-acetate derivative of glucose was dissolved in ethylacetate. A calibration graph using xylose as an internal standard was used for the determination of glucose concentration. The enrichment of [6,6-\(^2\)H\(_2\)]-glucose was determined by dividing the peak at M+2 by the total peak of the glucose aldonitril penta-acetate peak and correction for the natural abundance by subtracting the natural abundance of the M+2 enrichment from the measured M+2 enrichment. The deuterium enrichment at carbon 5 of glucose was measured as described by Ackermans et al.\(^c\). All isotopic enrichments were measured on a gas chromatograph mass spectrometer (Gas Chromatograph Model 6890 coupled to a model 5973 mass selective detector, equipped with an electron impact ionisation mode, Hewlet Packard, Palo Alto, CA).

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Plasma insulin concentration was determined by RIA (Insulin RIA 100, Pharmacia Diagnostic AB, Uppsala, Sweden; intra-assay coefficient of variation (CV) 3-5%, inter-assay CV 6-9%, detection limit 12 pmol/l (2mU/l)). Glucagon was determined by RIA (Linco Research, St Charles, MO, USA, intra-assay CV 3-5%, inter-assay 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%, inter-assay CV 3-7%, detection limit 50 nmol/l. Catecholamines were measured by an in-house HPLC method; norepinephrine: inter-assay CV 6-8%, intra-assay CV 7-10%, detection limit 0.05 nmol/l, and epinephrine: inter-assay CV 6-8%, intra-assay CV 7-12%, detection limit 0.05 nmol/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 for [6,6-\textsuperscript{2}H\textsubscript{2}]glucose were constant during each sampling phase of the study, calculations for steady state kinetics were applied, adapted for the use of stable isotopes\textsuperscript{22,23}. Data are reported as means ± s.e.m. All differences between malaria and controls were analyzed using an independent sample t-test at baseline (t=0h) and/or at the end of the study (t=6); differences in the (relative and absolute) decrease in glycogenolysis between malaria and control study were also analyzed by comparing the slopes of the curves in an independent sample t-test. Time points within either study (i.e. at t=0h and t=6h) were compared using a paired sample t-test. Statistical significance was set at p<0.05.

Results

Clinical data (see table 1)
The malaria patients had mild clinical signs of illness at admission meeting the WHO-criteria for non-severe malaria\textsuperscript{16}. The median duration of the illness before admission was 3 (range 1-10) days. Apart from the parasitaemia, the malaria patients did not differ anthropometrically or biochemically from the healthy controls.
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Table 1. Anthropometric and biochemical data, comparing baseline values (t=0h, 16 hrs of fasting) of six Vietnamese non-severe falciparum malaria patients, and six healthy Vietnamese controls.

<table>
<thead>
<tr>
<th>at baseline</th>
<th>malaria</th>
<th>p value</th>
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<td>n.s.</td>
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<tr>
<td>parasitaemia</td>
<td>5913±2185</td>
<td>-</td>
<td>-</td>
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<tr>
<td>creatinine μmol L.</td>
<td>116±7</td>
<td>n.s.</td>
<td>98±7</td>
</tr>
<tr>
<td>tot.bilirubin μmol L</td>
<td>21±3</td>
<td>n.s.</td>
<td>18±1</td>
</tr>
<tr>
<td>dir.bilirubin μmol L</td>
<td>6.8±1.5</td>
<td>n.s.</td>
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</table>

Glucose kinetics. (see figs 1 and 2.)
Glucose concentration did not differ significantly between patients and controls at baseline or at t=6h, and decreased significantly but not differently in both studies between t=0h and t=6h. (control: 5.1±0.13 to 4.8±0.15 mmol/l (p=0.03); malaria: 5.6±0.19 to 5.2±0.21 mmol/l (p=0.02)). Glucose production at baseline was higher in malaria than in controls (p=0.007), and between t=0h and t=6h, glucose production decreased in both studies (control: 17.3±0.48 to 13.3±0.21 μmol/kg/min (p<0.0001); malaria: 20±0.59 to 16.3±0.31 μmol/kg/min (p<0.0001))(fig.2). The decrease in glucose production was not different between the studies as indicated by the slopes of both curves (control: y= -0.57±0.04 and malaria : y= -0.53±0.06). Glycogenolysis at baseline was lower in malaria than in the healthy volunteers (p=0.001) and glycogenolysis decreased in both studies (control: 8.4±0.93 to 3.5±0.45 μmol/kg/min (p<0.0001); malaria: 2.3±0.37 to 0.6±0.35 μmol/kg/min (p=0.008)). The decrease in the absolute rate of glycogenolysis was different between the studies as reflected by the slopes of both curves (control:
y = -0.28±0.06 and malaria: y = -0.83±0.1 (p=0.001)(Fig 2a). The percentual decrease in glycogenolysis from baseline however was not different between the two studies (control: 100±0% to 41%±3%; malaria: 100±0% to 23±14%), as reflected by the (slopes of the curves control: y = -0.098±0.007 and malaria: y = -0.136±0.027 (Fig 2b).

**Fig. 1.** Glucose production in μmol/kg/min between 16 and 22 hr of fasting in six Vietnamese non-severe falciparum malaria patients (●) (slope: y = -0.53x+19), and six healthy Vietnamese controls (o)(slope: y = -0.57x+16). Data are presented as means ± SE.

**Fig 2.a** Glycogenolysis in μmol/kg/min between 16 and 22 hrs of fasting in six Vietnamese non-severe falciparum malaria patients (●)(slope: y = -0.83x+8.4) and six healthy Vietnamese controls (o)(slope: y = -0.28x+2.4).
Fig 2.b) percentual decrease in glycogenolysis from baseline, in malaria patients (●)(slope: y = -0.098x+0.99) and healthy controls (○)(slope: y = -0.14+1.10). Data are presented as means ± SE.

Glucoregulatory hormones (see table 2)
The concentrations of glucoregulatory hormones were not different between both groups at baseline except for glucagon, which was higher in malaria patients than in controls (p=0.03). Insulin did not decrease in malaria, but decreased in controls (p=0.006), glucagon, cortisol, epinephrine and norepinephrine did not change in time in either study.

Table 2. Glucoregulatory hormone concentrations at baseline (t=0h, 16hrs of fasting) and at the end of the study (t=6h, 22hrs of fasting) of 6 Vietnamese non-severe falciparum malaria patients, and 6 healthy Vietnamese controls.

<table>
<thead>
<tr>
<th>hormones</th>
<th>malaria</th>
<th>p value t=0h</th>
<th>healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t=0h</td>
<td>t=6h</td>
<td>malaria vs control</td>
</tr>
<tr>
<td>insulin, pmol/L</td>
<td>38±3</td>
<td>27±3</td>
<td>n.s.</td>
</tr>
<tr>
<td>glucagon, ng/L</td>
<td>54±5</td>
<td>66±8</td>
<td>0.03</td>
</tr>
<tr>
<td>cortisol</td>
<td>190±19</td>
<td>157±23</td>
<td>n.s.</td>
</tr>
<tr>
<td>epinephrine, nmol/L</td>
<td>0.23±0.06</td>
<td>0.43±0.11</td>
<td>n.s.</td>
</tr>
<tr>
<td>norepinephrine, nmol/L</td>
<td>0.97±0.17</td>
<td>0.95±0.21</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
Glycogenolysis in malaria

Discussion

Magnusson et al. suggested that during feeding liver glycogen may regulate its rate of breakdown in order to prevent accumulation of liver glycogen based on the finding that glycogen breakdown rates were higher in the fed (high liver glycogen) than in the fasted state (low liver glycogen). The aim of the present study was to investigate whether during short-term starvation (<24 hrs) glycogen breakdown adapts to a decreasing liver glycogen content in order to restrain glycogen depletion. From Magnusson's findings, we postulated that the lower rates of glycogenolysis found in non-severe falciparum malaria compared to healthy controls may reflect a lower liver glycogen content in malaria. We hypothesized that between 16 and 22 hr of fasting, the (lower) rate of glycogenolysis in malaria would decrease faster than in healthy controls, in order to restrain glycogen breakdown.

Our data show that the decrease in the rate of glycogenolysis was not faster but slower in malaria than in healthy controls between 16 and 22 hr of fasting, despite a much lower rate of glycogenolysis in malaria. The relative decrease in glycogenolysis from baseline in malaria was similar to that in controls. These data indicate that during fasting other factors than glycogen content dictate the change in the rate of glycogenolysis over time.

The decrease in glucose production rate observed in both studies is in agreement with previously published data. Glycogenolysis after an overnight fast was lower in malaria patients, as expected, and in agreement with data from literature. These data indicate that during fasting other factors than glycogen content dictate the change in the rate of glycogenolysis over time.

The above-mentioned findings cannot be explained by confounding variables such as differences in food intake in the days preceding the study, or anthropomorphic differences between patients and controls. The malaria patients and the healthy controls were equally well proportioned, not malnourished and had anamnestically eaten well as usual before and during their illness.

There were some differences in glucoregulatory hormone concentrations between the two studies. Insulin levels were not different at baseline or at the end of the study, although the decrease in insulin did not reach statistical significance in the malaria study. We believe it is unlikely that this difference influenced our results,
as glucose production was persistently higher in the malaria study, whereas insulin levels were not different. Glucagon was higher in malaria than in controls at baseline, and this difference was maintained throughout the time course of both studies: glucagon did not change over time in either of the studies. Glucagon is a strong and immediate stimulator of glycogenolysis.\(^{26,27}\) We cannot rule out a relationship between the higher rate of glucose production in malaria and their higher glucagon levels, but the lower rates of glycogenolysis in malaria are inconsistent with higher glucagon levels. We can also not rule out that the lesser decrease in the rate of glycogenolysis in malaria patients stands in relation to higher glucagon levels. Cortisol levels were higher in malaria at baseline and at the end of the study, although this difference did not reach statistical significance. As cortisol stimulates glycogenolysis, the same arguments as for glucagon apply to cortisol.\(^{28,29}\) Epinephrine and norepinephrine levels were not different between the studies. Epinephrine seems higher in the healthy controls, but these epinephrine levels stay below the threshold for an effect of epinephrine on glycogenolysis (0.55 nmol/l).\(^{30-32}\)

Our hypothesis is based on studies on the relation between glycogen content and the rate of glycogenolysis, with the implicit assumption that a lower rate of glycogenolysis is indicative for a lower glycogen content.\(^1,2\) Theoretically a dissociation between glycogen content and the rate of glycogenolysis is a possibility, but this seems unlikely. In living humans, glycogen content can only be measured reliably by \(^{13}\)C NMR spectroscopy. All available studies indicate or suggest a positive correlation between glycogen content and the rate of glycogenolysis, both in healthy subjects and in disease. After a meal, both type 2 diabetes patients and patients with liver cirrhosis synthesize less glycogen, and both also have a lower glycogen breakdown rate than healthy age- and BMI-matched subjects, as shown by the decrease in liver glycogen by \(^{13}\)C NMR spectroscopy.\(^5,6\) In healthy subjects, in a study comparing glycogen synthesis and breakdown after an overnight fast and in the fed state, glycogenolysis was higher in the fed state (high liver glycogen), than in the fasted state (glycogen content substantially lower).\(^2\) We therefore believe that the principle on which our hypothesis was built, i.e. glycogen content influences glycogenolysis, finds considerable support in literature. Nonetheless, despite a presumed lower liver glycogen content, glycogenolysis was not restrained in malaria patients compared to healthy subjects.
A potential error in the interpretation of the results depends on the way of calculating glycogenolysis. Glucose production and gluconeogenesis were measured, while glycogenolysis was calculated as the difference between glucose production and gluconeogenesis. Glucose is produced by the liver and the kidney. Gluconeogenesis takes place in liver and kidney, whereas glycogenolysis, as a direct source for plasma glucose, only takes place in the liver. The isotopic technique we used does not discriminate renal or hepatic glucose production and gluconeogenesis. Would malaria preferentially stimulate glucose production by the kidney, the contribution of glycogenolysis to glucose production by the liver could be underestimated, concomitantly with an underestimation of the presumed liver glycogen content. We cannot refute this, but there are no data in literature to support such a possibility.

It can be argued that comparing data obtained in patients and healthy subjects is inappropriate and that we should have compared healthy subjects with a normal and low glycogen content. Theoretically this is true. However it should be kept in mind that it is not possible to induce big differences in glycogen content in healthy humans without inducing other confounding variables. A low content can be induced by prolonged starvation or feeding a carbohydrate free diet. Prolonged starvation stimulates the secretion of the glucoregulatory hormones. Feeding a eucaloric carbohydrate free diet requires an increase in the fat content of the diet with the concomitant induction of insulin resistance. In the present study the feeding pattern was controlled and, except for a slight increase in plasma glucagons, glucoregulatory hormones were not different between patients and healthy subjects. We therefore consider our study design as appropriate as possible in humans.

In conclusion, Magnussen et al. described a mechanism for autoregulation of glycogen breakdown by glycogen content in humans in the fed and fasted state. Their results indicate that the influence of glycogen content on glycogen turnover may importantly limit the accumulation of liver glycogen during feeding. If a similar mechanism existed under conditions of lower glycogen content in order to preserve liver glycogen (e.g. during fasting), we would have expected the rate of glucose production to decline faster in the malaria patients than in healthy volunteers. We found that the relative decrease in glycogenolysis was the same in malaria and controls, and the absolute decrease in glycogenolysis was even slower.
in malaria patients, despite their lower initial rates of glycogenolysis, presuming a lower liver glycogen. It therefore seems that, during fasting, the regulation of the rate of glycogenolysis is not preferentially dictated by glycogen content but by the necessity to contribute to glucose output and maintain euglycaemia.

Acknowledgements

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References

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