Fasting in healthy subjects is associated with intrahepatic accumulation of lipids as assessed by $^1$H-magnetic resonance spectroscopy

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ABSTRACT

The impact of fasting on IHL (intrahepatic lipid) content in human subjects has not been investigated previously, but results indicate that it may change rapidly in response to metabolic cues. The aim of the present study was to measure IHL content after fasting and to correlate this with circulating lipid intermediates. A total of eight healthy non-obese young males were studied before and after 12 or 36 h of fasting. IHL content was assessed by $^1$H-magnetic resonance spectroscopy, and blood samples were drawn after the fasting period. IHL content increased significantly after the 36 h fasting period [median increase 156 % (range, 4–252 %); $P < 0.05$]. Furthermore, a significant positive correlation between this increase and 3-hydroxybutyrate concentration was detected ($P = 0.03$). No significant change in IHL content was demonstrated after the 12 h fasting period. The baseline median inter-individual variation in IHLs was 0.51 % (range, 0.25–0.72 %). The coefficient of variation of IHL measurements was 11.6 %; 25–30 % of the variation was of analytical origin and the remaining 70–75 % was attributed to repositioning. In conclusion, IHL content increases in healthy male subjects during fasting, which demonstrates that nutritional status should be accounted for when assessing IHLs in clinical studies. Moreover, the increase in IHLs was positively correlated with the concentration of 3-hydroxybutyrate.

INTRODUCTION

NAFLD (non-alcoholic fatty liver disease) is prevalent in obese subjects and is considered, by many, to be an important feature of the metabolic syndrome. Although the majority of such cases are asymptomatic, several reports underline an association between NAFLD and dyslipidaemia, hypertension, diabetes and cardiovascular disease [1]. The correlation between NAFLD and insulin resistance is close, and is independent of BMI (body mass index), gender and age [2–5]. Furthermore, the morbidity and mortality in patients with NAFLD is increased [6], which is attributed to cardiovascular disease, diabetes and liver cirrhosis [7–9].

Elevation of NEFAs (non-esterified fatty acids) appears to be pivotal in the development of NAFLD, but the
amount of IHLs (intrahepatic lipids) is also determined by factors such as the capacity of hepatocytes to uptake and turnover NEFAs, the release of TAG [triacylglycerol (triglyceride)] in VLDL (very-low-density-lipoprotein) particles and TAG storage in spherical droplets [10]. Donnelly et al. [11] have calculated that, in patients with NAFLD, 59 % of TAG in the liver derives from NEFAs, 26.2 % from de novo lipogenesis and 14.9 % from the diet.

Intervention studies reveal that the pool of IHLs may change rapidly, especially in response to dietary changes. In patients with Type 2 diabetes, 2 weeks on a hypocaloric diet or an 8 % weight reduction reduce IHLs by 27 and 81 % respectively [12,13]. Furthermore, 2 weeks on a low-fat diet has been shown to decrease IHLs by 20 % in overweight non-diabetic subjects [14]. In rats, as little as 3 days of high-fat feeding causes a 3-fold increase in IHLs [15].

It is well-described that fasting induces an increase in circulating levels of NEFAs and a decrease in both hepatic and peripheral insulin sensitivity. This is associated with an increase in both intramyocellular lipid content [16,17] and hepatic production of ketone bodies [18], both of which reflect mobilization and oxidation of lipid intermediates. Therefore fasting is probably associated with significant changes in IHLs in human subjects, but this remains to be verified experimentally.

The aim of the present study, therefore, was to measure changes in IHLs during fasting in normal subjects by $^1$H-MRS (magnetic resonance spectroscopy) and to correlate this with circulating levels of ketone bodies.

**MATERIALS AND METHODS**

**Subjects**

The study population comprised eight healthy non-obese young males [age, 23.6 ± 0.7 years; BMI, 22.8 ± 0.4 kg/m²; fasting plasma glucose, 4.3 ± 0.1 mmol/l; HbA1c (glycosylated haemoglobin A), 5.5 ± 0.1 %; ALT (alanine aminotransferase), 23.4 ± 2.8 units/l; TAG, 0.73 ± 0.08 mmol/l; total cholesterol, 4.2 ± 0.2 mmol/l; HDLs (high-density lipoproteins), 1.5 ± 0.1 mmol/l; and LDLs (low-density lipoproteins), 2.4 ± 0.2 mmol/l]. All subjects were students from the local university.

The exclusion criteria included a family history of Type 2 diabetes, use of any medication, alcohol consumption > 21 units/week, known liver disease, claustrophobia and the presence of magnetic implants.

All subjects were instructed to consume a diet with no major deviations from the national recommendations (i.e. a maximum of 30 % of energy from fat, 50–60 % from carbohydrates and 10–20 % from protein), to avoid high-fat meals and to abstain from alcohol for 3 days before each study period. During the fasting period they were allowed to drink tap or mineral water and to perform normal ambulatory activities, excluding any kind of exercise.

**Study design**

The subjects were randomly assigned to one of two groups: group 1, $^1$H-MRS examination at baseline and after 36 h of fasting ($n = 6$); and group 2, $^1$H-MRS examination at baseline and after 12 h of fasting ($n = 3$). Blood samples were drawn from both groups immediately after the fasting period. One of the subjects participated in both groups separated by 2 months.

The protocol was approved by the regional Ethics Committee, and the nature and potential risks were explained before participants gave written informed consent. The study was conducted according to the declaration of Helsinki (2000) of the World Medical Association.

**$^1$H-MRS**

$^1$H-MRS was performed using a Signa Excite 1.5 telsa twin speed scanner (GE Medical Systems). The subjects were allowed to eat and drink until the beginning of the study period. The baseline examination was performed at 20.00 hours. Group 1 was then fasted for 36 h and group 2 was fasted overnight (12 h), before re-examination at 08.00 hours.

During each visit, three $^1$H-MRS measurements (spectra 1–3) were made to enable calculations of the inter-individual variation in baseline IHLs and the intra-day CV (coefficient of variation) to be made. First, spectra 1 and 2 were obtained (with the same shimming and spatial position), and the subject was taken out of the machine, allowed to move around for a few minutes and then repositioned before spectra 3 was made. Subjects were positioned feet first in the supine position. A belt was strapped around the lower part of the thorax and upper part of abdomen to minimize respiratory movement of the diaphragm and liver. To generate the spectra, a standard whole-body coil was used for radio-frequency transmission and signal reception. The exact orientation of the liver was verified in a three-plane T2-weighted localizer pulse sequence.

An oblique plane T1 weighted gradient echo pulse sequence using a single breath-holding technique with a TR (repetition time) of 140 ms and a TE (echo time) of 2.2/4.4 ms was performed to enable identification of the area of interest.

The volume of interest (2 cm$^3$ × 2 cm$^3$ × 3 cm$^3$) was carefully positioned in the lower posterior part of the liver (area 6), avoiding the inclusion of costae, visible vessels and the bile duct. Auto-shimming was performed to optimize the magnetic field homogeneity. A water-suppressed point resolved spectroscopy sequence during free breathing (TE, 30 ms; TR, 2000 ms; number of acquisitions, 128) was applied, using water as the auto-centre frequency. FWHM (full width at
Figure 1  Spectra from subject number 4 before (baseline) and after the 36-h fast
(A) Suppressed water peak; (B) lipid peak.

half maximum) of the unsuppressed water peak was 10.2 ± 1.6 Hz. Each session lasted approx. 40 min.

Analytical procedures and calculations

RLC (relative lipid content)
The spectra were analysed using SAGE (version 7; GE Medical Systems). The height of the suppressed water signal intensity peak ($S_{\text{water}}$) was measured at approx. 4.8 p.p.m. and the lipid/(-CH2)-, signal intensity ($S_{\text{lipid}}$) peak at approx. 1.4 p.p.m. Representative spectra from subject number 4, before and after the 36-h fast, are shown in Figure 1. Peak height rather than the AUC (area under the curve) was used, as our FWHM was relatively low and constant (10.2 ± 1.6); moreover, using AUC resulted in a higher CV.

RLC was calculated using the following formula:

$$\text{RLC} = \frac{(S_{\text{lipid}} \times 100)}{S_{\text{water, corr}} + S_{\text{lipid}}}[19]$$

$$S_{\text{water, corr}} = \frac{(S_{\text{water}} \times 100)}{\text{WS\%}}$$

where WS\% is the percentage of water suppression.

Validation of the data

The CV was calculated by dividing the S.D. with the mean (of the three RLCs), and is presented as a percentage. To demonstrate the intra-day CV, the median CV of RLCs was determined.

Blood analysis

A blood sample was drawn at the end of the fasting period. Plasma glucose was measured in duplicate immediately after sampling on a Beckman Glucoanalyser (Beckman Instruments). Serum samples were frozen immediately and stored at −20° C. Insulin, growth hormone and cortisol were analysed using a time-resolved fluoroimmunoassay (AutoDELFIA; PerkinElmer), C-peptide was analysed by ELISA (DakoCytomation), and NEFAs were analysed using a commercial kit (Wako Chemicals). ALT was determined using a commercial method (Cobas Integra 800; Roche Diagnostics). Glycerol, lactate, alanine and 3-hydroxybutyrate were measured using a Cobas biocentrifugal analyser with fluorimetric attachment (Roche Diagnostics) [20].

Statistical analysis

Intercooled Stata 9.0 was used for the statistical analysis. To analyse the changes in IHLs, the mean of the three RLCs, at baseline and after the fasting period, were used as the best estimates of the ‘true’ values, and a paired Student’s $t$ test was used to determine statistical significance. For estimation of the different components of variation in the RLC measurements, two Bland–Altman analyses were made followed by a two-component ANOVA on the S.D. For statistical significant results, a $P$ value < 0.05 was required. As the distribution of the RLC data were skewed, data were log-transformed before applying the relevant statistical tests, and only the medians are shown. Unless otherwise stated, results are presented as means ± S.E.M.

RESULTS

Baseline variation in RLCs

The inter-individual variation in RLCs before the fasting periods ranged from 0.25 to 0.72 % (5th to the 95th percentile) with a median of 0.51 %. As one of the subjects participated in both the 12h- and 36-h fast, a mean of his baseline RLC was used.

Validation of RLC measured by $^1$H-MRS

The median CV for RLC measured by $^1$H-MRS was 11.6 %. None of the spectra were significantly different, as shown in Figure 2. After performing a two-component analysis, 25–30 % of the variation was found to be due to differences between spectra 1 and 2, attributed to elements of uncertainty in the analysis of data, equipment errors and respiration movement, with the remaining
Reproducibility of the three measurements of RLC obtained at the 18 magnetic resonance sessions

The broken line depicts the optimal correlation (slope = 1). (A) Correlation between RLC 1 and 2 (r = 0.97, P < 0.01); (B) correlation between RLC 1 and 3 (r = 0.95, P < 0.01); (C) correlation between RLC 2 and 3 (r = 0.93, P < 0.01).

70–75% being attributed to repositioning before the third spectra was made. This underlines the importance of precision when the voxel is placed.

Changes in IHLs during the fasting periods

The IHL increased significantly during the 36-h fast. Median RLC increased from 0.42 to 0.74% (95% confidence interval, 1.34–3.54%; P = 0.009) (Figure 3). The median RLC% (percentage change in RLC) during the 36-h fast was 156% (range, 4–252%; P < 0.05) (Table 1). No significant change in the IHL was observed after the 12-h fast (RLC, 0.72% before compared with 0.42% after fasting; P = 0.45).

Analytes in blood

The levels of the measured analytes at the end of each fasting period are shown in Table 2. Owing to the small sample size, a statistical comparison between the levels after the two fasting studies was not performed, but, as expected, the concentrations of all of the lipid intermediates increased with more prolonged fasting. There was a significant positive correlation between the increase in RLC% and the 3-hydroxybutyrate concentration after the 36-h fast (r = 0.85, P = 0.03), as shown in Figure 4. The RLC% as well as RLC after the 36-h fast did not correlate with either NEFA levels (P = 0.31, P = 0.60) or ALT levels (P = 0.68, P = 0.20).

DISCUSSION

The present study demonstrates for the first time that fasting is associated with an increase in IHLs in human subjects. Apart from extending our knowledge about...
In several studies, ALT levels have been used as a surrogate marker of IHLs [21,22], but, in the present study, ALT did not correlate with any indices of IHLs. This may reflect the short duration of the present study, but it is also plausible that the mechanisms underlying the increase in IHLs during fasting differ from those of NAFLD.

As the present study population consisted of lean and healthy adult males, without any evidence of hepatic disease or steatosis, one would anticipate IHLs to be low. We found a baseline median RLC of 0.51 %, with inter-individual variation ranging from 0.25 to 0.72 % (5th to 95th percentile). Compared with the median of 1.9 % recorded by Szczepaniak et al. [23] in a group with a low risk of steatosis, IHLs in our present subjects is indeed low. Several factors, including dietary habits, physical fitness, age and gender, are known to influence IHLs in otherwise healthy subjects. Westerbacka et al. [14], who measured IHLs by 1H-MRS in obese non-diabetic women before and after 2 weeks of an isocaloric high-fat or low-fat diet, recorded a 20 % decrease in IHLs in the low-fat diet group and a 35 % increase in the high-fat diet group; however, a single high-fat meal does not appear to influence IHLs [23]. A high level of habitual physical activity is associated with low IHLs [24]. Cross-sectional studies indicate that age is inversely related to IHLs [25,26], whereas the impact of gender remains controversial.

In conclusion, the present study shows for the first time that IHLs increase during fasting in healthy human subjects and that this increase correlates positively with circulating levels of 3-hydroxybutyrate. This contributes to our knowledge about the regulation of substrate metabolism during alterations in nutritional supply. It also emphasizes that nutritional status should be standardized when assessing IHLs in patients.

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**REFERENCES**