Fasting for 72 h increases intramyocellular lipid content in nondiabetic, physically fit men

S. R. STANNARD,1 M. W. THOMPSON,1 K. FAIRBAIRN,2 B. HUARD,3 T. SACHINWALLA,3 AND C. H. THOMPSON2

1School of Exercise and Sport Science, Faculty of Health Sciences, The University of Sydney, Lidcombe 1825; 2Human Nutrition Unit, Department of Biochemistry, Faculty of Science, The University of Sydney 2006; 3Rayscan Imaging, Liverpool 2170, New South Wales, Australia

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Stannard, S. R., M. W. Thompson, K. Fairbairn, B. Huard, T. Sachinwalla, and C. H. Thompson. Fasting for 72 h increases intramyocellular lipid content in nondiabetic, physically fit men. Am J Physiol Endocrinol Metab 283: E1185–E1191, 2002. First published July 30, 2002; 10.1152/ajpendo.00108.2002.—The purpose of this study was to determine changes in intramyocellular lipid (IMCL) content in the vastus lateralis of nondiabetic, physically fit males over 72 h of fasting. Six men, mean age 35 yr (range 23–55 yr), body mass index 23.7 kg/m2 (21.2–27.4 kg/m2), undertook a water-only fast for 84 h. Vastus lateralis IMCL content was determined using proton magnetic resonance spectroscopy after 12 and 84 h of fasting. Venous blood was sampled at 12-h intervals throughout the fast. IMCL-(CH2)n/water and IMCL-(CH2)n/total creatine ratios increased by 10.220.33. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

METHODS

Subjects. Six healthy, physically active men volunteered to participate in the study, which was approved by The University of Sydney Human Ethics Committee. Subject characteristics are presented in Table 1. None had any history of

Address for reprint requests and other correspondence: S. Stannard, School of Exercise and Sport Science, Faculty of Health Sciences, The Univ. of Sydney, Lidcombe 1825, NSW, Australia (E-mail: stevestannard@ozemail.com.au).

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diabetes, and their overnight-fasted plasma glucose concentrations averaged 4.7 mmol/l (range 4.1–5.5 mmol/l). All subjects had extensive experience as subjects in human physiological studies in the laboratory.

Study design. Subjects arrived at the laboratory on a Wednesday morning after an overnight (10–12 h) fast. Immediately after venous blood collection, 1H-MRS examination of the vastus lateralis muscle was conducted. No food or drink other than water was ingested until the completion of the fast the following Saturday morning. Venous blood (8 ml) was sampled every 12 h (7 AM and 7 PM) each day from the Wednesday morning until the fast was broken after a second MRS examination on Saturday morning. During the course of the fast, subjects were encouraged to participate in their normal daily routine but limit their exercise to walking required for essential activities. Twenty-four hour per day observer surveillance ensured compliance of subjects with the study protocol (frequent water ingestion only, with minimal physical activity). In total, the fasting period between the two MRS scans and first and last blood samples was 72 h and the total length of the fast ~84 h.

NMR spectroscopy. Image-guided, 1H-localized MRS and high-resolution T1-weighted imaging were performed on a 1.5 Tesla Gyroscan NT whole body system (Philips Medical Systems, Best, The Netherlands) by use of a combination of whole body and circular polarized standard extremity coil for radio frequency transmitting and signal receiving. Volumes of interest were centered within the vastus lateralis muscle at the level of midfemur, 3 cm medial to the axillary line. This position was chosen to avoid the vasculature and subcutaneous adipose depots and to ensure consistent orientation of the muscle fibers along the main magnetic field. Legs were fixed at the ankles, and subjects were instructed to lie as still as possible to prevent movement artifacts. A vitamin E capsule was taped to the skin to identify the area of interest on the MR images. During the initial scan, the leg was marked with indelible ink, and the mark was maintained until the second scan to ensure accurate repositioning of the 1H-MRS voxel. Image-guided spectra were acquired using the point resolved spectroscopy technique [repetition time (TR) = 5,000 ms, echo time (TE) = 32 ms, 32 measurements, 1,024 sample points, acquisition time 3 min]. Fully automated shimming was carried out on the voxel (5 × 1.5 × 1.5 cm) to ensure maximum field homogeneity, and excitation water suppression was used to suppress signal from water during data acquisition. The long repetition time was chosen to ensure a fully relaxed signal, minimizing T1 saturation effects. Unsuppressed water spectra were also acquired for use as an internal standard. Creatine was also used as a standard for quantitation, with signal measurements taken from the water-suppressed spectrum.

Spectral data processing. Spectral data were transferred offline for postprocessing with magnetic resonance user interface (MRUI) software [jMRUI version 1.1, EU Project “Advanced Signal Processing for Medical Magnetic Resonance Imaging and Spectroscopy,” TMR, FMRX-CT97–0160 (27)]. After Fourier transformation and manual phasing of the spectra, the water peak was identified and nominated 4.7 ppm. For the water-suppressed signal, the following steps were taken. 1) Residual water and any signals from 3.4 to 7.5 ppm were suppressed in the time domain with an HLSVD (Hankel Lanczos Singular Values Decomposition) filter; 2) an eight-resonance model was used, including carnitine (trimethyl: 3.2 ppm), total creatine (methyl: 3.0 ppm), lipid (C2 methylene: 2.25 ppm), lipid (aliphatic methane: 2.15 ppm), extramyocellular lipid (EMCL) ([CH2]: 1.45 ppm) and IMCL ([CH2]: 1.35 ppm), and EMCL (1H: 1.5) and IMCL (1H: 0.85 ppm); and 3) the signal amplitude was obtained in absolute units for each resonance by using AMARES, a nonlinear least squares quantitation algorithm. This "prior knowledge" that was applied incorporated known factors from prior publications (33) with the addition of soft constraints on carnitine and creatine resonance frequencies and line widths. Fitting of lipid resonances at 2.15 and 2.25 ppm also allowed more reliable fitting of EMCL and IMCL peaks. The resonances were fitted assuming a Lorentzian line shape for carnitine and creatine and a Gaussian line shape for all other resonances. The zero-order phase correction was manually estimated, and the first-order phase correction was fixed at zero.

The unsuppressed water signal was calculated as follows. 1) The water resonance was identified at center frequency and nominated at 4.7 ppm. 2) The signal amplitude was obtained in absolute units with AMARES. The resonance was fitted to a Lorentzian line shape. As for the other resonances in the water-suppressed signal, zero-order phase correction was manually estimated with first-order correction fixed at zero.

T2 relaxation times were measured in a subset of the subjects during a separate but identical 84-h fast. This was done to ensure 1) that there was no difference in T2 relaxation times of the metabolites considered under 12- and 84-h fast conditions and 2) to allow for T2 relaxation effect correction in quantitation under the study conditions. T2 relaxation times were measured for unsuppressed water, total creatine, CH3-EMCL, and CH3-IMCL resonances by use of the same positioning and volume of the region of interest (TR 1,500 ms, TE 40 ms). Ten measurements were made at 40, 65, 90, 120, 150, 205, 235, 265, and 295 ms. Rates of signal decay were plotted as ln amplitude vs. time in milliseconds, and the linear best line of fit was used to estimate the rate of signal decay.

Seventy-two-hour fasting induced no apparent changes in T2 (Table 2). Furthermore, the measured values are similar to those previously reported (37, 39).

Absolute quantification of metabolites. Signal amplitude of water, carnitine-N(CH3)2, creatine-CH3, EMCL-CH2, and IMCL-CH2, were corrected using appropriate T2 relaxation times available in the literature for leg skeletal muscle [H2O, 1,100 ms (37); IMCL-CH2, 300 ms; EMCL-CH2, 300 ms; creatine-CH3, 1,350 ms (10)] and the T2 relaxation times obtained under study conditions. The (CH2)n resonance was used for measurement of the intramuscular triglyceride content, given the higher signal intensity and narrower line

<table>
<thead>
<tr>
<th>Age, yr</th>
<th>Body Weight, kg</th>
<th>Height, cm</th>
<th>Body Fat, %</th>
<th>VO(2max)</th>
<th>ml O2/kg·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>35(23–55)</td>
<td>74.4(71.3–77.0)</td>
<td>178(171–186)</td>
<td>13.6(9.5–19.9)</td>
<td>65.8(55.3–79.2)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means; nos. in parentheses are ranges; n = 6. VO2max, maximal O2 uptake. Body weight and %body fat are for 14-h fasted. Body density was measured using hydrodensitometry, and %body fat was then estimated using the methods of Brozek et al. (9).
width compared with the CH₃ resonance (39). IMCL-(CH₂)n values were then expressed as the ratio of both IMCL-(CH₂)n to water and IMCL-(CH₂)n to creatine. Absolute concentrations of IMCL and total creatine were also calculated in millimoles per kilogram wet weight by use of the internal reference of muscle water concentration. Total creatine was calculated as follows (7): 1) total creatine CH₃ signal, measured at 3.00 ppm resonance, and unsuppressed water resonance, measured at 4.7 ppm corrected for T₁ and T₂ relaxation effects; 2) corrected signal amplitude, expressed as the ratio of total creatine to unsuppressed water; 3) the ratio corrected for the number of protons contributing to signal, three for total creatine and two for water; 4) the ratio converted to units of millimoles per kilogram wet weight, assuming a water concentration of 55 mmol/kg wet wt and a tissue water fraction of 0.81 kg/kg (39). IMCL and EMCL concentrations were similarly calculated using muscle water as the internal reference with the added considerations of 1) IMCL structure similar to trioleate (61.0 mmol 1H/ml triglyceride) and 2) tissue water fraction of 0.81 kg/kg and tissue density of 1.05 g/ml (39).

Although prior knowledge in the use of the MRUI peak-picking routine is intended to separate IMCL-(CH₂)n and EMCL-(CH₂)n content, the presence of EMCL within the volume of interest can cross-contaminate the IMCL signal, reducing the precision of IMCL estimation. Careful placement of the voxel wholly within the muscle ensured the exclusion of subcutaneous adipose tissue (Fig. 1). We also report the EMCL-(CH₂)n signals, corrected for T₁ and T₂ effects, both in relation to total creatine and water.

Blood analysis. Plasma glucose was determined immediately upon collection by use of an EML 105 autoanalyzer (Radiometer, Copenhagen, Denmark). Plasma FFA concentration was determined using a commercially available assay kit (Wako Pure Chemicals, Osaka, Japan), scaled down for the microplate. Serum insulin was determined with a microplate enzyme immunoassay by means of a fully automated procedure (AxSYM; Abbott Laboratories, Sydney, Australia). Deproteinized supernatant removed from a 1:2 blood-0.6 M perchloric acid mix was used for determination of both 3-hydroxybutyrate and glycerol by the use of methods previously described (6, 35). Plasma total triglycerides were determined from a commercially available kit (ThermoTrace, Noble Park, Australia).

Statistical analysis. Student’s paired t-test (two tailed) was used to compare anthropometric and MRS data at 12 and 84 h. One-way, repeated-measures ANOVAs were used to determine the effect of fasting time on blood biochemical variables. Pairwise comparisons between means were performed by post hoc contrasts. All statistical analysis was performed using a specialized statistical package (SPSS for Windows, version 10.0.1).

RESULTS

MRS. The IMCL-(CH₂)n-to-water ratio increased from 0.00623 ± 0.00065 to 0.0142 ± 0.0015 (mean ± SE, P = 0.002; Fig. 2A). The IMCL-(CH₂)n-to-creatine-CH₃ ratio increased from 6.82 ± 0.87 to 14.96 ± 1.73 (P = 0.001; Fig. 2B). Calculated absolute IMCL concentration increased from 8.95 ± 0.92 to 20.10 ± 2.05 mmol/kg wet wt (P = 0.002). Mean corrected unsuppressed water signal did not change significantly during fasting (6.54 × 10⁻¹ ± 4.11 × 10⁻² vs. 6.73 × 10⁻¹ ± 6.18 × 10⁻² for 12- and 84-h fasting, respectively, P = 0.713). Similarly, there was no significant change in total creatine concentration (29.6 ± 4.3 vs. 30.6 ± 4.0 mmol/kg wet wt, P = 0.817). Also, neither the EMCL-(CH₂)n-to-water nor the EMCL-(CH₂)n-to-creatine-CH₃ ratio was significantly changed by fasting (0.00633 ± 0.00123 vs. 0.00654 ± 0.00134, and 7.3 ± 1.63 vs. 7.52 ± 2.14, P = 0.851 and P = 0.885; Fig. 2, A and B, respectively). Calculated absolute EMCL-(CH₂)n concentration was also unchanged (9.08 ± 1.76 to 9.38 ± 1.91 mmol/kg, P = 0.853).

Biochemistry. Plasma FFA, serum triglyceride, and whole blood 3-hydroxybutyrate concentrations all significantly increased (Fig. 3) during the fasting period (P < 0.001, P < 0.05, P < 0.001, respectively), whereas plasma glucose (Fig. 4A) and serum insulin (Fig. 4B)

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**Table 2. T₂ relaxation time of various resonances in muscle**

<table>
<thead>
<tr>
<th>T₂</th>
<th>H₂O in Skeletal Muscle</th>
<th>IMCL-(CH₂)n</th>
<th>EMCL-(CH₂)n</th>
<th>Cr-(CH₃)</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>12-h fasted</td>
<td>49 ± 1</td>
<td>95 ± 12</td>
<td>89 ± 13</td>
</tr>
<tr>
<td></td>
<td>84-h fasted</td>
<td>53 ± 3</td>
<td>91 ± 4</td>
<td>84 ± 12</td>
</tr>
<tr>
<td></td>
<td>Szczepaniak et al. (39)</td>
<td>40</td>
<td>86</td>
<td>71</td>
</tr>
</tbody>
</table>

Values are means (± SD) expressed in ms. Subject nos. for T₂ relaxation data: n = 2 for H₂O, and n = 4 for IMCL-(CH₂)n, EMCL-(CH₂)n, and Cr-(CH₃). IMCL and EMCL, intra- and extramyocellular lipid, respectively; Cr, creatine.
concentrations decreased ($P < 0.001$ and $P < 0.001$, respectively).

**DISCUSSION**

The present investigation was conducted to test the hypothesis that a prolonged period of fasting would result in an increase in vastus lateralis IMCL content. Our results show a greater than twofold increase in IMCL, expressed in relation to either water or creatine or as calculated absolute concentration. This increase was associated with significant reductions in venous blood concentrations of glucose and insulin and concomitant significant elevations in concentrations of FFAs, triglycerides, and 3-hydroxybutyrate.

Greater adiposity will result in a large EMCL signal with the potential for greater contamination of the IMCL signal, making accurate IMCL quantification more difficult using the MRUI curve-fitting technique. Subjects in the present study, however, were relatively lean (Table 1), and careful voxel placement was able to exclude subcutaneous fat and minimize the possibility of EMCL contamination. Also, EMCL and IMCL peaks in most subjects were quite distinct, and changes were easily observed (Fig. 5). In addition, cross-contamination was unlikely, because there was no significant change in EMCL-(CH2):Cr/water ratios (Fig. 2).

Our results are in support of previous findings in animals, where triglyceride content, determined by biochemical methods, increased by 60% in the gastrocnemius muscle of rats (26) and 255% in the pectoralis muscles of pigeons (44) after 72 h of fasting. They are, however, in contrast with the findings of others (19), who observed a 50% decrease in three rat leg muscles during a fast of the same time period. Interestingly, in both these and other (28) rodent studies, diaphragm triglyceride content decreased significantly during fasting. The discrepancy between studies, and indeed

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**Fig. 2.** Effect of 72 h of water-only fasting on vastus lateralis intra- and extramyocellular lipid (IMCL and EMCL, respectively) content expressed relative to water (A) and relative to total creatine (Cr; B). Values are means ± SE. *$P < 0.01$ compared with 12-h fasting.

**Fig. 3.** Effect of 72 h of water-only fasting on plasma free fatty acid (FFA; A), serum triglyceride (B), and whole blood 3-hydroxybutyrate (C) concentrations. Values are means ± SE. *$P < 0.05$ compared with 12-h fasting.
between muscle groups in one study (26), may be a function of the level of contractile activity of the different muscle groups during the fasting period; IMCL is thought to be an important fuel during submaximal exercise (13). During the fasting intervention, the diaphragm is in continual use, but the legs may have undergone different levels of activity. The subjects in our study were habitual cyclists or runners, but during the fast their level of activity was severely curtailed. An alternative explanation of the discrepancies among the aforementioned animal studies could be that, in some cases, there was error due to the methodological difficulties in separating the contribution of intracellular and extracellular triglyceride from a muscle biopsy sample (48).

The observed increase in IMCL with fasting helps to explain the observations of Jensen et al. (20), which suggest a nonhepatic site of net fatty acid esterification over a 60-h fast. Such a rapid adaptation may serve to provide an immediate fuel supply to the muscle as endogenous carbohydrate stores are challenged. In an evolutionary context, a 72-h period of food deprivation is not long and has probably relatively often been experienced. Physical adeptness would possibly be more important when food was scarce, and thus preservation of the muscle and more immediate energy stores would be vital.

During the fasting period, serum insulin significantly decreased below basal levels. These findings refute others (8), who suggest that significant storage of IMCL occurs only with the combination of high concentrations of circulating FFAs and raised insulin levels.

Much of the fatty acid taken up by resting skeletal muscle is not immediately oxidized but is reesterified (29) for later lipolysis and oxidation (11). This may thus limit cytoplasmic fatty acyl concentration, high levels of which have the potential to disrupt cellular integrity (45). Thus we can think of IMCL as a buffer between the rate of fatty acid entry into the myocyte and the rate of fatty acid oxidation in the mitochondria.

The rate at which fatty acids enter the muscle cell is largely a function of the plasma/cytoplasmic gradient (46). Thus, if cytoplasmic concentrations are kept low, uptake is driven primarily by plasma FFA concentration. However, uptakes at high plasma concentrations appear limited by saturation kinetics (40), i.e., at lower concentrations in the untrained than in the trained (41). Indeed, the perfused muscles of endurance-trained rats have a greater rate of uptake for the same plasma concentration and a greater content of plasma membrane fatty acid-binding protein (FABP_{pm}) (43). Furthermore, 48-h fasting has been shown to increase

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**Fig. 4.** Effect of 72 h of water-only fasting on plasma glucose (A) and serum insulin (B) concentrations. Values are means ± SE. *P < 0.05 compared with 12-h fasting.

**Fig. 5.** Fasting spectra [12 (A) and 84 h (B)] from 1 subject.

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the FABP<sub>PM</sub> content in rat red skeletal muscle by 60% (42).

Therefore, in a situation where both the plasma/cyttoplasmic FFA gradient and transporter activity are high, such as in endurance-trained, fasted muscle, uptake will be near maximal. If, at the same time, the muscle cell is in a relative state of rest, the rate of oxidation is reduced so that, for a time at least, uptake is greater than oxidation and an increase in IMCL must ensue. Thus our well-trained subjects may have accumulated IMCLs during the fast when the untrained would not have. However, there is the paradox that endurance-trained (22) and insulin-resistant, obese persons (16, 24) exhibit higher levels of IMCLs than normals. In both situations, for a period of time at least, there is a mismatch, so that the rate of muscle fiber fatty acid uptake is greater than the rate of oxidation. In athletes at rest, it may be because the rate of cellular uptake is elevated compared with normals because of increased FABP and improved capilarization. In the insulin-resistant obese, it is more likely a function of reduced mitochondrial uptake (12) and oxidation (38), because muscle FABP<sub>PM</sub> is lower in type 2 diabetics compared with normals (2).

Although we did not compare glucose sensitivity before and after the fast, it is known that fasting inhibits insulin-mediated whole body oxidative glucose disposal (25, 47), presumably via operation of the glucose-fatty acid cycle (32). However, when FFAs are available in excess after lipid-heparin infusion, glucose 6-phosphate levels fall below control levels, suggesting inhibition of glucose transport/phosphorylation rather than via elevation of acetyl-CoA/CoA and NADH/NAD<sup>+</sup> (34). Hence, it is also unlikely that the glucose-fatty acid cycle operates when sufficient acetyl-CoA is available from ketone sources. Accordingly, Beylot et al. (1) observed no change in insulin-stimulated glucose disposal during euglycemic hyperinsulinemic clamp, when acetacetate was infused into normal subjects. These observations, taken together with the results of the present study and those of others who have shown an association between IMCL content and insulin resistance in normals (23, 24), suggest that the insulin resistance seen during fasting may be mediated, at least in part, by an increase in IMCL.

In conclusion, 72 h of water-only fasting results in accumulation of IMCL in the vastus lateralis of nondiabetic, nonobese, physically fit men. This accumulation of IMCL occurs in the presence of elevated circulating FFA, triglyceride, and 3-hydroxybutyrate but declining glucose and insulin concentrations. These results suggest that, in physically fit, nonobese diabetic men, it is the relationship between the rate of fatty acid availability and oxidation, not circulating insulin levels, that determines IMCL content.

REFERENCES


