Eur J Appl Physiol (2006) 98:341–354
DOI 10.1007/s00421-006-0279-5

ORIGINAL ARTICLE

Intramyocellular lipid stores increase markedly in athletes after 1.5 days lipid supplementation and are utilized during exercise in proportion to their content

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Accepted: 18 July 2006 / Published online: 11 August 2006

Abstract Intramyocellular lipids (IMCL) and muscle glycogen provide local energy during exercise (EX). The objective of this study was to clarify the role of high versus low IMCL levels at equal initial muscle glycogen on fuel selection during EX. After 3 h of depleting exercise, 11 endurance-trained males consumed in a crossover design a high-carbohydrate (7 g kg^{-1} day^{-1}) low-fat (0.5 g kg^{-1} day^{-1}) diet (HC) for 2.5 days or the same diet with 3 g kg^{-1} day^{-1} more fat provided during the last 1.5 days of diet (four meals; HCF). Respiratory exchange, thigh muscle substrate breakdown by magnetic resonance spectroscopy, and plasma FFA oxidation ([1-^{13}C]palmitate) were measured during EX (3 h, 50% W_{max}). Pre-EX IMCL concentrations were 55% higher after HCF. IMCL utilization during EX in HCF was threefold greater compared with HC ($P < 0.001$) and was correlated with aerobic power and highly correlated ($P < 0.001$) with initial content. Glycogen values and decrements during EX were similar. Whole-body fat oxidation (Fat_{ox}) was similar overall and plasma FFA oxidation smaller ($P < 0.05$) during the first EX hour after HCF. Myocellular fuels contributed 8% more to whole-body energy demands after HCF ($P < 0.05$) due to IMCL breakdown (27% Fat_{ox}). After EX, when both IMCL and glycogen concentrations were again similar across trials, a simulated 20-km time-trial showed no difference in performance between diets. In conclusion, IMCL concentrations can be increased during a glycogen loading diet by consuming additional fat for the last 1.5 days. During subsequent exercise, IMCL decrease in proportion to their initial content, partly in exchange for peripheral fatty acids.

Keywords High-carbohydrate diet · Fat supplement · Muscle glycogen · Magnetic resonance spectroscopy

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Introduction

Muscle glycogen availability is often a limiting factor for exercise at 60–85% aerobic power, hence carbohydrate loading strategies have been successful in improving physical performance (Ivy 2001). Lipid oxidation also contributes substantially to energy expenditure during sustained exercise and a significant contribution of intramyocellular lipids (IMCL) to the fuel mix has been suggested using different techniques (Watt et al. 2002a; Romijn et al. 1993; Johnson et al. 2003; White et al. 2003), even though quantitative and dynamic information is still scarce. Consequently, there has been a focus of interest on devising ways in which to increase the contribution of lipid energy during exercise, with the aim of sparing muscle glycogen.

High-fat feeding strategies (50% dietary energy or more) for periods of 3 days and more, with continued endurance training have been tried in order to adapt metabolic pathways for a more efficient use of fatty

acids (FA). A common observation of these studies was an increased oxidation of lipids during exercise. However, chronic exposure to high-fat diets did not generally lead to improvement in endurance performance in comparison with high-carbohydrate diets (for a review see Erlenbusch et al. 2005). Even when 5–6 days on a high-fat diet was followed by 1 day of carbohydrate restoration, endurance performance was not improved, in spite of the persistent increase in whole-body fat oxidation (e.g. Burke et al. 2000; Carey et al. 2001). With an exercise model that simulates race conditions, i.e. including high-intensity sprints, a decrement in performance after fat adaptation was clearly apparent (Havemann et al. 2006). Several confounding factors associated with high-fat feeding for prolonged periods have been proposed to explain the lower or unchanged exercise performance: reduction in muscle glycogen content, impaired ability to train hard (Helge et al. 2002), increased sympatho-adrenal activation (Jansson et al. 1982), downregulation of glycogenolysis (Stellingwerff et al. 2006), and decreased mood states (Achten et al. 2004).

On the other hand, postexercise replenishment of IMCL levels can be achieved within a day (Starling et al. 1997; Boesch et al. 1999; Décombaz et al. 2000, 2001; Larson-Meyer et al. 2002). The primary objective of this study in endurance-trained athletes was to investigate substrate oxidation during prolonged exercise after a rapid elevation of IMCL without carbohydrate restriction, using 1.5 days of lipid supplementation. Importantly, pre-exercise glycogen stores were kept equal. We hypothesized that an increased availability and use of IMCL during submaximal exercise would shift substrate oxidation towards that of lipids, thereby sparing muscle glycogen for a subsequent high intensity exercise.

Materials and methods

Subjects and preliminary testing

Eleven healthy, endurance trained male cyclists (road or cross-country cyclists, duathletes, and triathletes) with competitive experience participated in the study (Table 1). All subjects were training for endurance at least three times a week (2–3 h per session) and had a training history of at least 5 years. They gave written consent before admission into the study, which was approved by the institutional Ethic's Committee. At least 1 week prior to the main experiment, the subjects reported to the laboratory where anthropometric measurements and assessment of training status were made (Table 1). Body fat was evaluated from triplicate skin-fold thickness measurements at seven sites (Sinning et al. 1985). Maximal voluntary workload (W_{max}), aerobic power (VO_{2peak}; Oxycon ß, Jäger, Höchberg, Germany) and peak heart rate (Polar NV, Polar Electro, Kempele, Finland) were determined during an incremental test on a cycle ergometer (Ergometrics 900, Ergoline, Bitz, Germany). W_{max} was defined as the highest intensity that the subjects could maintain for at least 1 min with a pedal rate above 80 rpm. VO_{2peak} was the highest value reached in a 30-s period.

Table 1 Subject characteristics

Age (years)	31.4 ± 1.7
Height (cm)	181 ± 2
Weight (kg)	74.5 ± 2.5
Body fat (%)	7.6 ± 0.6
BMI (kg m^{-2})	22.6 ± 0.5
Power W_{max}	
W	365 ± 9
W kg^{-1}	4.9 ± 0.2
VO_{2peak}	
l min^{-1}	4.68 ± 0.13
ml min^{-1} kg^{-1}	63 ± 2
HR_{peak} (bpm)	186 ± 3

Values are mean ± SEM

HR heart rate

Study design

This was a reference-controlled crossover (two treatment, two period), balanced, masked, single center outpatient metabolic trial, the experimental design of which is illustrated in Fig. 1a. After a 3-day baseline period to normalize diet and physical activity, the subjects performed a 3-h exercise on a bicycle ergometer at 50% W_{max} (Ex1) intended to reduce their IMCL and glycogen stores. This was followed by a period of dietary intervention with either a high-carbohydrate, low-fat diet (HC diet) for 2.5 days or the same HC diet for 1 day followed by a high-carbohydrate, high-fat diet (HCF diet) for 1.5 days. At the end of the dietary treatment, a test exercise (Ex2) identical to Ex1 was carried out, during which indirect calorimetry and [1-^{13}C]palmitate infusion were used to estimate whole-body energy expenditure, substrate oxidation, and plasma FFA oxidation. IMCL and glycogen were measured in thigh muscles by nuclear magnetic resonance spectroscopy (MRS) three times: before diet (post-Ex1), after diet (pre-Ex2), and at the end of Ex2. The last MRS measurement was followed by a simulated 20-km time-trial (TT).

Run-in period

The purpose of the run-in period and initial exercise Ex1 was to ensure a reproducible physiological state

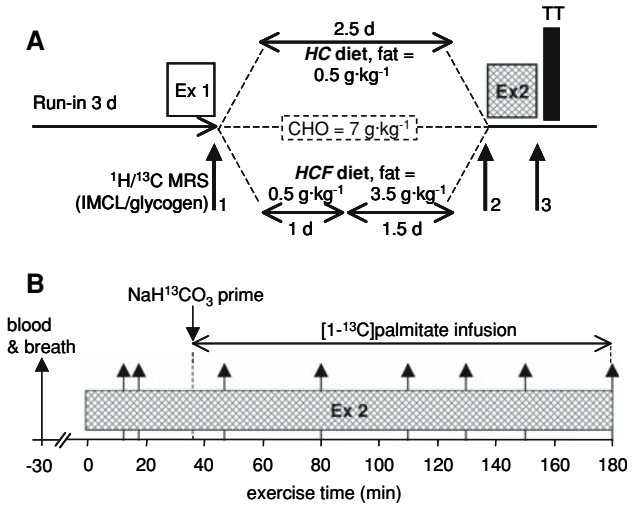

Fig. 1 **a** Study protocol with run-in period and depleting exercise Ex1, daily macronutrient intake during the high-carbohydrate *high*-fat (*HCF*) and high-carbohydrate *low*-fat (*HC*) diets, and main trial including Ex2 and the 20-km time-trial (TT) on a stationary bicycle. Ex1 = Ex2 = 3 h cycling at 50% W_{max}. **b** Infusion protocol during Ex2. Indirect calorimetry measurements (10-min periods) were contiguous with blood and breath samplings

and low energy stores at the start of both trials (Fig. 1a). The following instructions were given individually and replicated before the crossover trial: (1) maintenance of usual prolonged physical activity, mostly cycling (target >3 h in 3 days); (2) adherence to a moderately low-fat diet, based on avoidance of specified foods. Subjects kept an activity and nutritional diary over the period to help maintain similar activities and ensure dietary compliance. Ex1 was performed in the afternoon of day 3, at least 2 h after a light lunch. Briefly, oxygen consumption, heart rate, and perception of fatigue were recorded, and breath was sampled for assessing the drift in background $^{13}CO_2$ isotope enrichment with exercise (vide infra description of Ex2). After Ex1, the first MRS measurements were performed. The subjects then began one of the study diets.

Dietary intervention

Diet was strictly controlled and entirely provided. All food items were packed by meal and supplied on a subject and treatment basis for the 2.5 days between Ex1 and Ex2. Only calorie-free and caffeine-free beverages were allowed in addition to the prescribed foods. Diets were composed of regular food items, dishes, and ingredients in order to obtain high and similar glycogen concentrations in the presence of either high (HCF diet) or low (HC diet) IMCL levels before Ex2. Both diets provided equal amounts of protein (1 g kg^{-1} day^{-1}) and CHO (7 g kg^{-1} day^{-1}). On the first day (three meals, starting with the evening meal) both diets were isocaloric with only minimal fat content (~180 kJ kg^{-1} day^{-1}; 0.5 g kg^{-1} day^{-1}). For the last 1.5 days (four meals), the HCF diet provided more energy (~276 kJ kg^{-1} day^{-1}; 3.5 g kg^{-1} day^{-1}), all of which was in the form of supplementary fat. The proportion (%) of energy provided by the macronutrients protein/CHO/fat was 11/52/37 (HCF) and 14/73/12 (HC) when pooled over 2.5 days and 9/43/48 on the HCF diet during the last 1.5 days of fat-enriched feeding. Lipids in the HCF diet comprised >50% C18:1, >20% C18:2 and other polyunsaturated FA. Food items such as corn, pineapple, and soft drinks, known to be naturally rich in ^{13}C, were excluded from the treatment diets, as well as corn oil during the lipid supplement phase. Dietary treatments could not be made blind to the subjects, but their expectation about treatment effects was balanced as much as possible by explaining that everybody received their individually calculated energy requirements for endurance and for optimal performance in both tests. Empty boxes, wrappers, and eventual leftovers were returned to the investigators on the second visit to record and calculate energy intake. Nutrient intake was calculated from manufacturers' information and standard food tables (Souci et al. 2002). During the diet treatment, physical activity was restricted to sedentary occupations.

Submaximal exercise (Ex2)

Following diet treatment, the subject reported to the laboratory after an overnight fast. At 7.45 a.m., he ingested 100 g of white bread before being positioned in the magnet for the second sequence of MRS measurements. IMCL and glycogen concentration data were acquired between 8 and 9.30 a.m. After this, the subject voided his bladder, was weighed and lay down for the insertion of two catheters, one into a right forearm vein for isotope infusion, and the other into a left forearm vein, 10 cm from the wrist on the posterior side. Catheters were kept patent with a saline drip. Resting measurements were collected while the subject was sitting quietly on the ergometer. Exercise was then initiated (2.5–3 h after the morning snack and 1 h after the end of the MRS measurements). After the first 6 min of warm-up, steady state was maintained for 3 h at constant power (50% W_{max}) and pedaling frequency (70–75 rpm). The forefoot was placed on the pedal so that the calf and the thigh muscles were engaged in the exercise. Gas exchange and perceived exertion were measured periodically. Heart rate was recorded

continuously. Blood was sampled at rest 30 min before exercise, and at 15, 18, 45, 80, 110, 130, 150, and 180 min of exercise (total 90–100 ml per trial) for the determination of plasma metabolites and palmitate isotope enrichment (from 45 min onwards). At 25 min, blood samples were collected in duplicate to determine the initial background isotope enrichment during exercise, and at 35 min isotope infusion was started (Fig. 1b). During exercise the left forearm was maintained close to central body temperature by means of a cotton sleeve to obtain near arterialized blood samples. The subject was allowed to drink water ad libitum. Room temperature was maintained within 23.0–24.2°C and relative humidity within 30.4–31.2%. Immediately after exercise, catheters were withdrawn and total urine was collected for nitrogen analysis (Kjeldahl method). Weight loss was determined by pre- and post-exercise weighing to 0.1 kg. The subject then drank half a liter of an isotonic drink (25 g CHO) before being repositioned in the magnet within 20 min for the final MRS measurements.

Tracer infusion

At 35 min of exercise, a priming dose of [1-^{13}C]bicarbonate (1.05 μmol kg^{-1}) was injected over 30 s and a constant infusion of [1-^{13}C]palmitate was initiated (0.056 ± 0.004 μmol min^{-1} kg^{-1}, ~160 ml fluid h^{-1}). The exact infusion rate was determined by weighing the amount infused and dividing by the time. Sodium bicarbonate (^{13}C, 99%) and potassium palmitate (1-^{13}C, 99%) were obtained from Cambridge Isotope Laboratories (Andover, MA, USA) and sterile pathogen free solutions were prepared by the Lausanne University Hospital Pharmacy. Sodium [^{13}C]bicarbonate was conditioned in 0.9% NaCl ampoules (7 mg ml^{-1}) and potassium [1-^{13}C]palmitate (2.12 μmol ml^{-1}) was bound to 5% human albumin (Wolfe 1992). The final palmitate enrichment, measured in each individual infusate, averaged 91.7 ± 0.2 mol%.

Techniques

Respiratory gas exchange was measured at blood-draw time points by indirect calorimetry over 10-min periods (Vmax29C, SensorMedics, Bilthoven, The Netherlands), in breath-by-breath mode while using a mouthpiece. Flow and gas analyzers were calibrated before each experiment. Breath gas for assessing $^{13}CO_2$ isotope enrichment was sampled into aluminum bags from QuinTron (Milwaukee, WI, USA). A bag was filled with a single expiration, the upper respiratory dead space volume of which was automatically trapped in a plastic bag of lesser resistance. Duplicate samples were transferred into 7-ml air-evacuated glass tubes for later $^{13}CO_2$ analysis. Subjective perception of fatigue was assessed always by the same investigator using the Borg scale with levels 6–20 (Noble 1982).

Simulated cycling time trial

The simulated TT took place immediately after the final MRS measurements, approximately 1.5 h after Ex2 (Fig. 1a) and after complete rehydration. It was performed on a racing bicycle (Trek 1200 Alpha, Waterloo, WI, USA) with clipless pedals, the back wheel of which was fixed to an external magnetic brake (Carnielli, model Leader, Vittorio Veneto, I). A fan was positioned in front of the cyclist to allow circulation of air. A powermeter was connected to the bicycle and was calibrated before each simulated TT (SRM Training System, professional road version, Schoberer Rad Messtechnik, Jülich, Germany). The system calculates the external work produced from power and, independently, velocity using the number of revolutions and diameter of the rear wheel. After an individual warm up of at least 5 min the subject started the 20 km simulated TT in a resting saddle position. He was not allowed to stand up during the trial. Pedaling cadence could be freely selected. The distance covered was the only variable the subject was aware of. Neither the subject nor the investigator was aware of the elapsed time. Heart rate was monitored continuously. Time to completion was measured by the SRM system and checked with an additional stopwatch. Simulated TTs were performed at the same time of day and supervised by the same investigator. The amount of water the subject voluntarily consumed during the first test was consumed during the crossover test (0.5 ± 0.1 l). Ambient temperature and relative humidity were similar and stable during both trials. Indicative reproducibility: a reanalysis of data from a comparable TT protocol, although its end point was duration (1 h) rather than distance (Jeukendrup et al. 1996), gives an intra-individual reproducibility in performance equal to 3.08% (CV_{intra}).

Magnetic resonance

Muscle glycogen and IMCL stores in the *m. quadriceps femoris* of the right leg were measured sequentially, glycogen first, within the same session. An entire session consisted of image acquisition, ^{13}C- and ^{1}H-MRS and lasted about 90 min. Magnetic resonance measurements were performed using a 1.5T whole-body system (SIGNA, General Electrics, Milwaukee, WI,

USA), with which images and spectra were acquired using a $^{13}C/^{1}H$ double-tuned flexible coil (Medical Advance; ^{13}C: single turn 11.5×11.5 cm^2 quadratic coil; ^{1}H: Helmholtz design). The volunteers were placed on a specially designed mount to guarantee a reproducible positioning of the right leg for all MRS sessions (after Ex1, before and after Ex2). Repositioning of the volunteer and placement of the coil were controlled using the acquired images.

Determination of glycogen stores

Glycogen concentrations in *m. quadriceps femoris* were determined by ^{13}C-MRS using techniques similar to those described in detail in Rotman et al. (2000) and Décombaz et al. (2001). The spectra were evaluated using the *j*MRUI 1.3 software package (Naressi et al. 2001). Preprocessing of the spectra included zero- and first-order corrections as well as filtering of the signals from CH = CH double bonds, CH_2 and CH_3 of fatty acyl chains with HLSVD filtering (Coron et al. 2001). The spectra were fitted using AMARES (Vanhamme et al. 1997). Frequency shifts as well as linewidth of the creatine and glycogen signals were fixed relative to the respective values of the COOH signal from fatty acyl chains at 172 ppm. These values were determined using a summed spectrum of all measurements. Signal areas of total creatine (creatine and phosphocreatine) and glycogen were corrected for T_1 saturation and NOE effects in order to convert the signal ratio of glycogen and total creatine $Glyc/Cr_{tot}$ into the respective concentration ratio. Glycogen concentrations in units of millimole per kilogram wet weight (mmol kg$^{-1}_{ww}$) were finally calculated using total creatine as the muscle specific internal concentration standard. A mean concentration of total creatine of 32 mmol kg$^{-1}_{ww}$ was assumed for all volunteers (Bottomley et al. 1997). Absolute quantitation of glycogen concentrations was based on full MR-visibility of glycogen, the creatine concentration in thigh muscle as taken from the literature as well as on the correction factors for the creatine and glycogen signals, which were determined in separate experiments.

Determination of IMCL stores

The IMCL content was determined with single voxel ^{1}H-MRS. In contrast to ^{13}C-MRS, the recorded spectra originate from a predefined volume (voxel) that measured $18 \times 11 \times 12$ mm^3 (IS × RL × AP). The voxel was positioned in the *M. vastus intermedius* close to the femoral bone. Care was taken that no sign of fatty infiltration was visible inside the voxel that would be the source of contamination of the spectrum by a large extramyocellular lipid (EMCL) signal. Since optimal and reproducible placement of the ^{1}H-MRS-voxel is crucial, positioning as well as repositioning of the voxel was achieved using the image series. ^{1}H-MR-spectra were recorded using an optimized PRESS sequence (TR = 3 s, TE = 20 ms, 128 acquisitions, 16 phase rotating steps, 1,953 Hz, 1,024 pts) with water and additional outer volume suppression. The spectra were quantified using the fully relaxed, unsuppressed water signal as internal concentration standard. IMCL concentrations in mmol kg$^{-1}_{ww}$ were evaluated as described earlier (Boesch et al. 1999).

Analyses

Plasma metabolites

Insulin, glucose, and TAG were measured in plasma EDTA. Insulin was determined by ELISA enzyme immunoassays (Immuno-Biological Laboratories, Hamburg, Germany), TAG (BioMérieux, Genève, CH), and glucose by enzymatic tests (Roche Diagnostics, Berthoud, CH) on a Cobas Mira centrifugal analyzer (Roche, Basel, Switzerland). Plasma FFA concentrations were quantified in duplicate by gas chromatography (GC, Hewlett Packard 6890, Palo Alto, CA, USA) after addition of heptadecanoic acid as internal standard. Data on hormonal responses (ghrelin, leptin, GH and cortisol) have been published elsewhere (Christ et al. 2006).

Stable isotopes

Breath $^{13}CO_2$ was determined by isotope ratio mass spectrometry (Delta S, Finnigan MAT, Bremen, Germany). For each run, the system was calibrated with a carbon dioxide gas of known ^{13}C isotopic enrichment calibrated with a primary reference gas standard (^{13}C at –25‰, Messer GmbH, Krefeld, Germany) traceable to the international Pee Dee Belemnite standard (PDB). Duplicate breath samples were prepared and run in sequence with calibration curve standards. Two milliliters were injected into a HayesSep-D packed steel column heated at 80°C using helium as vector gas. After elution of breath gas components, water was removed by diffusion through a nafion tube and the remaining gas was directed to the MS analyzer where ion masses 44, 45, and 46 were quantified for measurement of ^{13}C labeled carbon dioxide enrichment (Atom % Excess, APE_{CO2}, or delta per mil, see Fig. 5a). The isotope enrichment of plasma palmitate was assessed using gas chromatography/mass spec-

trometry (GC/MS). Plasma FAs were converted to their methyl esters and injected into the GC, which was equipped with a 30 m × 0.25 mm capillary column (HP-5MS) and connected to a quadrupole MS (HP 5972) with electron impact ionization. Ions were selectively monitored at mass-to-charge ratios (m/z) of 270 and 271 to quantify isotopic enrichment in the plasma resulting from [1-^{13}C]palmitate infusion (m/z 271). Measured isotope enrichment of plasma [1-^{13}C]palmitate was 2.1 mol% or more over background.

Calculations

Protein oxidation during Ex2 was estimated from urinary nitrogen without correction for changes in the body urea pool. Energy expenditure and whole-body oxidations of CHO (CHO_{ox}) and fat (Fat_{ox}) were calculated from respiratory exchange data (Jéquier et al. 1987). Total FA oxidation was determined by converting Fat_{ox} to its molar equivalent using a mean molecular weight of 858 g mol^{-1} TAG. The contribution of IMCL and muscle glycogen during Ex2 was estimated on a commonly used assumption of 10 kg of active muscle mass per 70 kg of body weight (van Hall et al. 1999). IMCL decrement was converted from mole to weight and energy (Boesch et al. 1999). Glycogen decrement was converted from mole to weight (162 g mol^{-1} glycosyl unit) and to energy (17.6 kJ g^{-1} glycogen). The whole-body rates of palmitate appearance (R_a) and disappearance (R_d) were calculated (μmol min^{-1}) using the non-steady-state equations of Steele (1959), with

$$R_a = \left[\frac{i - V\left(\frac{C_1+C_2}{2}\right)\left(\frac{E_2-E_1}{t_2-t_1}\right)}{\left(\frac{E_1+E_2}{2}\right)}\right]$$

$$R_d = R_a - V\left(\frac{C_2 - C_1}{t_2 - t_1}\right)$$

where i (μmol min^{-1}) is the [1-^{13}C]palmitate infusion rate; V (l) the volume of distribution of FFA (40 ml kg^{-1} body mass); C_2 and C_1 (μmol l^{-1}) the plasma palmitate concentrations at times t_2 and t_1 (min), respectively; E_2 and E_1 the plasma palmitate isotopic enrichment over the pre-infusion baseline (assumed from the average value at 15th and 18th min of exercise) at t_2 and t_1, respectively. The rate of plasma palmitate oxidation (μmol min^{-1}) was calculated as

$$\text{Plasma palmitate oxidation} = \frac{\text{APE}_{CO_2} V\text{CO}_2}{0.85\left(\frac{E_1+E_2}{2}\right)}$$

where APE_{CO2} is the atom % excess in expired CO_2 over baseline (assumed from the background CO_2 enrichment measured during the 3rd hour of Ex1 in the absence of isotope infusion); VCO_2 (μmol min^{-1}) the expired CO_2 and 0.85, an assumed acetate recovery factor in breath of $^{13}CO_2$ resulting from the oxidation of the palmitate tracer at the present exercise intensity, as derived from Sidossis et al. (1995). Plasma FFA R_a, R_d and oxidation were derived from the fractional contribution of palmitate to the total FFA concentrations, which was stable at 27.0 ± 1.4 mol% throughout Ex2.

Statistical analysis

Single parameters in the two diet groups HCF and HC were compared by a paired, two-tailed t test and, if significantly different, indicated with asterisks (*). Differences over time between the two groups were analyzed by multiple analysis of variance (MANOVA) and, if significant, indicated with asterisks. Linear regression analyses were performed with standard procedures using Excel 2000 (Microsoft) and StatView 2002. Statistical significance was set at $P < 0.05$. Values are presented as mean ± SEM.

Results

Dietary compliance

During the treatment period, all subjects complied with the instructions and ate the meals as provided.

Endurance exercises

Ex1 and Ex2 were performed at an identical power output and similar heart rates (Ex2, see Table 2). Perceived exertion ranged between 11 (fairly light) after 30 min and 14 (>somewhat hard) after 180 min and there was no difference in any of the given numbers between exercises or trials. Weight loss during Ex2 was negligible (Table 2) as a consequence of adjusting water intake to the weight lost during Ex1.

Intramyocellular lipids and muscle glycogen

Intramyocellular lipids

There was no difference in postexercise IMCL concentrations after either Ex1 or Ex2 comparing the two groups (Fig. 2a). In contrast, IMCL concentration increased 70% with the HCF intervention (+3.2 ± 0.5 mmol kg$^{-1}_{ww}$; $P < 0.001$) whereas it remained

Table 2 Metabolic characteristics of exercise Ex2

	HCF diet	HC diet
Set power[a] (50% W_{max}) (W)	183 ± 4	183 ± 4
Duration[a] (min)	180	180
Heart rate (bpm)	131 ± 4	130 ± 3
VO_2 (l min^{-1})	2.40 ± 0.05	2.38 ± 0.06
Weight loss[b] (kg)	0.08 ± 0.48	0.05 ± 0.60
Protein oxidation (g min^{-1})	0.06 ± 0.003	0.05 ± 0.00
Energy expenditure (kJ min^{-1})	48.6 ± 1.0	48.5 ± 1.3
Lipid (% energy)	46 ± 3	41 ± 3
Carbohydrate (% energy)	52 ± 3	57 ± 3
Protein (% energy)	2 ± 0	2 ± 0
Nonprotein RER	0.86 ± 0.01	0.88 ± 0.01

There was no significant difference between diets for any variable. Values are means ± SEM over the 3 h of Ex2

[a] Set power and duration were the same as during the depleting exercise Ex1

[b] Water loss was balanced during Ex2 with water intake (1.9 ± 0.2 l) and saline infusion (1.0 ± 0.2 l)

stable after the HC diet. The rate of IMCL resynthesis over the 1.5 days of effective fat supplementation during HCF was about 2.13 mmol kg$^{-1}_{ww}$ day^{-1}. The reduction of IMCL during Ex2 was significant after both diets (HCF: $P < 0.001$; HC: $P < 0.05$) but after HCF subjects depleted significantly more in absolute (HCF: –3.2 ± 0.5; HC: –1.1 ± 0.5 mmol kg$^{-1}_{ww}$; $P < 0.001$) and relative terms (HCF: –41 ± 4%; HC: –21 ± 8%, $P < 0.01$). Figure 3a shows a strong correlation ($P < 0.001$) between IMCL levels before Ex2 and their reduction during Ex2. Associations were found between aerobic power and IMCL responsiveness to diet (storage) and to exercise (use): VO_{2peak} was correlated with IMCL concentration after HCF ($P < 0.01$) and with the amount of IMCL depleted during exercise Ex2 ($P < 0.05$). There was a positive correlation between the extent of IMCL reduction during Ex2 and Fat_{ox} after both HCF ($P < 0.01$) and HC diets ($P < 0.01$, Fig. 3b). The subjects who had the highest IMCL levels after HCF also had the highest ones after HC ($P = 0.01$).

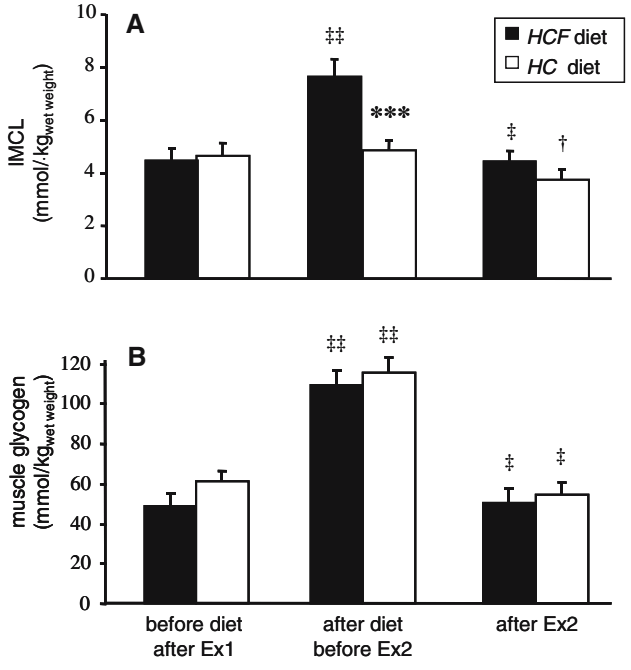

Fig. 2 IMCL (**a**) and muscle glycogen (**b**) concentrations before and after diet intervention, and after Ex2. ‡‡$P < 0.001$ for the effect of diet; ‡$P < 0.001$, †$P < 0.05$ for the effect of exercise; ***$P < 0.001$ for the difference between diets. Values are means ± SEM

Glycogen

There was no treatment difference in postexercise glycogen concentrations after either Ex1 or Ex2 (Fig. 2b). Furthermore, glycogen concentration increased significantly ($P < 0.001$) during both diet interventions, approximately doubling in value. Glycogen reduction during Ex2 was significant in both trials ($P < 0.001$) with no difference in either absolute (HCF: –59 ± 4; HC: –61 ± 6 mmol kg$^{-1}_{ww}$) or relative (HCF: –56 ± 4%; HC: –52 ± 4%) terms.

Energy metabolism during exercise Ex2

There were no significant treatment differences in overall energy expenditure and substrate oxidation over the 3 h (Table 2, Fig. 4), but there was a trend for a lower average RER (a higher percentage contribution of fat to energy) after HCF during the first hour ($P < 0.07$). Subjects hydrolyzed threefold more IMCL after HCF (0.15 ± 0.02 g min^{-1}) than after HC (0.05 ± 0.02 g min^{-1}; $P < 0.001$). Muscle glycogen breakdown was similar with the two diets (HCF 0.52 ± 0.03, HC 0.53 ± 0.05 g min^{-1}). Based on muscle substrate breakdown data, IMCL contributed 27 ± 3 and 8 ± 4 % lipid energy (HCF vs. HC, $P < 0.001$) and glycogen ~35% carbohydrate energy after both diets. Total muscle energy contribution to the energy demands of exercise was 8% larger after HCF than after HC (32 ± 2 and 24 ± 2%; $P < 0.05$) due to IMCL alone.

The rates of plasma FFA appearance (R_a) and disappearance (R_d) obtained during the last 2 h of exercise were similar after both diets (Table 3), but R_d tended to be less ($P < 0.1$) and plasma FFA oxidation was lower ($P < 0.05$) after HCF than after HC at 60 min—the first kinetic measurement point (Fig. 5). Plasma FFA oxidation increased with time until a plateau was reached at 2–2.5 h of exercise.

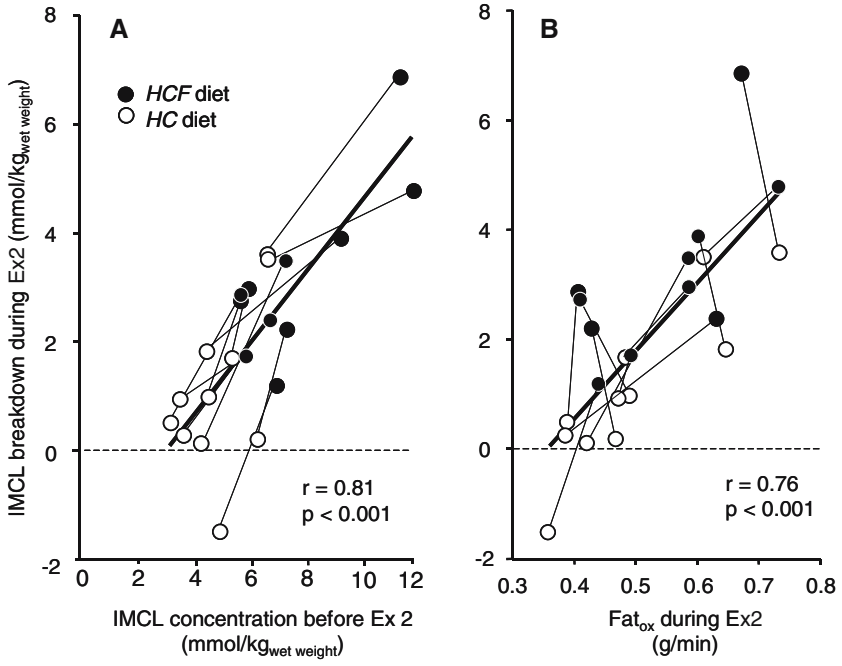

Fig. 3 Correlation of IMCL breakdown during Ex2 (**a**) with their pre-exercise concentrations; (**b**) with whole-body fat oxidation (Fat_{ox}). Within subjects' connecting lines between HCF and HC trials show (*panel a*) that every single subject used more IMCL after HCF compared to HC

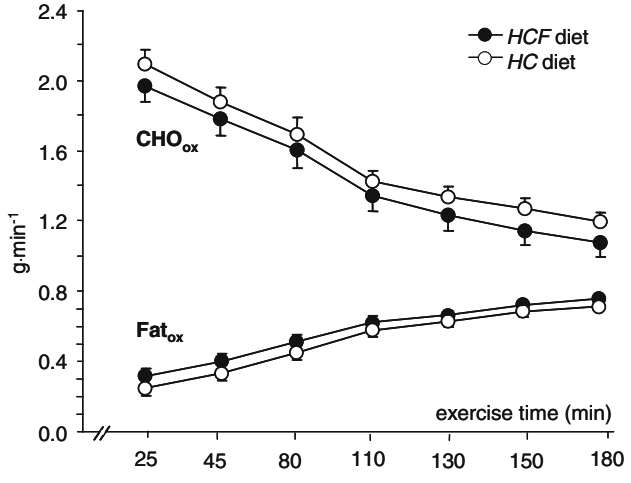

Fig. 4 Whole-body fat and carbohydrate oxidations (Fat_{ox} and CHO_{ox}) during Ex2. There was a trend ($P < 0.09$) for higher fat oxidation during the first hour of exercise after HCF compared to HC diet. Values are means ± SEM

Table 3 Lipid kinetics and metabolism during exercise Ex2 (last 2 h)

	HCF diet	HC diet
Plasma FFA R_a	8.5 ± 0.7	9.3 ± 0.9
Plasma FFA R_d	8.2 ± 0.7	9.1 ± 0.9
Total FA oxidation (Fat_{ox})	28.3 ± 1.4	26.4 ± 1.6
Plasma FFA oxidation	7.0 ± 0.7	8.6 ± 1.0
As % Fat_{ox}	25 ± 3%	32 ± 3%*

Values are means ± SEM in μmol kg^{-1} min^{-1}

*$P < 0.05$ for differences between diets

Plasma parameters

Pre-exercise, resting TAG concentrations were marginally lower after HCF (0.51 ± 0.04 mmol l^{-1}) compared with HC (0.71 ± 0.09 mmol l^{-1}, $P = 0.06$). In each group there were significant changes in plasma FFA and glucose (Fig. 6), and insulin over time. The FFA concentrations increased similarly after both diets, starting after 45 min of exercise ($P < 0.0001$). Glucose concentrations decreased modestly but significantly during exercise after both diets ($P < 0.01$), with no treatment difference. The lowest individual glucose values occurred during the last hour of exercise and were within normal limits (HCF 4.5 ± 0.1; HC 4.3 ± 0.1 mmol l^{-1}). Insulin concentrations decreased ($P < 0.0001$) in both conditions during exercise (pre-exercise, HCF 10.3 ± 2.7; HC 4.9 ± 0.9 μU ml^{-1}; end of exercise, HCF 0.7 ± 0.5; HC 0.2 ± 0.0 μU ml^{-1}). There was a significant diet × time interaction ($P < 0.03$), with elevated insulin concentrations after HCF that was apparent only during the first 45 min.

Simulated 20-km cycling time trial

Table 4 presents data collected during the simulated TT. One subject was excluded because of asthmatic problems. Body weights before the TT were the same. Subjects were 34 ± 31 s faster after HCF than after HC, but this was not significant ($P = 0.30$). The correlation between individuals' velocities, determined by the average of the two trials for each subject, and the improvement

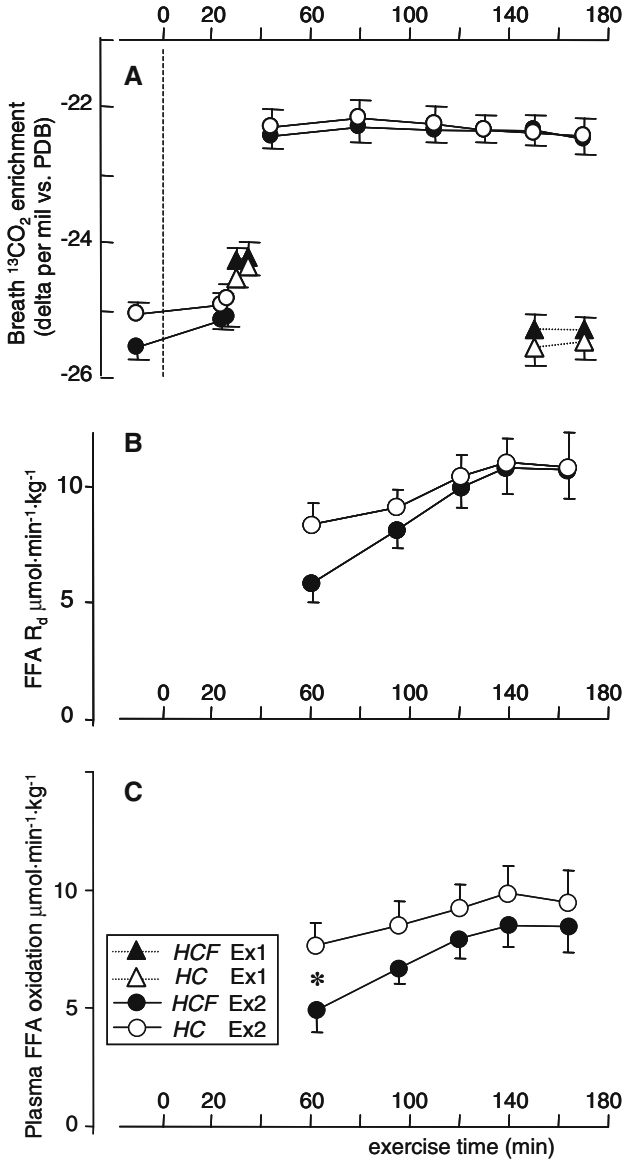

Fig. 5 **a** Breath $^{13}CO_2$ enrichment (delta per mil. with reference to PDB) during rest and exercise without (Ex1) and with (Ex2) isotope infusion. Plasma FFA rate of disappearance R_d (**b**) and oxidation (**c**) during the last 2 h of exercise Ex2 at 50% W_{max} (µmol min^{-1} kg^{-1}). *$P < 0.05$ for the difference between diets. Values are means ± SEM

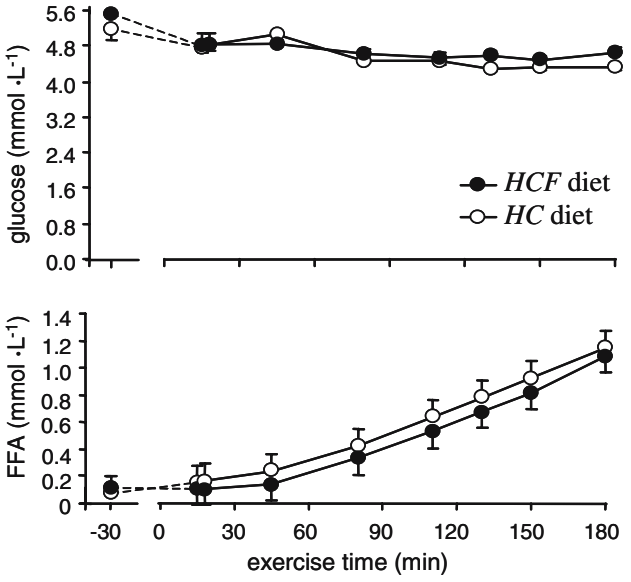

Fig. 6 Plasma concentrations of FFA and glucose during Ex2. Values are means ± SEM

Table 4 Performance achieved during the simulated 20-km time-trial

	HCF diet	HC diet
Distance (km)	20.00	20.00
Heart rate (min^{-1})	175 ± 3	173 ± 3
Time (min:s)	34:28 ± 1:32	35:02 ± 1:15
Speed (km h^{-1})	35.4 ± 1.6	34.5 ± 1.2
Power (W)	232 ± 10	231 ± 12
Work (kJ)	444 ± 13	457 ± 5
Revolution (rpm)	91 ± 2	93 ± 2

The simulated time trial took place after Ex2, rehydrated. Values are means ± SEM(10)

after *HCF* was significant ($P < 0.04$): in other words, the faster a subject was, the higher was the performance improvement after HCF. Figure 7 illustrates a negative correlation ($P < 0.01$) between the reduction of IMCL during Ex2 and the required time for the subsequent simulated TT for all subjects after both diets.

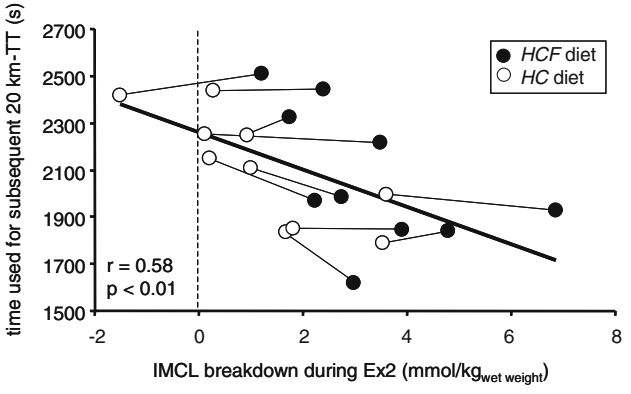

Fig. 7 Correlation of time used for the simulated 20 km TT with IMCL breakdown during the preceding Ex2. Note that IMCL had returned to equal levels in both trials before TT

Discussion

The main findings of this study are that (1) a short fat-supplemented high-carbohydrate feeding protocol

after exercise can replenish IMCL stores in addition to reloading glycogen, (2) IMCL utilization during prolonged exercise is directly proportional to the initial stores, and (3) whole-body fat oxidation during prolonged exercise is unchanged by prior short-term fat supplementation.

Diet and muscle fuel storage

The vast majority of studies on the role of lipid nutrition in exercise metabolism and performance of high-fat feeding have balanced energy intake across treatments, whether after a single meal (Okano et al. 1998), one day (Starling et al. 1997), 2 days (Zderic et al. 2004), 3 days (Stepto et al. 2002) or more, e.g. (Goedecke et al. 1999; Burke et al. 2000; Coyle et al. 2001). As a consequence, a more or less prolonged state of CHO deprivation was induced, making it difficult to assign a single cause (either glucose deficiency or abundance of FA) for the observed metabolic adaptations. Corrections of carbohydrate status before or during exercise testing in many of these studies have not rectified such markers as elevated whole-body fat oxidation (Carey et al. 2001) or pyruvate dehydrogenase inactivation (Stellingwerff et al. 2006). A unique feature of the present study was to ensure identical carbohydrate intake and equal muscle glycogen concentrations in the two trials prior to exercise testing. It was therefore possible to study the effect of IMCL stores on fuel use in isolation. The two-phase approach used—supplementing a relatively high-CHO diet with lipids for the last four meals only of a period of low physical activity—was based on the evidence that glycogen is replenished faster in the early postexercise period (Ivy 2001) and preserved during inactivity, whereas IMCL accumulation takes place with a certain delay (Bachmann et al. 2001; Boden et al. 2001; van Loon et al. 2003b), but can then be replenished within a day or two with high-fat feeding (Starling et al. 1997; Boesch et al. 1999; Décombaz et al. 2000, 2001; Larson-Meyer et al. 2002; Zderic et al. 2004). With a low-fat, high-CHO diet, IMCL repletion is impaired substantially as shown previously (Décombaz et al. 2000, 2001; van Loon et al. 2003b).

A shortcoming of the present approach is the caloric imbalance resulting from ingesting a surplus of fat (~240 g) over the last four meals. However, acute fat supplementation has very little influence on energy metabolism, at least at rest (Flatt et al. 1985), whereas the effects of carbohydrate restriction can be severe, e.g. (Johnson et al. 2006). In addition, a difference in fat mass gain of 0.24 kg is unlikely in itself to have a measurable influence on any of the whole-body variables being measured, which indicates that the option of maintaining CHO constant rather than total energy is a valid one.

Intramyocellular lipid breakdown

This study indicates that diet is a major determinant of, not only short-term IMCL storage, but also its subsequent utilization during exercise. IMCL depletion during exercise was strongly correlated with its absolute content prior to exercise (Fig. 3a), in keeping with previous studies in insulin dependent diabetic patients (Standl et al. 1980), healthy women and men (Steffensen et al. 2002; Roepstorff et al. 2006) or trained males (Zehnder et al. 2005b). Recent dietary intervention studies have reached similar conclusions using either indirect (Coyle et al. 2001) or direct (Johnson et al. 2003) measurements. Therefore, the present results substantiate the prevailing view and suggest that pre-exercise IMCL concentrations trigger IMCL breakdown during work independently of glycogen content.

It was observed that, in addition to diet, other individual factors also influenced IMCL storage and breakdown. One such factor might be a difference in the degree or the specificity of training between subjects. VO_{2peak} was correlated with the magnitude of IMCL storage after feeding and with the extent of IMCL breakdown during exercise. IMCL breakdown was correlated with Fat_{ox} (Fig. 3b). These relations indicate that even within a group of apparently homogenous athletes with similar VO_{2peak}, total body mass and lean body mass (CV ~ 3%), those that are better trained tend to have higher IMCL levels than those who are less well trained. We cannot rule out that the large inter-individual range in IMCL accumulation after HCF (from 15 to 109% increase) or in the ability to mobilize IMCL after HCF (from 17 to 61% decrease) was due in part to methodological variability. However, the fact that subjects with the highest IMCL concentration after the HCF diet were also those with the highest concentration after HC argues in favor of a biological component. The intensity of exercise in this study was adjusted relative to W_{max}, but fat oxidation is more closely related to the anaerobic threshold than to either W_{max} or VO_{2max} (Fox and Mathews 1981). In addition, as polyathletes the subjects practiced running and cycling endurance activities in varying proportions and their training specificity and the distribution of fiber types in leg muscles may have been distinctly different depending on their training focus (Clarkson et al. 1980). Hence differences in adaptive effects of habitual running and

cycling schedules on aerobic and fat metabolism (Schneider et al. 1990; Achten et al. 2003) and differences in IMCL content of different fiber types may have contributed to the disparity in IMCL storage and utilization rates.

Energy metabolism

In the presence of similar pre-exercise glycogen levels and similar plasma glucose, insulin and FFA responses during exercise, the large difference in muscle lipid content (Fig. 2) had a negligible influence on whole-body CHO_{ox} during exercise, as shown by the similar glycogen breakdown and substrate oxidation data after HCF and HC. This is in line with a primary role for CHO availability in determining the rate of Fat_{ox} during exercise (Sidossis et al. 1996), unless a prolonged adaptive period of CHO deprivation has taken place (Goedecke et al. 1999; Burke et al. 2000). At similar Fat_{ox}, the greater decline in IMCL after HCF was in line with a reduced rate of plasma FFA oxidation (Table 3), at least during the first half of the exercise. This suggests that the pivotal role played by intracellular FFA concentrations to respond to the metabolic demand of muscle contraction (Sacchetti et al. 2002) can be met by IMCL under different conditions, i.e. a surplus of IMCL or a shortage of plasma FFA (Watt et al. 2004).

As net IMCL breakdown occurs only in active muscles during intermediate intensity exercise (Sacchetti et al. 2002), IMCL breakdown can be translated into energy contribution to whole-body fat oxidation. However, the accuracy of the estimates, 27 and 8% of Fat_{ox} after HCF and HC, depends on the reliability of values for Fat_{ox}, IMCL concentrations and size of the active muscle mass. A reasonable 1% error on oxygen consumption would affect these values modestly by ±2 and ±1% units, respectively. The typical MRS measurement error on IMCL content (with repositioning of the subject in the magnet) can be as low as 6% in the best conditions (*m. tibialis ant.*) (Boesch et al. 1997), a variability which compares favorably with the value of ~12% given for the biochemical method in trained men (Watt et al. 2002b). Furthermore, the means of 5 (HC) to 8 (HCF) mmol kg^{-1} for IMCL concentration in this study fall within the range of 2–10 mmol kg^{-1} muscle TAG concentrations reported from studies using the biochemical assay in biopsy samples (van Loon 2004). The resting IMCL content of *m. vastus intermedius*, as well as IMCL depletion during exercise, is representative of values in other quadriceps muscles (Zehnder et al. 2005a). For these reasons, there should not be an important bias in the measured IMCL values. Another source of possible uncertainty is the size of the working muscle mass during cycling (10 kg per 70 kg of body weight). If as much as 16 kg muscles, a high limit proposed for cycling (van Hall et al. 1999), were working similarly, the IMCL contribution could increase up to 43% Fat_{ox} (HCF). In comparison, the range of published estimates for the contribution of IMCL during prolonged exercise, mostly by indirect methods is ~15–35% Fat_{ox} (Jeukendrup et al. 1998). Our study like others (e.g. De Bock et al. 2005) supports a large impact of diet on IMCL energy contribution and suggests that individual factors make prediction all the more difficult.

Peripheral FFA utilization was estimated during the last 2 h of the 3-h exercise (Table 3). Plasma FFA oxidation (25–35% Fat_{ox} collectively) was lower than in comparable investigations on trained subjects, from ~40% (Coyle et al. 1997; Coggan et al. 2000; Roepstorff et al. 2002) up to 65% Fat_{ox} (van Loon et al. 2003a). The absolute values (7–9 μmol kg^{-1} min^{-1}) were lower than expected at comparable workloads (range ~10–15 μmol kg^{-1} min^{-1}). The reason why plasma FFA oxidation is lower in this study than in comparable ones could not be explained. The data nevertheless suggest that IMCL degradation may occur preferentially during the first hour of the exercise (smaller plasma FFA oxidation at 60 min, tendency for higher Fat_{ox}), as proposed by others (Sacchetti et al. 2002; Watt et al. 2002b), but this is speculative given the lack of information on IMCL and FFA kinetics during this period.

Performance (TT)

The hypothesis that IMCL utilization spares glycogen to enable better performance in the simulated TT could not be tested, because IMCL and glycogen stores had returned to identical levels, respectively, after Ex2 in both groups before the TT. It is therefore not surprising if performance was not significantly different. Nevertheless, two correlative observations are worthy of notice. The subjects who were faster during the simulated TT from the average of both trials (i.e. the fitter) were those who tended to improve performance after the HCF diet. They were also those (Fig. 7) who reduced IMCL the most during the previous 3-h exercise. This may simply indicate that subjects with a higher aerobic power are able to turnover IMCL stores more rapidly than those with a lower aerobic power and that they are better performers, independently of the fat content of the diet. Whether IMCL status is a critical factor in the performance of these athletes remains to be established.

Conclusion

In conclusion, the present study of endurance-trained athletes describes a short-term dietary strategy by which it is possible to increase both of the muscle energy stores, glycogen and IMCL, prior to exercise. The diet involves two phases that take into account the kinetics of glycogen and IMCL repletion after exhaustive exercise: a high-CHO diet for 1 day (phase 1), which is continued for a further 1.5 days during which supplemental fat is added (phase 2). In the presence of equally high endogeneous carbohydrate stores, a dietary-induced increase in IMCL leads to a larger IMCL contribution to the energy demand of submaximal exercise, possibly at the expense of peripheral FAs, but does not significantly alter the overall fat to carbohydrate fuel ratio. IMCL appear to play a relatively modest and variable role in the overall energy budget of prolonged exercise, but their influence on lipid fuel partitioning during the first hour of exercise warrants further study. Furthermore, aerobic capacity appears to be a factor favoring IMCL storage and utilization, suggesting that both higher IMCL and a better training condition contribute to increasing IMCL mobilization during moderate intensity exercise.

Acknowledgments We thank the subjects of this study for their time and effort, Charles Schindler for preparing the sterile, pyrogene-free isotope solutions, Stéphane Berger, Anny Blondel, Bernard Decarli, Irina Monnard Jean-Marc Schneider, and Irène Zbinden for their skilled technical assistance. For evaluation of the glycogen spectra, the MRUI software package was kindly provided by the participants of the EU Network programs: Human Capital and Mobility, CHRX-CT94-0432 and Training and Mobility of Researchers, ERB-FMRX-CT970160. We appreciate Karin Zwygart for data acquisition. This work was supported by a grant from the Swiss National Science Foundation (3100-065315).

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