High dietary fat intake increases fat oxidation and reduces skeletal muscle mitochondrial respiration in trained humans

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Abbreviations List

100SS	100 minute steady state ride
ACC	Acetyl CoA carboxylase
ADP	Adenosine diphosphate
AMPK	AMP-activated protein kinase
AU	Arbitrary unit
βHB	βeta-hydroxybutyrate concentration
BIOPS	Biopsy preservation solution
BM	Body mass
CHO	Carbohydrate
CI	Mitochondrial complex I
CII	Mitochondrial complex II
CIII	Mitochondrial complex III
CIV	Mitochondrial complex IV
CV	Mitochondrial complex V
CPT1a	Carnitine palmitoyltransferase-1
CS	Citrate synthase
DM	Dry mass
DTT	Dithiothreitol
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EI	Energy intake
ETF	Electron-transferring flavoprotein (leak state respiration)
ETFp	Electron-transferring flavoprotein (ADP stimulated oxidative phosphorylation)
ETS	Electron transport system (respiratory capacity during uncoupled state)
FA	Fatty acid
FAT/CD36	Fatty acid translocase / CD36
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HDL	High density lipoprotein

HR	Heart rate
LDL	Low density lipoprotein
mTOR	Mammalian target of rapamycin
OXPHOS	Oxidative phosphorylation
PDH	Pyruvate dehydrogenase
PPO	Peak power output
PO	Power output
RER	Respiratory exchange ratio
RPE	Rate of perceived exertion
RPS6	S6 ribosomal protein
SUIT	Substrate-uncoupler-inhibitor titration
TT	Time-trial
$\dot{V}O_2peak$	Peak oxygen uptake
$\dot{V}CO_2$	Volume of carbon dioxide produced
$\dot{V}O_2$	Volume of oxygen consumed

Abstract

High-fat, low-carbohydrate (CHO) diets increase whole-body rates of fat oxidation and down-regulate CHO metabolism. We measured substrate utilization and skeletal muscle mitochondrial respiration to determine if these adaptations are driven by high-fat or low-CHO availability. In a randomized crossover design, eight male cyclists consumed five days of a high-CHO diet (HCHO, > 70% energy intake (EI)), followed by five days of either an isoenergetic high-fat (HFAT, > 65% EI) or high-protein diet (HPRO, > 65% EI) with CHO intake 'clamped' at < 20% EI. During the intervention, participants undertook daily exercise training. On day six, participants consumed a high-CHO diet, prior to undertaking 100 min of submaximal steady state cycling plus a ~30 min time trial. Following five days of HFAT, skeletal muscle mitochondrial respiration supported by octanoylcarnitine and pyruvate as well as uncoupled respiration was decreased at rest, and rates of whole-body fat oxidation were higher during exercise compared to HPRO. Following one day of HCHO intake, mitochondrial respiration returned to baseline values in HFAT while rates of substrate oxidation returned towards baseline in both conditions. These findings demonstrate that high dietary fat rather than low-CHO intake contributes to reductions in mitochondrial respiration and increases in whole-body rates of fat oxidation following a high-fat, low-CHO diet.

Key words: Carbohydrate oxidation, substrate oxidation rates, mitochondrial adaptations, cycling

Introduction

High-fat, low-carbohydrate (CHO) diets have increased in popularity over the past two decades with regards to their efficacy for improving both metabolic health profiles [1] and athletic performance [2, 3]. Short-term (1-3 week) ingestion of a high-fat, low-CHO diet when compared with an isoenergetic high-CHO diet for the same duration increases rates of whole-body and muscle fat utilization and decreases the rate of muscle glycogenolysis during submaximal exercise [2, 4-6]. Such metabolic perturbations are robust and persist in the face of high-CHO availability from both endogenous and exogenous sources [4, 7, 8]. Impaired glycogenolysis as a consequence of high-fat, low-CHO diets has been explained by decreased pyruvate dehydrogenase (PDH) activation [5], suggesting impaired metabolic flexibility in skeletal muscle. A range of alterations in the activities of regulatory enzymes and/or signaling proteins in the pathways underlying skeletal muscle fat and CHO metabolism are likely to explain the changes observed with adaptation to a high-fat diet. However, to date it has not been possible to determine whether such adaptations are driven by high-fat or low-CHO availability as the protocols used in previous studies involved changes to both macronutrients simultaneously [2, 4, 5, 7]. Therefore, in order to elucidate the underlying mechanisms driving changes in metabolic flexibility, high-fat dietary intake must be compared to an isoenergetic diet, where CHO intake is clamped in both dietary interventions. Few studies have determined changes to skeletal muscle in well-trained humans following a high-fat diet and to date, no study has assessed mitochondrial respiration in this population to determine if this could explain changes in metabolic flexibility.

Therefore, the current investigation aimed to determine whether the metabolic perturbations induced by a high-fat diet are a result of high-fat or low-CHO availability. Well-trained humans were fed five days of either a high-fat diet or an isoenergetic high-protein diet (~65% energy intake (EI)) with CHO intake 'clamped' to < 20% of total daily EI (2.6 g·kg^{-1} body mass). We utilized whole-body expired gas measures together with assessment of skeletal muscle substrates, mitochondrial respiration and signaling proteins with putative roles in substrate metabolism in an effort to identify mechanisms underlying changes in the patterns of substrate oxidation observed following a high-fat diet. We hypothesized that whole body rates of fat oxidation would be greater following high-fat compared to a high-protein diet due to high-fat rather than low-CHO availability driving the shifts in fuel utilization and skeletal muscle mitochondrial respiration.

Materials and Methods

Ethical Approval

This study conformed to the standards set by the *Declaration of Helsinki* and was approved by the Human Research Ethics Committee of Australian Catholic University and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12616000433404). Participants completed a medical history questionnaire to ensure they were free from illness and injury before commencing the study and were informed of all experimental procedures and possible risks prior to providing their written, informed consent.

Overview of study design

Eight well-trained male cyclists with a history of endurance training and riding > 200 km$\cdot$week^{-1} were recruited for this study. Participant characteristics were: age, 25 ± 4 (SD) y; body mass (BM), 77.3 ± 7.0 kg; $\dot{V}O_2$peak, 64.0 ± 3.5 mL$\cdot$kg$\cdot$min^{-1}; peak power output (PPO), 380 ± 36 W. An overview of the study design is shown in Figure 1. Each participant completed two experimental conditions in a block randomized, crossover design while undertaking supervised training. There was a ~14 day wash out period between conditions. It was not possible to blind participants to the dietary interventions. However, the principal researchers completing the data collection and performance measures were blinded to the order of experimental trials.

Preliminary testing

Each participant completed an incremental test to volitional fatigue on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands) to determine $\dot{V}O_2$peak and PPO [9]. During the maximal test and all subsequent experimental trials, expired gas was collected every 30 s via open-circuit spirometry (TrueOne 2400; Parvo Medics, Sandy, UT) and the instantaneous rates of O_2 consumption ($\dot{V}O_2$) and CO_2 production ($\dot{V}CO_2$) were used to calculate the respiratory exchange ratio (RER). Before each test, gas analyzers were calibrated with commercially available gases (16% O_2, 4% CO_2) and volume flow was calibrated using a 3 L syringe. An individual's $\dot{V}O_2$peak was determined as the highest 30-s average. These data were used to calculate the work rate corresponding to 63% and 80% of PPO for the two experimental rides.

Experimental trials

Participants followed a 'controlled' high-CHO diet (72% EI), 10 $g \cdot kg^{-1}$ BM [HCHO]) for five days prior to an experimental trial (see Table 1). Participants reported to the lab on the 5th day after an overnight fast and a resting blood sample (6 mL) was collected from an antecubital vein. Participants were then provided a standardized breakfast (2 $g \cdot kg^{-1}$ BM CHO). Two hours following breakfast, participants were weighed and a second blood sample was collected before they completed a 20 min continuous ride at 63% PPO. Expired gas and measures of heart rate (HR) and rating of perceived exertion (RPE) were collected during the last 5 min of the ride [10]. Water was consumed *ad libitum* and upon completion of the ride, a third blood sample was collected prior to participants leaving the lab for the final (5th) day of the HCHO diet.

The following morning, participants reported to the lab overnight fasted and a cannula (22G; Terumo, Tokyo, Japan) was inserted into an antecubital vein and a resting blood sample (6 mL) was collected. A resting muscle biopsy was then taken from the vastus lateralis using the percutaneous biopsy technique with suction applied [11]. Participants then repeated the 20 min continuous ride at 63% PPO in the fasted state, before commencing a high-intensity interval session (HIIT) (8 x 5 min at 80% PPO), as previously described [12]. The purpose of this interval session was to reduce muscle glycogen stores in both conditions prior to the dietary intervention.

Diet and Training Intervention

Participants commenced five days of either a high-fat (HFAT) or a high-protein (HPRO) diet. The HFAT and HPRO diets comprised 67% EI from fat or protein and 19% EI from CHO (Table 1). Protein was provided as an alternative macronutrient to meet energy requirements, while CHO was 'clamped'. Total EI was 0.22 $MJ \cdot kg^{-1}$ BM. The HFAT diet was comprised of ~55% saturated and 45% unsaturated (mono and polyunsaturated) fats. Fiber intake was matched for both diets. All meals, snacks and energy-containing fluids were provided to participants in pre-prepared packages, with diets individualized for food preference. Participants completed a daily food checklist to maximize compliance and recorded all fluid (water) consumed on a daily basis during both trials. Caffeine ingestion was not permitted 24 h prior to an experimental trial and participants refrained from alcohol during the intervention period. During this time, participants followed a prescribed training program described previously [2] that closely matched each individual's habitual road cycle training volume. Training was matched for each experimental treatment and

participants were instructed to ride at a rating of perceived exertion (RPE) that corresponded to 11-13 [10] during each on-road session. Participants reported to the lab on day 4 and completed the same HIIT session as on day 1. On the morning of day 6, participants reported to the lab in a fasted state and a resting blood sample (6 mL) and muscle biopsy were collected before they completed a 20 min ride at 63% PPO. Participants were then provided with 1 day of a high-CHO diet (10 g$\cdot$kg^{-1} BM CHO) (Table 1).

Performance ride

After an overnight fast participants reported to the laboratory to complete a performance ride consisting of 100 min steady state (100SS) cycling at 63% PPO, followed by a 7 kJ$\cdot$kg^{-1} BM time trial (TT). On arrival at the laboratory, a cannula was inserted into an antecubital vein and a fasted blood sample (10 mL) was collected. A muscle biopsy was then taken 2-3 cm distal from the previous incision. Participants then consumed breakfast (2 g$\cdot$kg^{-1} BM CHO) and rested for 120 min. Immediately prior to exercise participants were weighed and a second blood sample was collected. During exercise blood samples (10 mL) and measures of RPE and HR were collected every 20 min, with expired gas collected at 15, 35, 55, 75, and 95 min. Participants were provided with CHO in the form of isotonic gels (SiS GO Isotonic Gel; Blackburn, UK) and a 6% CHO solution (933 mL fluid, 2 gels total) every 20 min throughout the ride at a rate of 60 g$\cdot$h^{-1} and water was consumed *ad libitum* during each trial. Immediately upon completion of the 100SS ride, a further muscle biopsy was taken. Participants then voided their bladder and had a 3 min rest prior to commencing the TT. Participants were instructed to complete the TT as fast as possible with visual feedback of cadence and verbal feedback of elapsed work as a percentage of the total work (every 10%). Participants were only provided the results of their TT performance upon study completion. Blood samples were collected immediately before and after the TT.

Rates of substrate oxidation and total energy expenditure

Whole body rates of CHO and fat oxidation (g$\cdot$min^{-1}) were calculated from respiratory gas samples collected during rides using the non-protein RER equations [13] which are based on the assumption that $V\dot{O}_2$ and $VC\dot{O}_2$ accurately reflect tissue O_2 consumption and CO_2 production:

CHO oxidation $(g \cdot min^{-1}) = 4.585\ \dot{V}CO_2\ (L \cdot min^{-1}) - 3.226\ \dot{V}O_2\ (L \cdot min^{-1})$

Fat oxidation $(g \cdot min^{-1}) = 1.695\ \dot{V}O_2\ (L \cdot min^{-1}) - 1.701\ \dot{V}CO_2\ (L \cdot min^{-1})$.

Rates of CHO and fatty acid oxidation $(\mu mol \cdot kg \cdot min^{-1})$ were calculated by converting the rates of oxidation $(g \cdot kg \cdot min^{-1})$ to their molar equivalent. It was assumed that 6 moles of O_2 is consumed and 6 moles of CO_2 is produced for each mole of CHO (180 g) oxidized and that the molecular mass of human triacylglycerol is $855.3\ g \cdot mol^{-1}$. The molar rates of triacylglycerol oxidation were multiplied by 3 because each molecule contains 3 moles fatty acid.

Blood sampling and analyses

Blood samples (6-10 mL) were collected into vacutainers containing EDTA and immediately analyzed for blood lactate (YSI 2900 STAT Plus, Yellow Springs, OH, USA) and total cholesterol, high density lipoproteins (HDL), low density lipoproteins (LDL) and triglycerides (Cobas b 101, Roche Diagnostics Ltd, Basel, Switzerland). The remaining sample was then centrifuged at 1,500 *g* for 10 min at 4 °C, and aliquots of plasma were stored at -80 °C for later analysis of FFA (Wako Pure Chemical Industries, Ltd, Osaka, Japan), glycerol (Sigma-Aldrich, Ltd, Australia), insulin (R-Biopharm – Laboratory Diagnostics Pty Ltd, NSW, Australia), βeta-hydroxybutyrate (βHB) (Sigma-Aldrich, Ltd, Australia) and glucose (Melbourne Pathology, Vic, Australia) concentration.

Mitochondrial respiration analyses

Vastus lateralis muscle biopsies were excised and 10-20 mg was immediately placed into 3 mL of ice-cold biopsy preservation solution (BIOPS) [2.77 mM CaK_2 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2 \cdot 6\ H_2O$, 20 mM taurine, 15 mM Na_2Phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol (DTT), 50 mM MES hydrate; pH 7.1]. Muscle fibers were mechanically separated in ice-cold BIOPS to maximize fiber surface area and transferred into ice-cold BIOPS supplemented with saponin $(50\ \mu g \cdot mL^{-1})$ for 30 min with agitation to permeabilize the sarcolemma and allow diffusion of substrates. Fibers were then washed 3 times via agitation in ice-cold MiR05 respiration medium (20 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 0.5

mM EGTA, 10 mM KH_2PO_4, 3 mM $MgCl_2 \cdot 6H_2O$, 60 mM lactobionic acid, 20 mM taurine, 110 mM D-sucrose, 1 $g \cdot L^{-1}$ bovine serum albumin (BSA); pH 7.1). Fiber bundles were divided and weighed on a microbalance (1.5-3 mg each) for respirometry analysis in duplicate. All respiration analyses were commenced within 1 h of sampling.

Electron transport system (ETS) and oxidative phosphorylation (OXPHOS) respiration were measured by the Oxygraph O2k high resolution respirometer (Oroboros Instruments, Innsbruck, Austria) via a substrate-uncoupler-inhibitor titration (SUIT) protocol at 37 °C in MIR05 respiration medium with magnetic stirring at 750 rpm. Briefly, after fibers were added and O_2 was injected to the respiration chamber (maintained between 300 and 500 pmol), the sequential addition SUIT protocol commenced with titrations of malate (2 mM final concentration) and octanoylcarnitine (0.2 mM) to determine leak electron-transferring flavoprotein (ETF) respiration. OXPHOS ETF (ETFp) respiration was assessed by addition of adenosine diphosphate (ADP; 5 mM), complex I (CI) substrate pyruvate (5 mM) and complex II (CII) substrate succinate (10 mM). Cytochrome c (10 μM) was added to confirm mitochondrial membrane integrity, and titrations of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; 0.025 μM) were added to determine uncoupled respiratory flux. Complex-specific respiration was inhibited by the addition of rotenone (1 μM) and antimycin A (5 μM) to CI and complex III (CIII), respectively. Finally, complex IV (CIV) capacity was measured during oxidation of N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD; 0.5 mM) with ascorbate (2 mM). O_2 flux due to auto-oxidation of these chemicals was determined after inhibition of complex IV (CIV) with sodium azide (15 mM) then subtracted from the raw CIV O_2 flux. Chamber O_2 concentration was maintained between 300 and 450 $μmol^. L^{-1}$. Mass-specific O_2 flux was determined from steady-state flux normalized to tissue wet weight and adjusted for instrumental background and residual O_2 consumption.

Muscle glycogen concentration

Muscle glycogen concentration was determined as described previously [14]. In brief, ~20 mg of muscle was freeze-dried and powdered, with all visible connective tissue removed under a microscope. Glycogen was then extracted from the freeze-dried sample and glycogen concentration was determined via enzymatic analysis [15].

Citrate synthase activity

Whole skeletal muscle lysates were prepared at a concentration of 2 mg·mL^{-1} and 5 µL of sample was loaded onto a 96-well microtiter plate with 40 µL of 3 mM acetyl CoA, and 25 µL of 1 mM 5,5'-dithiobis [2-nitrobenzoic acid] (DTNB) in 165 µL of 100 mM Tris buffer (pH 8.3). Subsequently, 15 µL of 10 mM oxaloacetic acid was added to each well and immediately analyzed using a SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA). Absorbance was read at 412 nm and was recorded every 15 s for 3 min after 30 s of linear agitation. Maximal activity was recorded with citrate synthase activity reported in mol·h·kg^{-1} protein.

Protein analyses

For generation of whole skeletal muscle lysates, ~40 mg of skeletal muscle was homogenized in buffer containing 50 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM DTT, 10 µg/mL trypsin inhibitor, 2 µg·mL^{-1} aprotinin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Samples were spun at 16,000 g for 30 min at 4°C and supernatant was collected. After determination of protein concentration via bicinchoninic acid protein assay (Pierce, Rockford, IL), lysates were resuspended in Laemmli sample buffer and 10 µg protein of each sample was loaded into 4–20% Mini-PROTEAN TGX Stain-Free Gels (Bio-Rad Laboratories, California, USA). For OXPHOS antibody cocktail, 8.5 µg protein from unboiled lysates were loaded into 12% polyacrylamide gels. Following electrophoresis, gels were activated according to the manufacturer's instructions (Chemidoc; Bio-Rad Laboratories, Gladesville, Australia) and transferred to polyvinylidine fluoride (PVDF) membranes. After transfer, a Stain-Free image was obtained for protein loading normalization before rinsing membranes briefly in distilled water, blocking for 1 h with 5% nonfat milk, washing three times (5 min each wash) with 10 mM Tris·HCl, 100 mM NaCl, and 0.02% Tween 20 solution (TBST) and incubating with primary antibody diluted in TBST (1:1,000) overnight at 4°C on a shaker. Membranes were incubated for 1 h the next day with a secondary antibody diluted in TBST (1:2,000) and proteins were detected via enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce

Biotechnology) and quantified by densitometry (Chemidoc; Bio-Rad Laboratories). Time points and both diets for each subject were run on the same gel.

Antibodies against fatty acid translocase (FAT/CD36) (no. 14347), Carnitine palmitoyltransferase-1 (CPT1A) (no. 12252), AMP-activated protein kinase (AMPKα) (no. 2532), phospho-AMPK^{Thr172} (no. 2531), Acetyl CoA Carboxylase (ACC) (no.3662), phospho-ACC^{Ser79} (no.3661), mammalian target of rapamycin (mTOR) (no. 2972), phospho-mTOR^{Ser2448} (no. 2971), S6 Ribosomal Protein RPS6 (no. 2217), phospho-RPS6$^{Ser235/236}$ (no. 2211), Citrate Synthase (no. 14309), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (no. 2118) were purchased from Cell Signaling Technology (Danvers, MA) and total OXPHOS (no. 110411) purchased from Abcam (Cambridge, UK). Volume density of each target band was normalized to total protein loaded into each lane using Stain-Free technology (Bio-Rad Laboratories), excluding OXPHOS cocktail which was normalized to GAPDH imaged from the same membrane following the addition of stripping buffer (Thermo Fisher Scientific) to the OXPHOS membrane and re-probing for GAPDH. Following protein loading normalization, each phosphoprotein was then normalized to its respective total protein.

Statistics

Statistical analysis was undertaken using SPSS (Version 20 for Windows, SPSS Inc, Chicago, IL). Data from the two experimental conditions were analyzed using a linear mixed model (treatment × time) and subsequent *post hoc* comparisons were completed within the linear mixed model based on least significant difference. Separate analysis was completed to compare day five of high-CHO diet to 100SS (fed) and day one of HFAT or HPRO to five days post-diet (fasted). Normality was visually assessed using the linear model residuals. Differences in TT performance between trials were compared using a Student's paired t-test. Statistical significance was considered at $P < 0.05$. All data are represented as mean ± SD.

Results

All participants complied with the prescribed dietary (Table 1) and training intervention for both conditions. No difference was reported across the 5-day intervention periods for distance covered or RPE during training for either diet (HFAT, 222 ± 23 km, 13 ± 0.5; HPRO, 196 ± 29 km, 13 ± 0.7 respectively).

Muscle glycogen concentrations

There was a significant main effect of time for muscle glycogen concentration ($P < 0.001$) (Figure 2). Muscle glycogen was reduced in both HFAT and HPRO conditions pre- to post-diet ($P < 0.001$). Following one day of high-CHO diet, muscle glycogen increased by ~45% in both HFAT and HPRO conditions ($P < 0.001$) but was not restored back to pre-diet values in HFAT ($P < 0.001$). Following 100SS, muscle glycogen was reduced in both HFAT and HPRO conditions (526 ± 86 to 411 ± 62 mmol·kg^{-1} dry mass (DM), P=0.033; 637 ± 87 to 420 ± 92 mmol·kg^{-1} DM, $P < 0.001$, respectively); however, no difference in the percentage change from pre- to post-exercise was measured between conditions.

Rates of substrate oxidation

There was a significant interaction effect for RER and rates of CHO and fat oxidation (all P = 0.001) (Figure 3A, B, C) after five days of either HFAT or HPRO diet. RER was reduced pre- to post-diet for both HFAT and HPRO (0.90 ± 0.02 to 0.79 ± 0.02; 0.90 ± 0.03 to 0.86 ± 0.02, $P \leq$ 0.001 respectively) and was lower post-diet in HFAT compared to HPRO ($P < 0.001$). Rates of fat oxidation increased after five days of HFAT and HPRO and were greater in HFAT compared to HPRO post-diet (55 ± 7 vs. 36 ± 6 μmol·kg·min^{-1}, $P < 0.001$). Concomitantly, rates of CHO oxidation were reduced pre- to post-diet in both conditions and were lower in HFAT than HPRO post-diet (106 ± 20 μmol·kg·min^{-1} vs. 169 ± 17 μmol·kg·min^{-1}, $P < 0.001$). Following one day of high-CHO diet, RER values returned to baseline in the HPRO trial during the first 20 min of 100SS, but remained lower than baseline in the HFAT trial (HFAT 0.93 ± 0.02 to 0.90 ± 0.03, P = 0.002). RER was lower in HFAT compared to HPRO during the first 40 min of 100SS ($P <$ 0.04). Following one day of high-CHO diet, rates of CHO oxidation were lower than baseline during 100SS following HFAT ($P < 0.001$) and were significantly lower during 100SS in HFAT

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compared to HPRO (213 ± 35 vs. 241 ± 31 µmol·kg·min^{-1}, P = 0.025, respectively). Rates of CHO oxidation declined throughout 100SS in HPRO (from 241 ± 31 to 215 ± 26 µmol·kg·min^{-1}, P < 0.001), but remained stable in HFAT (~208 µmol·kg·min^{-1}). Despite one day of high-CHO diet, rates of fat oxidation remained elevated above baseline in the HFAT trial during the first 20 min of 100SS (P = 0.002) but returned to baseline in HPRO. During the first 20 min of 100SS, rates of fat oxidation were significantly higher in HFAT than HPRO (0.53 ± 0.11 vs. 0.38 ± 0.18 µmol·kg·min-1, P = 0.010) and remained higher than HPRO after 40 and 80 min of exercise. Rates of fat oxidation increased during 100SS in both HFAT and HPRO (P < 0.05).

Blood metabolites pre- and post-diet

There was a main effect of time for FFA concentration (P < 0.001) pre- to post-diet. FFA concentration was greater following exercise post-diet compared to pre-diet in both HFAT (0.31 mM to 0.58 mM, P < 0.001) and HPRO (0.33 to 0.56 mM, P < 0.001). There was a significant main effect of time (P = 0.013) and condition (P = 0.048) for LDL cholesterol. LDL cholesterol increased pre- to post-diet in HFAT (2.44 ± 0.63 to 2.93 ± 0.75 mM) and was higher than HPRO post-diet (2.93 ± 0.75 vs 2.55 ± 0.71, P = 0.025). There was a significant interaction for HDL cholesterol and triglycerides (P = 0.010, 0.042, respectively) between HFAT and HPRO. HDL cholesterol increased (1.01 ± 0.20 to 1.30 ± 0.23 mM, P < 0.001) pre-to post-diet to be greater than HPRO (1.08 ± 0.20 mM), while triglycerides decreased (1.26 ± 0.46 to 0.69 ± 0.36 mM, P = 0.001) to be lower than HPRO (1.06 ± 0.45 mM). No difference in total cholesterol was measured between conditions from pre- to post-diet.

Blood metabolites during the performance ride

There was a significant interaction for plasma glycerol concentration between HFAT and HPRO (P = 0.035; Figure 4A). Glycerol concentration increased significantly from rest after 60 min of exercise in HFAT and remained elevated until after the TT. Glycerol concentrations were significantly higher in HFAT than HPRO after 40 min of 100SS. There was a significant effect of time for plasma FFA (P < 0.001), although no differences were observed between diets (Figure 4B). FFA concentrations decreased 2 h following CHO breakfast in both conditions and were

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elevated from resting values after 60 min of 100SS until completion of the TT. Plasma βHB concentrations increased following CHO breakfast and remained stable during 100SS in both conditions until after the TT (Figure 4C). There was a main effect of time for blood lactate, blood glucose and plasma insulin concentrations ($P < 0.001$) during 100SS, but no differences between diets (Figure 4D-F). Blood glucose concentration decreased following CHO breakfast in both diets but following 40 min of exercise, glucose concentrations had increased back to resting values. Plasma insulin concentrations increased in both conditions after breakfast and remained elevated 2 h after ingestion. After onset of exercise, insulin concentrations were reduced in both conditions and were similar to pre-breakfast values throughout 100SS. Following 100SS, participants ingested a CHO drink which increased insulin concentrations in both conditions, but this increase was abolished following the onset of the TT. Blood lactate concentrations remained stable throughout 100SS in both conditions and were higher post TT compared to rest in HFAT (3.1 ± 1.1 mM) and HPRO (3.1 ± 1.0 mM).

TT Performance

There was no difference in TT performance between diet conditions ($30:59 \pm 2:55$ vs. $30:10 \pm 2:70$ min:sec for HFAT and HPRO, respectively). Mean PO during the TT were 299 ± 34 W and 304 ± 35 W ($P = 0.41$) and HR averaged 168 ± 9 bpm and 166 ± 7 b.min^{-1} in HFAT and HPRO, respectively. A significant reduction in BM was observed pre- to post-exercise ($P < 0.04$) for both HFAT (-0.86 ± 0.79 kg) and HPRO (-0.61 ± 0.83 kg), although there were no differences between conditions. No difference in RPE was reported between conditions during 100SS although RPE increased throughout the exercise in both HFAT and HPRO (from 11 ± 1 to 14 ± 1, $P < 0.001$).

Skeletal muscle mitochondrial respiration

Based on differences in whole-body substrate oxidation rates pre- to post-diet and during prolonged exercise between HFAT and HPRO (Figure 3), we next tested if skeletal muscle mitochondrial substrate utilization was contributing to this outcome using a sequential addition SUIT protocol. Absolute O2k respiration measures taken from permeabilized skeletal muscle fiber bundles analyzed in duplicate from each participant at each time point and under each diet are

reported in Table 2. To portray the effects of diet and exercise on mitochondrial respiration, percentage change data are represented in Figure 5. Although HFAT and HPRO absolute O2k values were not significantly different following either diet (Table 2), there was a significant interaction in percentage change of CI + ETFp respiration following HFAT and HPRO (P = 0.042; Figure 5A). The diet-induced reduction in CI + ETFp respiration following the addition of octanoylcarnitine and pyruvate was significantly greater following HFAT compared to HPRO. Despite no differences in absolute O2k values, percentage change of ETS uncoupled respiration (Figure 5A; ETS CI + CII + ETF; ETS CII) was significantly reduced following HFAT but not HPRO. Absolute and percentage change ETS CII uncoupled respiration remained unchanged following one day of high-CHO diet in HPRO but percentage change was significantly increased in HFAT compared to HPRO (P = 0.032, Figure 5B). ETFp respiration was significantly reduced following 100SS in HFAT but not HPRO (Figure 5C). Percentage change of post-exercise CI + ETFp, CI + CII + ETFp, ETS CI + CII + ETF, and ETS CII respiration was significantly reduced in both HFAT and HPRO (Figure 5C) despite no differences in absolute O2k values. The reduction in percentage change of CI + ETFp and CI + CII + ETFp respiration was greater in HFAT than HPRO following 100SS (P = 0.024, 0.019, respectively). There were no significant differences in skeletal muscle CS activity across time or between diets (~ 20 mol·h·kg^{-1}) (Figure 5D).

Immunoblot analyses

Total protein contents of citrate synthase (Figure 5E) and OXPHOS complexes I-V (Figure 6A-F) were not different between HFAT and HPRO at any time point during intervention. OXPHOS complex III showed a trend towards a main effect for time (P = 0.073) with a decrease from pre- to post HFAT diet. There was a significant interaction for FAT/CD36 protein content (P < 0.001) from pre-diet to after one day of high-CHO diet (Figure 7A). FAT/CD36 protein content was higher pre- and post-diet and pre- and post-100SS in HFAT compared to HPRO. There were no differences in total CPT1a from pre- to post high-CHO diet in either HFAT or HPRO (Figure 7B). No main effects were found for AMPK Thr172 phosphorylation levels relative to total AMPK, although a trend towards a main effect of time was observed (P = 0.06) with an increase following HFAT (Figure 7C). There was an effect of time for ACC Ser79 phosphorylation relative to total ACC (P = 0.015). ACC Ser79 relative to total ACC was greater in HFAT following 100SS

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compared to post HFAT (Figure 7D). There were no differences in mTOR Ser2448 phosphorylation relative to total mTOR pre-diet compared to after one day of high-CHO (Figure 7E). There was a significant effect of time for RPS6 Ser235/236 phosphorylation relative to total RPS6 ($P < 0.05$). RPS6 Ser235/236 phosphorylation increased following 100SS in HPRO compared to pre- and post-diet (Figure 7F). RPS6 Ser235/236 phosphorylation was also higher post-exercise in HPRO compared to HFAT ($P = 0.034$).

Discussion

This is the first study to manipulate dietary fat and protein content while simultaneously 'clamping' dietary CHO intake during a short-term period of intense exercise training in well-trained humans. Such an experimental design is essential in an effort to pinpoint potential mechanisms underlying the high rates of fat oxidation reported following short-term adaptation to fat-rich diets, which persist even after one day of glycogen restoration with high-CHO intake [2, 7] and/or high exogenous CHO availability [4, 5, 7]. The results of the present study provide novel insights into the mechanisms governing patterns of substrate oxidation in response to diet-exercise interactions. We report that compared to an isoenergetic high-protein diet, five days' adaptation to a high-fat diet results in greater whole-body rates of fat oxidation during submaximal cycling and impairments in mitochondrial respiration.

A series of independent studies over the past two decades [2, 4, 5, 7, 8] have compared high-fat versus high-CHO diets and shown that short-term (< 7 days) high-fat diets result in peak rates of whole-body fat oxidation of ~ 1 g·min^{-1} (~ 50 umol·kg·min^{-1}), values that are typically two-fold greater than after isoenergetic high-CHO diets [2, 7]. The rates of fat oxidation in the present investigation (1.2 g·min^{-1}) after an identical period of a fat-rich diet were similar to those reported previously. However, the first novel finding from the present study was that rates of fat oxidation were 33% greater than after five days of a low-CHO, HPRO diet (0.8 g·min^{-1}). We report an increase in post-exercise plasma FFA concentration from pre-diet interventions in HFAT and HPRO, which likely contributed to the increased rates of fat oxidation after both conditions. However, the higher rates of fat oxidation in HFAT compared to HPRO are likely associated with altered rates of whole body lipolysis and subsequent storage of triglycerides [6, 16, 17]. Previous

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work has shown higher rates of whole-body lipolysis, determined by elevated glycerol concentration, and this increase was associated with elevated intramuscular triglyceride (IMTG) concentration following fat-adaptation [6, 18]. Limited muscle biopsy sample did not permit IMTG measurements in the current study. It is also known that low-CHO availability reduces circulating insulin concentrations which could increase rates of whole-body fat oxidation. Although CHO intake was identical in both dietary conditions, it is likely that a proportion of protein in HPRO was converted to glucose via gluconeogenesis [19], which may explain slightly higher muscle glycogen concentrations post-diet in HPRO compared to HFAT. Higher availability of muscle glycogen likely contributes to lower rates of whole-body fat oxidation in HPRO compared to HFAT. Accordingly, the higher rates of fat oxidation measured after HFAT are likely driven by higher fat rather than higher CHO availability.

Despite CHO intake being 'clamped' in both dietary conditions, rates of CHO oxidation were lower following five days HFAT compared to HPRO. Rates of CHO oxidation were reduced by 50% (3 g·min^{-1} to 1.5 g·min^{-1}) following HFAT compared to a 25% decline (2.3 g·min^{-1}) following HPRO. The greater oxidation of CHO-based fuels may be explained by the slightly higher muscle glycogen concentration post-diet in HPRO compared to HFAT, which has previously been shown to increase reliance on CHO-based fuels during subsequent exercise [20]. Without a protein tracer in the current study, we are unable to detect the proportion of protein that is being converted to glucose. Following a 6-day high-fat diet (63% EI from fat), Peters [21] observed a decrease in the active form of the rate limiting enzyme in CHO metabolism, pyruvate dehydrogenase (PDH), and consequently a reduction in rates of CHO oxidation during exercise, which was not observed following a moderate CHO diet (52% EI CHO). Therefore, a decrease in PDH activity may contribute towards the observed reduction in whole-body rates of CHO oxidation in the present study [5]; however, limitations in muscle biopsy sample did not permit assessment of PDH activity.

To further determine potential mechanisms for reduced CHO oxidation, we assessed skeletal muscle mitochondrial respiration to measure dietary effects on substrate flux and utilization. We found that respiration supported by octanoylcarnitine and pyruvate (CI + ETFp)

was significantly reduced after five days of HFAT, but not HPRO, when CIII and/or CIV are operating at or near maximal activity. In our interpretations of mitochondrial respiration data obtained using the Oroboros O2k SUIT protocol, it is important to note that supra-physiological mitochondrial substrate concentrations and a sequential addition protocol are used. Therefore, interpretations of substrate-specific effects on respiration must be made with caution because this protocol does not allow us to pinpoint whether the addition of a particular substrate alone or any previously added substrate in the protocol are responsible for the effect. Nonetheless, it was surprising that FFA (i.e. octanoylcarnitine)-driven mitochondrial respiration (ETF leak) was not subsequently increased with HFAT. Decreased respiration observed following the addition of octanoylcarnitine and pyruvate is in line with previous studies reporting high-fat diets reduce the amount of PDH (in its active form [PDHa]) and PDHa activity at rest but not after a moderate CHO diet [21]. Alterations in PDH activity have further been identified as a mechanism underlying regulation of metabolic flexibility in isolated rodent skeletal muscle mitochondria in response to altered substrate availability induced by high-fat feeding [22]. In addition, the reduction in respiration after five days of HFAT persisted after uncoupling (ETS CI + CII + ETF and ETS CII), suggesting that the functional reductions in respiration occurred either at the level of CI/CII or downstream at CIII/CIV but not at CV (ATP-synthase). In line with the observed reductions in uncoupled respiration, Skovbro [23] observed that ETFp and uncoupled respiration were decreased following a longer high-fat feeding period (i.e. 16 days; 55-60% fat) compared to a moderate CHO diet (i.e. 55-60% CHO) [23]. The mitochondrial effects of HFAT in the present study may have been more pronounced following a longer dietary intervention period. Additionally, the type of the dietary fat intake has previously shown to affect mitochondrial function and morphology [24]. Lionetti [24] has shown that high saturated fat intake was associated with greater mitochondrial dysfunction compared to unsaturated fat in rodents. The current study provided a 65% fat diet, which was made up of 55% saturated fat, and whether the reductions in mitochondrial respiration would be observed with a different dietary fat composition requires further investigation. As no changes in mitochondrial respiration were observed following five days of HPRO, this suggests that the primary driver of these skeletal muscle adaptations is high dietary fat availability. Based on the evidence in the present study, the biochemical explanation of why HFAT results in increased whole body fat oxidation despite reduced skeletal muscle mitochondrial respiration is inconclusive and warrants future investigation of mitochondria in other tissues. Given that potential HFAT-

induced changes in the delivery and transport of fatty acids across sarcolemma are removed in the ex vivo analysis of mitochondrial respiration, these additional variables may also contribute to the observed increases in fat oxidation at the whole body level.

Although we observed changes in respiration following the addition of octanoylcarnitine and pyruvate and uncoupled respiration with HFAT, we detected no differences in skeletal muscle protein content of the five mitochondrial OXPHOS complexes after either dietary condition. Additionally, neither citrate synthase protein content nor maximal activity was changed. Given that changes in complex I and citrate synthase activity have strong associations with mitochondrial content (i.e. volume and/or density), this suggests that content is not affected by either short-term HFAT or HPRO availability [25]. Instead, we speculate that changes in enzyme activities regulating mitochondrial substrate flux likely contribute towards the reduced respiration observed after short-term adaptation to HFAT.

To determine alternative enzymes and signaling pathways impacted by the HFAT and HPRO, we measured putative transporters with roles in skeletal muscle FA uptake, and two energy sensing metabolic signaling pathways, AMPK and mTOR. There was a 12% increase in FAT/CD36 protein observed following HFAT, suggesting potential increased capacity for sarcolemmal and/or mitochondrial membrane FA uptake, although this increase in FAT/CD36 did not reach statistical significance. No change in mitochondrial CPT1 was observed following five days of HFAT or HPRO. These findings are in agreement with previous work which reported that a high-fat diet together with an intensive training program resulted in significantly greater (i.e. 17% increase) protein abundance of FAT/CD36 without change in gene expression of CPT1 [16]. Low CHO diets together with periods of endurance training have previously been shown to increase AMPK activation and signaling to its downstream substrate ACC [26]. However, no significant change was reported in AMPK Thr172 phosphorylation relative to total AMPK, or its substrate ACC Ser79 phosphorylation relative to total ACC following the dietary interventions. No differences post-diet were found in mTOR Ser2448 phosphorylation relative to total mTOR and phosphorylation of its substrate RPS6 Ser235/236 relative to total RPS6 in HFAT or HPRO. Together these findings suggest that FA transporter abundance and activation of these energy-

sensing pathways were unaffected by the two diet interventions, perhaps as a result of the high training status of the cyclists and the ability to cope with the demand of the dietary overload. Further investigation is required to uncover alternative protein signaling pathways associated with changes in substrate metabolism that may underpin the dietary effects on skeletal muscle mitochondrial respiration.

After CHO restoration strategies (e.g. 1 day of high-CHO diet, a pre-exercise CHO-rich breakfast and CHO intake during exercise) muscle glycogen increased in both HFAT and HPRO, but did not reach pre-intervention values in HFAT. This may be a result of the brief (24 h) CHO restoration period compared to five days of high CHO intake prior to baseline measures. Rates of fat oxidation and CHO oxidation returned towards baseline values during 100 min SS cycling in both HFAT and HPRO, and were similar to the results seen in the pre-diet protocol. However, the CHO restoration and exercise feeding protocols involved aggressive strategies to promote high CHO availability from both exogenous and endogenous sources, compared with overnight fasted and water fed conditions on the pre-diet protocol. Therefore, rates of fat oxidation were higher and CHO oxidation lower than expected during the 100 min steady state protocol on day 7, particularly with HFAT. Indeed, although the present study did not include a direct comparison to a chronic high-CHO diet as in our prior investigations [2, 4, 7], our results are consistent with previous observations that muscle adaptation during chronic periods of a low-CHO diet, especially in the case of the HFAT, is sufficiently robust to persist despite the restoration of CHO [2, 4, 7]. For example, CHO oxidation rates following HFAT or HPRO and CHO restoration in the current study, were lower than those reported in previous studies following a controlled (chronic high-CHO) diet (200-220 umol·kg·min^{-1} vs. 250-300 umol·kg·min^{-1}) [7]. Muscle glycogen utilization in the current study was ~100 mmol·kg DM lower than those previously reported with HFAT intervention [2] and was slightly higher in HPRO compared to HFAT. This difference may be related to higher pre-exercise muscle glycogen in HPRO. Overall, this reduced capacity for CHO oxidation in HFAT, despite the availability of exogenous and endogenous stores was previously associated with persistent downregulation of PDH activity [5].

Whole body rates of fat oxidation after CHO restoration remained slightly higher after HFAT compared to HPRO, but this resulted in only a small difference in the total fat oxidized

during 100 min SS cycling (~ 15 g over 100 min). No difference was measured for FFA concentrations between the two dietary conditions, although plasma glycerol concentrations were greater during exercise following HFAT compared to HPRO. The elevated glycerol concentration following HFAT indicates a greater rate of whole-body lipolysis which could be associated with greater IMTG utilization and/or liberation of FFA into the blood, contributing to the minor variation in rates of whole-body fat oxidation between HFAT and HPRO. Corresponding to total fat utilization, there were only minor differences in total CHO oxidized between HFAT and HPRO (~ 30 g) during 100 min of exercise.

Although one day of high CHO availability in the current study failed to fully reverse the differences in whole-body rates of substrate oxidation, it was sufficient to restore the decreased mitochondrial respiration (CI + ETFp) from HFAT, back to pre-diet values. This indicates that there may be an additional underlying mechanism regulating changes in substrate oxidation (i.e. downregulating CHO oxidation) and mitochondrial respiration. As the current study precluded investigation of a high-CHO trial due to the high number of biopsies that would have been required, we are unable to speculate whether differences in mitochondrial respiration would have been observed between the three dietary conditions. We also reported greater post-exercise reductions in CI + ETFp and CI + CII + ETFp respiration in HFAT than HPRO and this was not attributable to reductions to citrate synthase protein or activity. These effects of exercise following the HFAT may be attributable to mitochondrial adaptations at the cessation of exercise that impact ETC, including changes in signaling, mitochondrial membrane dynamics and/or buffering of reactive oxygen species. It should also be noted that there could have been damage to myofibers during separation and permeabilization. This could limit the interpretation of the respiratory values prior to the addition of cytochrome C in the SUIT protocol since it occasionally increased O_2 flux more than 10% above CI+CII+ETFp (Table 2). Despite this, we are confident that the data prior to the addition of cytochrome C is still meaningful since the effect was consistent across all trials. To determine the effect of potential shifts in substrate utilization on exercise performance, previous studies have included a cycling TT after a bout of steady state exercise, following the high-CHO intake. Burke [2] reported similar TT performance between high-fat and high-CHO trials and in the present study we did not detect any difference in TT performance between HFAT and HPRO

after one day of CHO restoration; however, we cannot compare this performance to a high-CHO condition and therefore the shifts in substrate utilization require further investigation.

In conclusion, the results of the present investigation demonstrate that whole-body rates of fat oxidation increase to a greater extent in trained humans following high dietary fat intake compared to a high-protein diet, with CHO 'clamped' at 20% of energy intake. High dietary fat also reduced mitochondrial respiration supported by octanoylcarnitine and pyruvate as well as uncoupled respiration. These reductions in mitochondrial 'function' may be compensatory, and not solely 'driving' fuel regulation under the conditions of our investigation. Further mechanistic investigation into potential underlying diet-induced differences in mitochondrial membrane dynamics, mitochondrial complex subunits and additional enzymes regulating mitochondrial substrate flux is warranted. The acute but aggressive restoration of endogenous and exogenous CHO availability was unable to completely restore normal rates of substrate oxidation but was able to reverse the fat-induced disruption of mitochondrial respiration. Together these findings demonstrate the impact a high-fat diet has on metabolic flexibility and skeletal muscle mitochondrial respiration in trained cyclists.

Author Conflict

No competing interests declared

Funding

Figure Legends

Figure 1: An overview of the study design showing the five days high-CHO diet followed by five days high-fat or high-protein diet (D1 to D7) with one day of a high-CHO diet. CHO, carbohydrate; HIIT, high-intensity interval training; TT, time-trial.

Figure 2: Resting muscle glycogen levels following five days high-CHO (Pre-diet D1), five days high-fat or high-protein (Post-diet D6), one day of a high-CHO diet (D7 Pre Ex) and following 100 min SS cycling at 63% PPO (D7 Post Ex) (A) and muscle glycogen percent change from Pre-diet (D1) to Post-diet (D6), from Post-diet (D6) to D7 Pre Ex and from D7 Pre Ex to D7 Post Ex (B) . Values are mean ± SD for n=6. a HFAT different to Pre-diet (D1) within condition; b HPRO different to Pre-diet (D1) within condition; c HFAT different to D7 Pre Ex and D7 Post Ex within condition; d HPRO different to D7 Pre Ex and D7 Post Ex within condition.

Figure 3: RER (A) and rates of CHO (B) and fat oxidation (C) following five days high CHO (Baseline and Pre-diet D1), five days high-fat or high-protein (Post-diet D6) during 20 min cycling and following one day of a high-CHO diet (D720-100) during 100 min SS cycling at 63% PPO. Values are mean ± SD. # HFAT different to HPRO at time point; e HFAT different to Baseline within condition; f HPRO different to Baseline within condition; a HFAT different to Pre-diet (D1); b HPRO different to Pre-diet (D1) within condition.

Figure 4: Effect of five days high-fat diet or high-protein and one day of a high-CHO diet on plasma glycerol (A), FFA (B), βHB (C), blood glucose (D), plasma insulin (E) and blood lactate concentration (F) before and during 100 min cycling at 63% PPO. Values are mean ± SD. # HFAT different to HPRO at time point; g HFAT different to T=0 within condition; h HPRO different to T=0 within condition; $ different to all time points within each condition.

Figure 5: Effect of five days high-fat or high-protein diet (A), one day of a high-CHO diet (B), 100 min SS cycling at 63% PPO (C) on mitochondrial respiration using a sequential addition protocol. Respiratory states are supported by single or convergent electron input via complex I (CI), complex II (CII) and/or electron transfer flavoprotein (ETF) under non-phosphorylating (Leak) conditions, state-3 oxidative phosphorylation in the presence of ADP (indicated by "p"), or with an uncoupler (FCCP) to assess maximal electron transport system (ETS) activity. Values are mean ± SD as a fold change from respective day indicated in each figure panel. Differences in

respiratory fluxes were not due to altered overall mitochondrial content as indicated by citrate synthase activity (D) or protein content (E). * O_2 flux per mass different from Pre-Diet D1 (A); Post-Diet D6 (B); Pre-Ex D7 (C) of the respective high-fat or high-protein diet. # HFAT different to HPRO condition at time point.

Figure 6: Skeletal muscle protein content (A-E) and representative images (F) of five OXPHOS complexes following five days high-CHO diet, five days high-fat or high-protein diet, one day of a high-CHO diet and following 100 min SS cycling at 63% PPO . Values are mean ±SD as a fold change relative to resting pre-diet D1 values.

Figure 7: Skeletal muscle protein content and representative blots of fat (CD36, CPT1a) (A & B), fat/CHO (p- Thr172 AMPK/Total, p-Ser79 ACC/Total) (C & D) and protein regulatory signaling pathways (p-Ser2448 mTOR/Total, p-Ser235/236 RPS6/Total) (E & F) following five days high-CHO, five days high-fat or high-protein diet, one day of a high-CHO diet and following 100 min SS cycling. Values are mean ± SD as a fold change relative to resting pre-diet D1 values. # HFAT different to HPRO at time point; i HFAT different to D7 Post Ex within condition, j HPRO different to D7 Post Ex within condition.

Tables

Table 1: Macronutrient content of the high-carbohydrate (HCHO), high-fat (HFAT) and high-protein (HPRO) diets consumed. Total Energy Intake (TEI).

	Energy		Carbohydrate			Protein			Fat		
	MJ	MJ/kg	Total (g)	g/kg	% TEI	Total (g)	g/kg	%TEI	Total (g)	g/kg	% TEI
HCHO	17.0 ± 1.4	0.22 ± 0.0	769.4 ± 63.4	10.0 ± 0.1	73.0 ± 0.5	140.5 ± 11.8	1.8 ± 0.0	14.2 ± 0.4	58.2 ± 6.3	0.8 ± 0.0	12.8 ± 0.8
HFAT	17.0 ± 1.5	0.22 ± 0.0	196.1 ± 19.7	2.5 ± 0.1	18.3 ± 0.7	138.7 ± 10.7	1.8 ± 0.1	13.7 ± 0.4	315.5 ± 26.8	4.1 ± 0.1	68.0 ± 0.6
HPRO	17.0 ± 1.5	0.22 ± 0.0	200.3 ± 19.5	2.6 ± 0.1	18.5 ± 1.0	679.8 ± 66.7	8.8 ± 0.5	66.7 ± 0.9	69.3 ± 7.4	0.9 ± 0.1	14.8 ± 0.7

Table 2: Effects of five days high-fat or high-protein diet, one day of a high-CHO diet and 100 min SS cycling at 63% PPO on mitochondrial respiration. Respiratory states are supported by single or convergent electron input via complex I (CI), complex II (CII) and/or electron transfer flavoprotein (ETF) under non-phosphorylating (Leak) conditions, state-3 oxidative phosphorylation in the presence of ADP (indicated by "p"), or with an uncoupler (FCCP) to assess maximal electron transport system (ETS) activity. Values are mean ± SD. a HFAT different to D7 Post Ex; b HPRO different to D7 Post Ex within condition; c HFAT different to D6.

O2k respiration measure; O2 flux per mass (pmol/s/mg)); mean ± SD)							
	ETF Leak	ETFp	CI + ETFp	CI + CII + ETFp	cyt-c test	ETS CI + CII + ETF	ETS CII
Substrate/Uncoupler/Inhibitor	malate, octanoylcarnitine	ADP	pyruvate	succinate	cytochrome c	FCCP	Rotenone
HFAT Pre-Diet (D1)	20.2 ± 5.9	43.8 ± 5.8	62.5 ± 11.2 [a]	116.8 ± 22.6 [a c]	136.6 ± 29.5 [a c]	144.4 ± 32.2 [a c]	92.5 ± 19.0 [a c]
HPRO Pre-Diet (D1)	17.5 ± 4.1	47.7 ± 5.6	66.1 ± 15.2	125.0 ± 31.2 [b]	147.1 ± 37.0 [b]	154.9 ± 37.3 [b]	100.6 ± 30.3 [b]
HFAT Post-Diet (D6)	21.0 ± 4.9	42.8 ± 7.0	55.7 ± 13.5	103.5 ± 27.2 [a]	119.1 ± 39.2 [a]	124.0 ± 38.4	77.2 ± 30.5 [a]
HPRO Post-Diet (D6)	19.1 ± 3.8	50.7 ± 4.5	68.4 ± 15.4 [b]	122.0 ± 26.9 [b]	140.3 ± 34.4 [b]	148.2 ± 37.2 [b]	91.3 ± 21.5 [b]
HFAT D7 Pre Ex	24.6 ± 5.7	52.4 ± 7.2	64.3 ± 13.9 [a]	120.1 ± 29.0 [a]	141.1 ± 39.7 [a]	145.9 ± 38.8 [a]	92.9 ± 27.3 [a]
HPRO D7 Pre Ex	18.7 ± 5.9	49.8 ± 12.8	64.8 ± 20.3	120.0 ± 36.9 [b]	142.9 ± 47.3 [b]	151.9 ± 51.0 [b]	97.3 ± 33.6 [b]
HFAT D7 Post Ex	24.6 ± 4.9	44.7 ± 9.8	47.6 ± 13.8	84.0 ± 23.8	99.0 ± 26.7	108.1 ± 31.0	60.8 ± 20.5
HPRO D7 Post Ex	21.7 ± 5.9	47.9 ± 11.3	56.8 ± 18.3	101.1 ± 30.2	105.4 ± 35.4	115.0 ± 37.4	65.4 ± 27.0

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