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

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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 whether these adaptations are driven by high fat or low CHO availability. In a randomized crossover design, 8 male cyclists consumed 5 d of a high-CHO diet (>70% energy intake (EI)), followed by 5 d 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 d 6, participants consumed a high-CHO diet before performing 100 min of submaximal steady-state cycling plus an ~30-min time trial. After 5 d 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 with HPRO. After 1 d of high-CHO diet intake, mitochondrial respiration returned to baseline values in HFAT, whereas rates of substrate oxidation returned toward baseline in both conditions. These findings demonstrate that high dietary fat intake, rather than low-CHO intake, contributes to reductions in mitochondrial respiration and increases in whole-body rates of fat oxidation after a consuming a high-fat, low-CHO diet.—Leckey, J. J., Hoffman, N. J., Parr, E. B., Devlin, B. L., Trewin, A. J., Stepto, N. K., Morton, J. P., Burke, L. M., Hawley, J. A. High dietary fat intake increases fat oxidation and reduces skeletal muscle mitochondrial respiration in trained humans. FASEB J. 32, 2979–2991 (2018). www.fasebj.org

KEY WORDS: carbohydrate · substrate utilization · adaptation · exercise · metabolism

ABBREVIATIONS: 100SS, 100-minute steady-state; ACC, acetyl CoA carboxylase; BIOPS, biopsy-preservation solution; BM, body mass; CI, mitochondrial complex I; CII, mitochondrial complex II; CIII, mitochondrial complex III; CIV, mitochondrial complex IV; CHO, carbohydrate; CPT1a, carnitine palmitoyltransferase-1; DM, dry mass; EI, energy intake; ETF, electron-transferring flavoprotein, leak state respiration; ETFp, electron-transferring flavoprotein, ADP-stimulated oxidative phosphorylation; ETS, electron transport system; FFA, free fatty acid; FAT/CD36, fatty acid translocase/CD36; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HFAT, high-fat diet; HPRO, high-protein diet; HR, heart rate; IMTG, intramuscular triglyceride; mTOR, mammalian target of rapamycin; oxphos, oxidative phosphorylation; PDH, pyruvate dehydrogenase; PPO, peak power output; RER, respiratory exchange ratio; RPE, rate of perceived exertion; RPS6, S6 ribosomal protein; SUIT, substrate-uncoupler-inhibitor titration; TBST, Tris-buffered saline/Tween 20; TT, time trial; VO2peak, peak oxygen uptake

As a result of their efficacy for improving both metabolic health profiles (1) and athletic performance (2, 3), high-fat, low-carbohydrate (CHO) diets have increased in popularity during the past 2 decades. Short-term (1–3 wk) ingestion of a high-fat, low-CHO diet 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), which suggests impaired metabolic flexibility in skeletal muscle. A range of alterations in the activities of regulatory enzymes and/or signaling proteins in the pathways that underlie skeletal muscle fat and CHO metabolism are likely to explain the...
changes observed with adaptation to a high-fat diet (HFAT); 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, to elucidate the underlying mechanisms that drive changes in metabolic flexibility, high-fat dietary intake must be compared with an isoenergetic diet during which CHO intake is clamped in both dietary interventions. Few studies have determined changes to skeletal muscle in well-trained humans after an HFAT, and, to date, no study has assessed mitochondrial respiration in this population to determine whether this could explain changes in metabolic flexibility.

Therefore, the current investigation aimed to determine whether the metabolic perturbations induced by HFAT are a result of high fat or low CHO availability. Well-trained humans were fed 5 d of either an HFAT or an isoenergetic high-protein diet [HPRO; ~65% energy intake (EI)], with CHO intake clamped to <20% of total daily EI (2.6 g/kg body mass). We utilized whole-body expired gas measures, together with an assessment of skeletal muscle substrates, mitochondrial respiration, and signaling proteins with putative roles in substrate metabolism in an effort to identify mechanisms that underlie changes in the patterns of substrate oxidation observed after HFAT. We hypothesized that whole-body rates of fat oxidation would be greater after HFAT compared with HPRO as a result of high fat availability rather than low CHO availability driving 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 (ACTRN1261600433404). 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 before providing their written informed consent.

Overview of study design

Eight well-trained male cyclists with a history of endurance training and riding >200 km/wk were recruited for this study. Participant characteristics were as follows: age, 25 ± 4 (SD) yr; body mass (BM), 77.3 ± 7.0 kg; $V_{O2}\text{peak}$ (peak $O_2$ uptake), 64.0 ± 3.5 mL/kg/min; and peak power output (PPO), 380 ± 36 W. An overview of the study design is shown in Fig. 1. Each participant completed 2 experimental conditions in a block randomized, crossover design while undertaking supervised training. There was an ~14 d washout period between conditions. It was not possible to blind participants to the dietary interventions; however, the principal researchers who completed 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, The Netherlands) to determine $V_{O2}\text{peak}$ 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, USA) and instantaneous rates of $O_2$ consumption ($V_{O2}$) and $CO_2$ production ($V_{CO2}$) 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 by using a 3-L syringe. An individual’s $V_{O2}\text{peak}$ was determined as the highest 30-s average. These data were used to calculate the work rate that corresponded to 63 and 80% of PPO for the 2 experimental rides.

Experimental trials

Participants followed a controlled high-CHO diet [72% EI; 10 g/kg BM (high-CHO diet)] for 5 d before an experimental trial (Table 1). Participants reported to the laboratory on the fifth 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/kg BM CHO). After breakfast (2 h), 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 rate 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 before participants left the laboratory for the final (fifth) day of the high-CHO diet.

<table>
<thead>
<tr>
<th>Days</th>
<th>-5 to 0</th>
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<td>Diet</td>
<td>High-CHO</td>
<td>High-fat or high-protein</td>
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<td>Training or lab trial</td>
<td>Habitual Training</td>
<td>Rest Day</td>
<td>Fed 20 min</td>
<td>Fasted 20 min + HIIT</td>
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<td>Lab trial</td>
<td>Blood Sample pre and post exercise</td>
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Figure 1. An overview of the study design showing the 5 d of high-CHO diet followed by 5 d of HFAT or HPRO diet (d 1–5) with 1 d of high-CHO diet. HIIT, high-intensity interval training.
The next morning, participants reported to the laboratory after an overnight fast, and a cannula (22 G; 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 by using the percutaneous biopsy technique with suction applied (11). Participants then repeated the 20-min continuous ride at 63% PPO in the fasting state before commencing a high-intensity interval training session (8 × 5 min at 80% PPO), as previously described (12). The purpose of this interval session was to reduce muscle glycogen stores in both conditions before the dietary intervention.

**Diet and training intervention**

Participants commenced 5 d of either HFAT or HPRO. HFAT and HPRO diets consisted of ~67% EI from fat or protein and 19% EI from CHO (Table 1). Protein was provided as an alternative macronutrient to meet energy requirements and CHO was clamped. Total EI was 0.22 MJ/kg BM. HFAT diet consisted 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 previously 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 before an experimental trial, and participants refrained from alcohol during the intervention period. During this time, participants followed a prescribed training program, as 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 an RPE that corresponded to 11–13 (10) during each on-road session. Participants reported to the laboratory on d 4 and completed the same high-intensity interval training session as on d 1. On the morning of d 6, participants reported to the laboratory in a fasting 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 d of a high-CHO diet (10 g/kg BM CHO; Table 1).

**Performance ride**

After an overnight fast, participants reported to the laboratory to complete a performance ride that consisted of 100 min of steady-state (100SS) cycling at 63% PPO, followed by a 7 kJ/kg BM time trial (TT). Upon arrival at the laboratory, a cannula was inserted into an antecubital vein, and a fasting blood sample (10 ml) was collected. A muscle biopsy was taken 2–3 cm distal from the previous incision. Participants then consumed breakfast (2 g/kg BM CHO) and rested for 120 min. Immediately before 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; Science In Sport, Blackburn, United Kingdom) and a 6% CHO solution (933 ml fluid, 2 gels total) every 20 min throughout the ride at a rate of 60 g/l, and water was consumed ad libitum during each trial. Immediately upon completion of the 100SS ride, an additional muscle biopsy was taken. Participants then voided their bladder and had a 3-min rest before 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/min) were calculated from respiratory gas samples that were collected during rides by using nonprotein RER equations (13), which are based on the assumption that $V_{O_2}$ and $V_{CO_2}$ accurately reflect tissue $O_2$ consumption and CO2 production:

$$\text{CHO oxidation (g/min)} = 4.585 \times \frac{V_{CO_2} (L/min)}{3.226}$$

$$\text{Fat oxidation (g/min)} = 1.695 \times \frac{V_{CO_2} (L/min)}{1.701}$$

Rates of CHO and fatty acid oxidation ($\mu$mol/kg/min) were calculated by converting the rates of oxidation (g/kg/min) to their molar equivalent. It was assumed that 6 mol of $O_2$ is consumed and 6 mol 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/mol. The molar rates of triacylglycerol oxidation were multiplied by 3 because each molecule contains 3 molecules of fatty acid.

**Blood sampling and analyses**

Blood samples (6–10 ml) were collected into vacutainers that contained EDTA and immediately analyzed for blood lactate (YSI 2900 STAT Plus; YSI Life Sciences, Yellow Springs, OH, USA) and total cholesterol, HDL, LDL, and triglyceride concentration (Cobas b 101; Roche, Basel, Switzerland). The remaining sample was then centrifuged at 1500 g for 10 min at 4°C, and aliquots of plasma were stored at −80°C for later analysis of free fatty acid (FFA; Wako Pure Chemical Industries, Osaka, Japan), glycero1 (Sigma-Aldrich, St. Louis, MO, USA), insulin (R-Biopharm Laboratory Diagnostics, Taren Point, NSW, Australia), 2-hydroxybutyrate (Sigma-Aldrich), and glucose (Melbourne Pathology, Kilmore, 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$_2$EGTA, 7.23 mM K$_2$EGTA, 5.77 mM Na$_2$ATP, 6.56 mM MgCl$_2$, 7.23 mM K$_2$EGTA, 110 mM NaCl, 110 mM NaHCO$_3$, 20 mM taurine, 20 mM imidazole, 0.5 mM DTT, 10 mM Na$_2$phosphocreatine, 20 mM imidazole, 0.5 mM DTT, 50 mM 2-(N-morpholino)ethanesulfonic acid (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 that was supplemented with saponin (50 µg/ml) for 30 min with agitation to permeabilize the sarcolemma and allow the diffusion of substrates. Fibers were then washed 3 times with ice-cold MiR05 respiration medium (20 mM HEPES, 0.5 mM EGTA, 10 mM KH$_2$PO$_4$, 3 mM MgCl$_2$, 6-H$_2$O, 60 mM lactobionic acid, 20 mM taurine, 110 mM d-sucrose, 1 g/L bovine serum albumin; 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 using 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. In brief, 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. ETFp [ETF (ADP-stimulated oxphos)] respiration was assessed by the addition of ADP (5 mM), mitochondrial complex I (CI) substrate pyruvate (5 mM), and mitochondrial complex II (CII) substrate succinate (10 mM). Cytochrome c (10 µM) was added to confirm mitochondrial membrane integrity, and titrations of cyanide-4- (trifluoromethoxy)phenyllahydrozone (0.025 mM) 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 mitochondrial complex III (CIII), respectively. Finally, mitochondrial complex IV (CIV) capacity was measured during oxidation of $N,N,N',N''$-tetramethyl-p-phenylenediamine dihydrochloride (0.5 mM) with ascorbate (2 mM). $O_2$ flux as a result of the auto-oxidation of these chemicals was determined after the inhibition of 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 µM. 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, 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) 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 by using a SpectraMax Paradigm plate reader (Molecular Devices, Sunnyvale, CA, USA). 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 protein.

**Protein analyses**

For the generation of whole skeletal muscle lysates, ~40 mg of skeletal muscle was homogenized in buffer that contained 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 aprotinin, 1 mM benzamidine, and 1 mM PMSF. Samples were spun at 16,000 g for 30 min at 4°C and supernatant was collected. After the determination of protein concentration via bicinchoninic acid protein assay (Pierce, Rockford, IL, USA), lysates were resuspended in Laemmli sample buffer, and 10 µg of protein of each sample was loaded into 4–20% Mini-Protein TGX Stain-Free Gels (Bio-Rad, Hercules, CA, USA). For oxphos Ab cocktail, 8.5 µg of protein from unbaked lysates was loaded into 12% polyacrylamide gels. After electrophoresis, gels were activated.
according to manufacturer instructions (Chemidoc; Bio-Rad,) and transferred to 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 3 times (5 min each wash) with 10 mM Tris HCl, 100 mM NaCl, and 0.02% Tween 20 solution (TBST), and incubating with primary Ab diluted in TBST (1:1000) overnight at 4°C on a shaker. Membranes were incubated for 1 h the next day with a secondary Ab diluted in TBST (1:2000), and proteins were detected via ECL (Amersham Biosciences, Little Chalfont, United Kingdom) and quantified by densitometry (Chemidoc). Time points and both diets for each participant were run on the same gel.

Abs against fatty acid translocase (FAT/CD36; 14347), carnitine palmitoyltransferase-1 (CPT1A; 12252), AMPK (AMPKα; 2532), phospho-AMPKThr172 (2531), acetyl CoA carboxylase (ACC; 3661), AMPK (AMPKα; 2532), phospho-ACCSer79 (3661), mTOR (2972), phospho-mTORSer2448 (2971), S6 ribosomal protein (RPS6; 2217), phospho-RPS6Ser235/236 (2211), citrate synthase (14309), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 2118) were purchased from Cell Signaling Technology (Danvers, MA, USA), and total oxphos (110411) purchased from Abcam (Cambridge, United Kingdom). The volume density of each target band was normalized to its respective total protein.

**Statistics**

Statistical analysis was undertaken using SPSS for Windows (v.20; SPSS, Chicago, IL, USA). Data from the 2 experimental conditions were analyzed by a linear mixed model (treatment × time), and subsequent post hoc comparisons were completed within the linear mixed model on the basis of the least significant difference. Separate analysis was completed to compare d 5 of high-CHO diet with 100SS (fed) and d 1 of HFAT or HPRO with 5 d postdiet (fasting). Normality was visually assessed by using the linear model residuals. Differences in TT performance between trials were compared by using a paired Student’s t test. Values of P < 0.05 were considered statistically significant. All data are represented as means ± SD.

**RESULTS**

All participants complied with the prescribed dietary (Table 1) and training intervention for both conditions. No difference was reported across the 5-d intervention periods for distance covered or RPE during training for either diet (HFAT, 222 ± 23 km, 13 ± 0.5; and 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; Fig. 2). Muscle glycogen was reduced in both HFAT and HPRO conditions pre-to postdiet (P < 0.001). After d 1 of high-CHO diet, muscle glycogen increased by ~45% in both HFAT and HPRO conditions (P < 0.001), but was not restored to prediet values in HFAT (P < 0.001). After 100SS, muscle glycogen was reduced in both HFAT and HPRO conditions (526 ± 86 to 411 ± 62 mmol/kg dry mass (DM), P = 0.033; 637 ± 87 to 420 ± 92 mmol/kg DM, P < 0.001, respectively); however, no difference in the percentage change from pre-to postexercise 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; Fig. 3A–C) after 5 d of either HFAT or HPRO diet. RER was reduced pre-to postdiet for both HFAT and HPRO (0.90 ± 0.02 to 0.79 ± 0.02; 0.90 ± 0.03 to 0.86 ± 0.02, P ≤ 0.001, respectively) and...
different than Prediet (d 1) within condition.
a within condition;
e different than HPRO at time point;
f different than HPRO postdiet (55 ± 35 μmol/kg/min vs. 241 ± 31 μmol/kg/min; 
P = 0.025, respectively). Rates of CHO oxidation declined pre- to postdiet in both conditions, 
and were lower in HFAT than in HPRO postdiet (106 ± 20 μmol/kg/min vs. 169 ± 17 μmol/kg/min; 
P = 0.001).

After 1 d 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 with HPRO diet during the first 40 
min of 100SS (P < 0.04). After 1 d of high-CHO diet, rates of CHO oxidation were lower than baseline during 100SS 
after HFAT (P < 0.001) and were significantly lower during 100SS in HFAT compared with HPRO 
(213 ± 35 μmol/kg/min vs. 241 ± 31 μmol/kg/min; 
P = 0.025, respectively). Rates of CHO oxidation declined 
throughout 100SS in HPRO (from 241 ± 31 μmol/kg/min to 
215 ± 26 μmol/kg/min; 
P < 0.001), but remained 
stable in HFAT (~208 μmol/kg/min). Despite 1 d 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 in HPRO (0.53 ± 0.11 μmol/kg/min vs. 0.38 ± 0.18 μmol/kg/min; 
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 postdiet

There was a main effect of time for FFA concentration (P < 0.001) pre- to postdiet. FFA concentration was greater after 
exercise postdiet compared with prediet in both HFAT (0.31 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 postdiet in HFAT (2.44 ± 0.63 to 
2.93 ± 0.75 mM) and was higher than HPRO postdiet 
(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 and P = 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 postdiet to be greater than in 
HPRO (1.08 ± 0.20 mM), whereas triglycerides decreased 
(1.26 ± 0.46 to 0.69 ± 0.36 mM; 
P = 0.001) to be lower than in 
HPRO (1.06 ± 0.45 mM). No difference in total cholesterol 
was measured between conditions from pre- to postdiet.

Blood metabolites during the performance ride

There was a significant interaction for plasma glycerol concentration between HFAT and HPRO diets (P = 0.035; 
Fig. 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 in 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 (Fig. 4B). FFA concentrations decreased 2 h 
after CHO breakfast in both conditions and were elevated 
from resting values after 60 min of 100SS until the 
completion of the TT. Plasma β-hydroxybutyrate 
concentrations increased after CHO breakfast and remained 
stable during 100SS in both conditions until after the TT.

Figure 3. RER (A) and rates of CHO (B) and fat oxidation (C) 
after 5 d of high-CHO (Baseline and Prediet d 1), 5 d of HFAT 
or HPRO diet (Postdiet d 6) during 20 min cycling, and 
after 1 d of a high-CHO diet (D7 20-100) during 100SS cycling 
at 63% PPO. Values are presented as means ± se. 1HFAT 
different than HPRO at time point; 2HFAT different than 
Baseline within condition; 3HPRO different than Baseline 
within condition; 4HFAT different than Prediet (d 1); 5HPRO 
different than Prediet (d 1) within condition.

was lower postdiet in HFAT compared with HPRO diet 
(P < 0.001). Rates of fat oxidation increased after 5 d of 
HFAT and HPRO diet, and were greater in HFAT 
compared with HPRO postdiet (55 ± 7 μmol/kg/min vs. 36 ± 
6 μmol/kg/min; 
P < 0.001). Concomitantly, rates of CHO 
oxidation were reduced pre- to postdiet in both conditions, 
and were lower in HFAT than in HPRO postdiet (106 ± 
20 μmol/kg/min vs. 169 ± 17 μmol/kg/min; 
P < 0.001).
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 (Fig. 4D–F). Blood glucose concentration decreased after the CHO breakfast in both diets, but after 40 min of exercise, glucose concentrations had returned to resting values. Plasma insulin concentrations increased in both conditions after breakfast and remained elevated 2 h after ingestion. After the onset of exercise, insulin concentrations were reduced in both conditions and were similar to pre-breakfast values throughout 100SS. After 100SS, participants ingested a CHO drink that increased insulin concentrations in both conditions, but this increase was abolished after the onset of the TT. Blood lactate concentrations remained stable throughout 100SS in both conditions and were higher post-TT compared with rest in HFAT (3.1 ± 1.1 mM) and HPRO (3.1 ± 1.0 mM) diets.

**TT performance**

There was no difference in TT performance between diet conditions (30:59 ± 2:55 vs. 30:10 ± 2:70 min:s for HFAT and HPRO, respectively). Mean power output during the TT was 299 ± 34 W and 304 ± 35 W ($P = 0.41$) and HR averaged 168 ± 9 bpm and 166 ± 7 bpm in HFAT and HPRO, respectively. A significant reduction in BM was observed pre- to postexercise ($P < 0.04$) for both HFAT

![Figure 4](https://www.fasebj.org)
respiration after HFAT and HPRO diets (significant interaction in the percentage change of CI + ETFp significantly different after either diet (Table 2), there was a
portray the effects of diet and exercise on mitochondrial
time point and under each diet are reported in
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 Fig. 5. Although HFAT and HPRO absolute O2k values were not significantly different after either diet (Table 2), there was a significant interaction in the percentage change of CI + ETFp respiration after HFAT and HPRO diets (P = 0.042; Fig. 5A). The diet-induced reduction in CI + ETFp respiration after the addition of octanoylcarnitine and pyruvate was significantly greater after HFAT compared with HPRO. Despite no differences in absolute O2k values, the percentage change of ETS uncoupled respiration (Fig. 5A; ETS CI + CII + ETF; ETS CII) was significantly reduced after HFAT, but not HPRO. Absolute and percentage change ETS CII uncoupled respiration remained unchanged after 1 d of high-CHO diet in HPRO, but percentage change was significantly increased in HFAT compared with HPRO diets (P = 0.032; Fig. 5B). ETFp respiration was significantly reduced after 100SS in HFAT, but not HPRO (Fig. 5C). Percentage change of postexercise CI + ETFp, CI + CII + ETFp, ETS CI + CII + ETF, and ETS CII respiration was significantly reduced in both HFAT and HPRO diets (Fig. 5C), despite no differences in absolute O2k values. The reduction in the percentage change of CI + ETFp and CI + CII + ETFp respiration was greater in HFAT than in HPRO diets after 100SS (P = 0.024 and P = 0.019, respectively). There were no significant differences in skeletal muscle citrate synthase activity across time or between diets (~20 mol/h/kg; Fig. 5D).

### Skeletal muscle mitochondrial respiration

On the basis of differences in whole-body substrate oxidation rates pre- to postdiet and during prolonged exercise between HFAT and HPRO diets (Fig. 3), we next assessed whether skeletal muscle mitochondrial substrate utilization was contributing to this outcome by using a sequential ad-

### Immunoblot analyses

Total protein contents of citrate synthase (Fig. 5E) and oxphos complexes I–V (Fig. 6A–F) were not different between HFAT and HPRO diets at any time point during the intervention. Oxphos CIII demonstrated a trend toward 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 prediet to after 1 d of high-CHO diet (Fig. 7A). FAT/CD36 protein content was higher pre- and postdiet and pre- and post-100SS in HFAT compared with HPRO diet. There were no differences in total CPT1a from pre- to post-high-CHO diet in either HFAT or HPRO diets (Fig. 7B). No main effects were found for AMPK Thr172 phosphorylation levels relative to total AMPK, although a trend toward a main effect of time was observed (P = 0.06), with an increase after HFAT (Fig. 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 after 100SS compared with post-HFAT diet (Fig. 7D). There were no differences in mTOR Ser2448 phosphorylation relative to total mTOR prediet compared with after 1 d of high-CHO diet (Fig. 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 after 100SS in HFAT compared with pre- and postdiet (Fig. 7F). RPS6 Ser235/236 phosphorylation was also higher postexercise in HPRO compared with HFAT diet (P = 0.034).

### Table 2. Effects of 5 d of HFAT or HPRO, 1 d of high-CHO diet, and 100SS cycling at 63% PPO on mitochondrial respiration

<table>
<thead>
<tr>
<th>Diet</th>
<th>ET leak (malate, octanoylcarnitine)</th>
<th>ETFp (ADP)</th>
<th>CI + ETFp (pyruvate)</th>
<th>CI + CII + ETFp (succinate)</th>
<th>cytochrome oxidase test (cytochrome c)</th>
<th>ETS CI + CII + ETF (FCCP)</th>
<th>ETS CII (rotenone)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prediet (d 1)</td>
<td>HFAT 20.2 ± 5.9</td>
<td>43.8 ± 5.8</td>
<td>62.5 ± 11.2*</td>
<td>116.8 ± 22.6*</td>
<td>136.6 ± 29.5*</td>
<td>144.4 ± 32.2*</td>
<td>92.5 ± 19.0*</td>
</tr>
<tr>
<td>HFAT</td>
<td>17.5 ± 4.1</td>
<td>47.7 ± 5.6</td>
<td>66.1 ± 13.2</td>
<td>125.0 ± 31.2</td>
<td>147.1 ± 37.0*</td>
<td>154.9 ± 30.3*</td>
<td>100.6 ± 30.3*</td>
</tr>
<tr>
<td>HPRO</td>
<td>21.0 ± 4.9</td>
<td>42.8 ± 7.0</td>
<td>55.7 ± 13.5</td>
<td>103.5 ± 27.2*</td>
<td>119.1 ± 39.2*</td>
<td>124.0 ± 38.4</td>
<td>77.2 ± 30.5*</td>
</tr>
<tr>
<td>Postdiet (d 6)</td>
<td>HFAT 20.1 ± 3.8</td>
<td>50.7 ± 4.5</td>
<td>68.4 ± 15.4*</td>
<td>122.0 ± 26.9*</td>
<td>140.3 ± 34.4*</td>
<td>148.2 ± 37.2*</td>
<td>91.3 ± 21.5*</td>
</tr>
<tr>
<td>HPRO</td>
<td>19.1 ± 3.8</td>
<td>50.7 ± 5.6</td>
<td>68.4 ± 15.4*</td>
<td>122.0 ± 26.9*</td>
<td>140.3 ± 34.4*</td>
<td>148.2 ± 37.2*</td>
<td>91.3 ± 33.6*</td>
</tr>
<tr>
<td>Preexercise (d 7)</td>
<td>HFAT 24.6 ± 5.7</td>
<td>52.4 ± 7.2</td>
<td>64.3 ± 15.9</td>
<td>120.1 ± 29.0*</td>
<td>141.1 ± 39.7*</td>
<td>145.9 ± 38.8*</td>
<td>92.9 ± 27.3*</td>
</tr>
<tr>
<td>HPRO</td>
<td>18.7 ± 5.9</td>
<td>49.8 ± 12.8</td>
<td>64.8 ± 20.3</td>
<td>120.0 ± 36.9*</td>
<td>142.9 ± 47.3*</td>
<td>151.9 ± 51.0*</td>
<td>97.3 ± 33.6*</td>
</tr>
<tr>
<td>Postexercise (d 7)</td>
<td>HFAT 24.6 ± 4.9</td>
<td>47.4 ± 9.8</td>
<td>47.6 ± 13.8</td>
<td>84.0 ± 23.8</td>
<td>99.0 ± 26.7</td>
<td>108.1 ± 31.0</td>
<td>60.8 ± 20.5</td>
</tr>
<tr>
<td>HPRO</td>
<td>21.7 ± 5.9</td>
<td>47.9 ± 11.3</td>
<td>56.8 ± 18.3</td>
<td>101.1 ± 30.2</td>
<td>105.4 ± 35.4</td>
<td>115.0 ± 37.4</td>
<td>65.4 ± 27.0</td>
</tr>
</tbody>
</table>

O2k respiration measure; O2 flux per mass (pM/s/mg). Terms in parentheses represent substrate/uncoupler/inhibitor. Respiratory states are supported by single or convergent electron input via CI, CII, and/or ETF under nonphosphorylating (leak) conditions, state-3 oxphos in the presence of ADP (indicated by “p”), or with an uncoupler (carboxylic acid or complex III inhibitors). There were no differences in skeletal muscle citrate synthase activity across time or between diets (~20 mol/h/kg; Fig. 5D).
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 the effort to pinpoint potential mechanisms that underlie the high rates of fat oxidation that have been reported after short-term adaptation to fat-rich diets, which persist even after 1 d 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 that govern patterns of substrate oxidation in response to diet–exercise interactions. We report that, compared with an isocaloric high-protein diet, 5 d of adaptation to an HFAT diet results in greater whole-body rates of fat oxidation during submaximal cycling and impairments in mitochondrial respiration.

A series of independent studies during the past 2 decades (2, 4, 5, 7, 8) have compared HFAT vs. high-CHO diets and demonstrated that short-term (<7 d) HFAT diets result in peak rates of whole-body fat oxidation of ~1 g/min (~50 µmol/kg/min), values that are typically 2-fold greater than after isocaloric high-CHO diets (2, 7). Rates of fat oxidation in the present investigation (1.2 g/min) 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 5 d of a low-CHO, HPRO diet (0.8 g/min). We report an increase in postexercise plasma FFA concentration from prediet interventions in HFAT and HPRO that likely contributed to the increased rates of fat oxidation after both conditions; however, the higher rates of fat oxidation in HFAT compared with HPRO diet are likely associated with altered rates of whole-body lipolysis and the subsequent storage of triglycerides (6, 16, 17). Previous work has demonstrated higher rates of whole-body lipolysis, determined by elevated glycerol concentration, and this increase was associated with an elevated intramuscular triglyceride (IMTG) concentration after 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 diet was converted to glucose via gluconeogenesis (19), which may explain the slightly higher muscle glycogen concentrations postdiet in HPRO compared with HFAT diet. Greater availability of muscle glycogen likely contributed to lower rates of whole-body fat oxidation in HPRO compared with HFAT diet. Accordingly, the higher rates of fat oxidation measured after HFAT are likely driven by higher fat, rather than low CHO, availability.

Despite CHO intake being clamped in both dietary conditions, rates of CHO oxidation were lower after 5 d of HFAT compared with HPRO diet. Rates of CHO oxidation were reduced by 50% (3 to 1.5 g/min) after HFAT diet compared with a 25% decline (2.3 g/min) after HPRO diet.
The greater oxidation of CHO-based fuels may be explained by the slightly higher muscle glycogen concentration postdiet in HPRO compared with HFAT diet, which has previously been demonstrated to increase the reliance on CHO-based fuels during subsequent exercise (20). Without a protein tracer in the current study, we were unable to detect the proportion of protein that was converted to glucose. After a 6-d HFAT diet (63% EI from fat), Peters et al. (21) observed a decrease in the active form of the rate-limiting enzyme in CHO metabolism, PDH, and, consequently, a reduction in rates of CHO oxidation during exercise, which was not observed after a moderate CHO diet (52% EI from CHO). Therefore, a decrease in PDH activity may have contributed to the observed reduction in whole-body rates of CHO oxidation in the present study (5); however, limitations in muscle biopsy sample did not permit the assessment of PDH activity.

To further determine potential mechanisms for reduced CHO oxidation, we assessed skeletal muscle mitochondrial respiration to measure the dietary effects on substrate flux and utilization. We found that respiration supported by octanoylcarnitine and pyruvate (CI + ETFp) was significantly reduced after 5 d of HFAT diet, but not HPRO diet, when CIII and/or CIV were operating at or near maximal activity. In our interpretations of mitochondrial respiration data obtained by using the Oroboros O2k SUIT protocol, it is important to note that supraphysiologic mitochondrial substrate concentrations and a sequential addition protocol were 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 diet. Decreased respiration observed after the addition of octanoylcarnitine and pyruvate is in line with previous studies that have reported that HFAT 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 additionally been identified as a mechanism that underlies the 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 5 d of HFAT diet persisted after uncoupling (ETS CI + CII + ETF, and ETS CII), which suggests that the functional reductions in respiration occurred either at the level of CI/CII or downstream at CIII/CIV, but not at complex V (ATP synthase). In line with the observed reductions in uncoupled respiration, Skovbro et al. (23) have reported that ETFp and uncoupled respiration were decreased after a longer high-fat feeding period (i.e., 16 d; 55–60% fat) compared with a moderate CHO diet (i.e., 55–60% CHO) (23). The mitochondrial effects of HFAT diet in the present study may have been more pronounced after a longer dietary intervention period. In addition, the type of the dietary fat intake has previously been demonstrated to affect mitochondrial function and morphology (24). Lionetti et al. (24) have demonstrated that high saturated fat intake was associated with greater mitochondrial dysfunction compared with intake of unsaturated fat in rodents. The current study provided a 65% fat diet, made up of 55% saturated fat, and whether the reductions in mitochondrial respiration would
be observed with a different dietary fat composition requires additional investigation. As no changes in mitochondrial respiration were observed after 5 d of HPRO diet, this suggests that the primary driver of these skeletal muscle adaptations is high dietary fat availability. On the basis of the evidence in the present study, the biochemical explanation of why HFAT diet 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 diet-induced changes in the delivery and transport of fatty acids across the 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 after the addition of octanoylcarnitine and pyruvate and uncoupled respiration with HFAT, we detected no differences in the skeletal muscle protein content of the 5 mitochondrial oxphos complexes after either dietary condition. In addition, neither citrate synthase protein content, nor maximal activity was changed. Given that changes in CI 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 that regulate mitochondrial substrate flux likely contribute to the reduced respiration observed after short-term adaptation to HFAT diet.

To determine the alternative enzymes and signaling pathways that are impacted by HFAT and HPRO diets, we measured putative transporters with roles in skeletal muscle fatty acid uptake and 2 energy-sensing metabolic signaling pathways, AMPK and mTOR. There was a 12% increase in FAT/CD36 protein observed after HFAT, which suggests potential increased capacity for sarcolemmal and/or mitochondrial membrane fatty acid uptake, although this increase in FAT/CD36 did not reach statistical significance. No change in mitochondrial CPT1 was observed after 5 d of HFAT or HPRO diets. These findings are in agreement with previous works that reported that an HFAT diet, together with an intensive training program, resulted in significantly greater (i.e., a 17% increase) protein abundance of FAT/CD36 without a 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 in its substrate ACC Ser79 phosphorylation relative to total ACC after the dietary interventions. No differences postdiet were observed in mTOR Ser2448 phosphorylation relative to total.
mTOR and in phosphorylation of its substrate RPS6 Ser235/236 relative to total RPS6 in HFAT or HPRO diets. Together, these findings suggest that fatty acid transporter abundance and activation of these energy-sensing pathways were unaffected by the 2 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. Additional investigation is required to uncover alternative protein signaling pathways that are associated with changes in substrate metabolism that may underpin the dietary effects on skeletal muscle mitochondrial respiration.

After CHO restoration strategies (e.g., 1 d of high-CHO diet, a pre-exercise CHO-rich breakfast, and CHO intake during exercise), muscle glycogen increased in both HFAT and HPRO diets, but did not reach preintervention values in the HFAT diet. This may be a result of the brief (24 h) CHO restoration period compared with 5 d of high CHO intake before baseline measures. Rates of fat oxidation and CHO oxidation returned to baseline values during 100SS cycling in both HFAT and HPRO diets, and were similar to the results observed in the prediet 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-fasting and water-fed conditions in the prediet protocol. Therefore, rates of fat oxidation were higher and CHO oxidation lower than expected during the 100SS protocol on d 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 diet, is sufficiently robust to persist despite the restoration of CHO (2, 4, 7). For example, CHO oxidation rates after HFAT or HPRO diet and CHO restoration in the current study were lower than those reported in previous studies following a controlled (chronic high CHO) diet (200–220 μmol/kg/min vs. 250–300 μmol/kg/min) (7). Muscle glycerol utilization in the current study was ~100 mmol/kg DM lower than that previously reported with HFAT intervention (2), and was slightly higher in HPRO compared with HFAT diet. This difference may be related to higher pre-exercise muscle glycogen in HPRO. Overall, this reduced capacity for CHO oxidation in HFAT diet, despite the availability of exogenous and endogenous stores, was previously associated with the persistent down-regulation of PDH activity (5).

Whole-body rates of fat oxidation after CHO restoration remained slightly higher after HFAT compared with HPRO, but this resulted in only a small difference in the total fat oxidized during 100SS cycling (~15 g over 100 min). No difference was measured for FFA concentrations between the 2 dietary conditions, although plasma glycerol concentrations were greater during exercise after HFAT compared with HPRO. Elevated glycerol concentration after HFAT diet indicates a greater rate of whole-body lipolysis that could be associated with greater IMTG utilization and/or liberation of FFA into the blood, thereby contributing to the minor variation in rates of whole-body fat oxidation between HFAT and HPRO diets. Corresponding to total fat utilization, there were only minor differences in total CHO oxidized between HFAT and HPRO diets (~30 g) during 100 min of exercise.

Although 1 d of high CHO availability in the current study failed to fully reverse the differences in the whole-body rates of substrate oxidation, it was sufficient to restore the decreased mitochondrial respiration (CI + ETP) from HFAT to prediet values. This indicates that there may be an additional underlying mechanism that regulates changes in substrate oxidation (i.e., down-regulating CHO oxidation) and mitochondrial respiration. As the current study precluded the investigation of a high-CHO trial as a result of 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 3 dietary conditions. We also reported greater postexercise reductions in CI + ETP and CI + CII + ETP respiration in HFAT diet than in HPRO diet, and this was not attributable to reductions in citrate synthase protein or activity. These effects of exercise after HFAT diet 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 before the addition of cytochrome c in the SUIT protocol as it occasionally increased O2 flux >10% above CI + CII + ETP (Table 2). Despite this, we are confident that the data before the addition of cytochrome c is still meaningful because 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 after high-CHO intake. Burke et al. (2) reported similar TT performance between HFAT and high-CHO trials, and in the present study, we did not detect any difference in TT performance between HFAT and HPRO diet after 1 d of CHO restoration; however, we cannot compare this performance with a high-CHO condition, and the shifts in substrate utilization therefore require additional investigation.

In summary, the results of the present investigation demonstrate that whole-body rates of fat oxidation increase to a greater extent in trained humans after high dietary fat intake compared with an HPRO diet, when CHO is clamped at 20% of energy intake. High dietary fat intake also reduced mitochondrial respiration supported by octanoyl-carnitine and pyruvate as well as uncoupled respiration. These reductions in mitochondrial “function” may be compensatory, and note solely “driving” fuel regulation under the conditions of our investigation. Additional mechanistic investigation into potential underlying diet-induced differences in mitochondrial membrane dynamics, mitochondrial complex subunits, and additional enzymes that regulate 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 that an HFAT diet has
on metabolic flexibility and skeletal muscle mitochondrial respiration in trained cyclists.

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AUTHOR CONTRIBUTIONS

L. M. Burke and J. A. Hawley supported the research; N. K. Stepto, J. P. Morton, L. M. Burke, and J. A. Hawley supervised the research; J. J. Leckey, N. J. Hoffman, E. B. Parr, B. L. Devlin, and A. J. Trewin performed the research; all authors analyzed and interpreted data; J. J. Leckey, N. J. Hoffman, L. M. Burke, and J. A. Hawley wrote the manuscript; and all authors designed the study protocol, and critically revised, contributed, and approved the final manuscript.

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