ABSTRACT

HAMMOND, K. M., S. G. IMPEY, K. CURRELL, N. MITCHELL, S. O. SHEPHERD, S. JEROMSON, J. A. HAWLEY, G. L. CLOSE, L. D. HAMILTON, A. P. SHARPLES, and J. P. MORTON. Postexercise High-Fat Feeding Suppresses p70S6K1 Activity in Human Skeletal Muscle. Med. Sci. Sports Exerc., Vol. 48, No. 11, pp. 2108–2117, 2016. Purpose: This study aimed to examine the effects of reduced CHO but high postexercise fat availability on cell signaling and expression of genes with putative roles in regulation of mitochondrial biogenesis, lipid metabolism, and muscle protein synthesis. Methods: Ten males completed a twice per day exercise model (3.5 h between sessions) comprising morning high-intensity interval training (8 × 5 min at 85% VO2peak) and afternoon steady-state (SS) running (60 min at 70% VO2peak). In a repeated-measures design, runners exercised under different isonitrogenic dietary conditions consisting of high-CHO (HCHO: 10 g·kg−1·CHO, 2.5 g·kg−1·protein, and 0.8 g·kg−1·fat for the entire trial period), reduced-CHO but high-fat availability in the postexercise recovery periods (HFAT: 2.5 g·kg−1·fat for the entire trial period) or reduced-CHO but high-fat availability in the postexercise recovery periods (HFAT: 2.5 g·kg−1·CHO, 2.5 g·kg−1·protein, and 3.5 g·kg−1·fat for the entire trial period). Results: Muscle glycogen was lower (P < 0.05) at 3 h (251 vs 301 mmol·kg−1·dry weight) and 15 h (182 vs 312 mmol·kg−1·dry weight) post-SS exercise in HFAT compared with HCHO. Adenosine monophosphate-activated protein kinase α2 activity was not increased post-SS in either condition (P = 0.41), although comparable increases (all P < 0.05) in PGC-1α, p53, citrate synthase, Tfam, peroxisome proliferator-activated receptor, and estrogen-related receptor α mRNA were observed in HCHO and HFAT. By contrast, PDK4 (P = 0.003), CD36 (P = 0.05), and carnitine palmitoyltransferase 1 (P = 0.03) mRNA were greater in HFAT in the recovery period from SS exercise compared with HCHO. Ribosomal protein S6 kinase activity was higher (P = 0.08) at 3 h post-SS exercise in HCHO versus HFAT (72.7 ± 51.9 vs 44.7 ± 27 fmol·min−1·mg−1). Conclusion: Postexercise high-fat feeding does not augment the mRNA expression of genes associated with regulatory roles in mitochondrial biogenesis, although it does increase lipid gene expression. However, postexercise ribosomal protein S6 kinase activity is reduced under conditions of high-fat feeding, thus potentially impairing skeletal muscle remodeling processes. Key Words: AMPK-α2, PGC-1α, p53, GLYCOGEN, MITOCOHALDRIAL BIOGENESIS

Traditional nutritional strategies for endurance athletes have largely focused on ensuring high-CHO (HCHO) availability before, during, and after each training session (2). However, accumulating data from our laboratory (7,29) and others (12,16,17,23,39) have demonstrated a potent effect of CHO restriction (the so-called train-low paradigm) in augmenting the adaptive responses inherent to endurance training. Indeed, reduced CHO availability before (33), during (1), and after (32) training sessions augments the acute cell signaling pathways and downstream gene expression responses associated with regulating training adaptation. Accordingly, reduced CHO availability during short-term periods of endurance training augments markers of mitochondrial biogenesis (16,29,39), increases both whole body (39) and intramuscular lipid metabolism (17), and also improves exercise capacity and performance (16,24). In the context of nutrient–gene interactions, it is therefore apparent that the
acute molecular regulation of cell signaling processes provides a theoretical basis for understanding the molecular mechanisms underpinning chronic training adaptations.

In addition to manipulation of CHO availability, many investigators have also demonstrated a modulatory role of high-fat availability in augmenting components of training adaptation (10). For example, the acute elevation in circulating free fatty acid (FFA) availability during exercise regulates key cell signaling kinases and transcription factors that modulate the expression of genes regulating both lipid and CHO metabolism (31,40). In addition, 5–15 d of high-fat feeding increases resting intramuscular triglyceride (IMTG) stores (38), hormone-sensitive lipase (38), carnitine palmitoyltransferase 1 (CPT1) (15), adenosine monophosphate-activated protein kinase α2 (AMPK-α2) activity (38), and protein content of fatty acid translocase (FAT/CD36) (11). Such adaptations undoubtedly contribute to the enhanced rates of lipid oxidation observed during exercise following “fat adaptation” protocols (10). Taken together, these data suggest carefully chosen periods of reduced CHO, but concomitant high-fat availability may therefore represent a strategic approach for which to maximize both the training-induced skeletal muscle mitochondrial biogenesis and the enhanced capacity to use lipid sources as fuels during exercise.

However, such a feeding strategy is not without potential limitations, especially if performed on consecutive days. Indeed, reduced CHO availability impairs acute training intensity (17,39) and 5 d of high-fat feeding reduces pyruvate dehydrogenase (PDH) activity (35), thus potentially leading to a detraining effect, reduced capacity to oxidize CHO, and ultimately, impaired competition performance (17,39). Moreover, although many endurance training–induced skeletal muscle adaptations are regulated at a transcriptional level, the turnover of myofibrillar (i.e., contractile) proteins are largely regulated through the translational machinery and the mechanistic target of rapamycin complex and ribosomal protein S6 kinase 1 (p70S6K1) signaling axis (28). In this regard, recent data suggest high circulating FFA availability impairs muscle protein synthesis (MPS) despite the intake of high quality protein, albeit examined via lipid and heparin fusion and euglycemic hyperinsulemic clamp conditions (36).

With this in mind, the aim of the present study was to examine the effects of reduced CHO but high postexercise fat availability on the activation of key cell signaling kinases and expression of genes with putative roles in the regulation of mitochondrial biogenesis, lipid metabolism, and MPS. In accordance with the original train-low investigations (16,17,29,39), we used a twice per day exercise model whereby trained male runners completed a morning high-intensity interval training (HIT) session followed by an afternoon training session consisting of steady-state (SS) running. Runners completed the exercise protocols under two different dietary conditions (both energy and protein matched) consisting of HCHO availability in the recovery period after both training sessions (i.e., best practice nutrition) or, alternatively, reduced CHO but high-fat availability in the postexercise recovery periods (HFAT).

We specifically hypothesized that our high-fat feeding protocol would enhance cell signaling and the expression of those genes with putative roles in the regulation of mitochondrial biogenesis and lipid metabolism but would also impair the activity of MPS-related signaling.

METHODS

Subjects

Ten trained male runners volunteered to participate in the study (mean ± SD; age = 24 ± 1.5 yr, body mass = 75.9 ± 6 kg, height = 177.3 ± 7.2 cm, VO_{2peak} = 60 ± 3.6 mL·kg⁻¹·min⁻¹). All subjects gave written informed consent before participation after all experimental procedures and potential risks had been fully explained. None of the subjects had any history of musculoskeletal or neurological disease, nor were they under any pharmacological treatment during the testing period. Subjects were instructed to refrain from any strenuous physical activity, alcohol, and caffeine consumption in the 48 h before each experimental trial. The study was approved by the ethics committee of Liverpool John Moores University.

Design

In a repeated-measures, randomized, crossover design separated by 7 d, subjects completed a twice per day exercise model under two different dietary conditions (both energy and protein matched) consisting of HCHO availability in the recovery period after both training sessions (i.e., best practice nutrition) or, alternatively, reduced CHO but HFAT. The twice per day exercise model comprised a morning (9:00–10:00 a.m.) high-intensity interval (HIT) training session (8 × 5 min at 85% VO_{2peak}) followed by an afternoon (1:30–2:30 p.m.) training session consisting of steady-state (SS) running (60 min at 70% VO_{2peak}). To promote training compliance during the HIT protocol in both the HCHO and the HFAT trials, subjects adhered to a standardized HCHO breakfast before this session. However, during the 3.5-h recovery between the HIT and the SS session and in the recovery period upon completion of the SS exercise protocol until the subsequent morning, subjects adhered to either an HCHO or an HFAT feeding protocol. Muscle biopsies were obtained from the vastus lateralis muscle immediately pre-HIT, immediately post-SS, and at 3 and 15 h post-SS. An overview of the experimental design and the nutritional protocols are shown in Figure 1.

Preliminary Testing

At least 7–10 d before the first main experimental trial, subjects performed a maximal incremental running test to volitional fatigue on a motorized treadmill (h/p/Cosmos, Nussdorf-Traunstein, Germany) to determine maximal oxygen uptake. After a 10-min warm-up at a self-selected treadmill speed, the maximal incremental test commenced, beginning...
with a 2-min stage at a treadmill speed of 10 km·h⁻¹. Running speed was then increased by 2 km·h⁻¹ every 2 min until a speed of 16 km·h⁻¹ was reached, after which the treadmill inclined by 2% every 2 min until volitional exhaustion. \( \dot{V}O_2 \) peak was defined as the highest \( \dot{V}O_2 \) value obtained during any 10-s period and was stated as being achieved by two of the following criteria: 1) HR was within 10 bpm of age-predicted maximum, 2) respiratory exchange ratio > 1.1, and 3) plateau of oxygen consumption despite increased workload. On their second visit to the laboratory (approximately 3 d later), subjects completed a running economy test to determine their individual running speeds for subsequent experimental trials. After a warm-up, the test began with a 5-min stage at a treadmill speed of 8 km·h⁻¹ with 1% incline, and speed was then increased by 1 km·h⁻¹ every 5 min thereafter. The test was stopped when 90% of the previously determined \( \dot{V}O_2 \) peak was reached. These measurements were recorded via breath-by-breath gas measurements obtained continuously throughout both tests using a CPX Ultima series online gas analysis system (Medgraphics, St. Paul, MN). The test–retest reliability of this system in our laboratory when quantified using 95% limits of agreement is 0.29 ± 2.4 mL·kg⁻¹·min⁻¹ (data were compiled from comparison of the oxygen uptake during the HIT protocols in the HCHO and HFAT trials undertaken in the present study).

HR (Polar, Kempele, Finland) was also recorded continuously during exercise.

**Experimental Protocols**

**HIT protocol.** In the 24-h preceding each main experimental trial, subjects consumed a standardized HCHO diet in accordance with typical nutritional recommendations (8 g·kg⁻¹·CHO, 2 g·kg⁻¹·protein, and 1 g·kg⁻¹·fat). On the morning of each experimental trial, subjects reported to the laboratory at ~7:00 a.m., where they were given a standardized HCHO breakfast (2 g·kg⁻¹·CHO, 0.3 g·kg⁻¹·protein, and 0.1 g·kg⁻¹·fat). At 2-h postprandial, a venous blood sample was then collected from an antecubital vein in the anterior crease of the forearm, and a muscle biopsy sample was taken from the vastus lateralis muscle. Subjects were then fitted with an HR monitor, and nude body mass (SECA, Hamburg, Germany) was recorded before commencing the high-intensity interval running (HIT) protocol, which lasted ~1 h. The HIT protocol consisted of 8 × 5-min bouts running at a velocity corresponding to 85% \( \dot{V}O_2 \) peak interspersed with 1 min of recovery at walking pace. The intermittent protocol started and finished with a 10-min warm-up and cooldown at a velocity corresponding to 50% \( \dot{V}O_2 \) peak, and a further venous blood sample was obtained immediately.
upon completion of the protocol. Water was given ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. HR was measured continuously during exercise (Polar), and RPE (9) were obtained upon completion of each HIT bout. To determine substrate use during exercise (20), expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, Medgraphics) for the final 2 min of each 5-min interval.

**SS protocol.** During the 3.5-h recovery period between the HIT and the SS protocols, subjects consumed either the HCHO (2.5 g·kg⁻¹ CHO, 1 g·kg⁻¹ protein, 0.3 g·kg⁻¹ fat) or the HFAT (0 g·kg⁻¹ CHO, 1 g·kg⁻¹ protein, 1 g·kg⁻¹ fat) feeding protocols (the pattern and frequency of feeding is shown in Fig. 1). After the recovery period, another venous blood sample was obtained immediately before commencing the afternoon SS exercise protocol. After a 5-min warm-up at a self-selected treadmill speed, subjects subsequently commenced the 60-min SS running protocol at a velocity corresponding to 70% VO₂peak. During exercise, subjects also consumed 60 g·h⁻¹ of CHO (SiS GO Istonic Gels; Science in Sport, Blackburn, UK) in HCHO, whereas no form of energy was consumed in the HFAT trial. Water was given ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Expired gases were also collected for 5 min at 15-min intervals throughout the exercise trial (CPX Ultima, Medgraphics) and substrate use again determined according to Jeukendrup and Wallis (20). HR was measured continuously during exercise (Polar), and RPE (9) were obtained every 15 min during exercise. Upon completion of the SS protocol until sleep, subjects consumed either the HCHO (3.6 g·kg⁻¹ CHO, 1.5 g·kg⁻¹ protein, 0.4 g·kg⁻¹ fat) or the HFAT (0.2 g·kg⁻¹ CHO, 1.5 g·kg⁻¹ protein, 2.3 g·kg⁻¹ fat) feeding protocols, where the pattern and frequency of feeding is shown in Figure 1. Vastus lateralis muscle biopsies and venous blood samples were also collected immediately postcompletion and at 3 and 15 h postcompletion (i.e., ~8:00 a.m. and in a fasted state) of the SS exercise protocol. The total energy intake across the entire trial period (i.e., 7:00 a.m.–9:00 p.m.) was ~10 g·kg⁻¹ SS exercise protocol. The total energy intake across the entire trial period (i.e., 7:00 a.m.–9:00 p.m.) was ~10 g·kg⁻¹ SS exercise protocol.

### Muscle Biopsies

Muscle biopsy samples (~50 mg) were obtained from the lateral portion of the vastus lateralis muscle using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge × 10 cm length (Bard Biopsy Systems, Tempe, AZ). Samples were obtained from separate incision sites 2–3 cm apart under local anesthesia (0.5% Marcaine) and immediately frozen in liquid nitrogen and stored at −80°C for later analysis.

### Analysis of Muscle Glycogen

Muscle glycogen concentration was determined according to the methods described by van Loon et al. (37). Approximately 3–5 mg of freeze dried muscle was powdered, and all visible blood and connective tissue were removed. The freeze-dried sample was then hydrolyzed by incubation in 500 μL of 1 M HCl for 3 h at 100°C. After cooling to room temperature for ~20 min, samples were neutralized by the addition of 250 μL 0.12 mol·L⁻¹ Tris/2.1 mol·L⁻¹ KOH saturated with KCl. After centrifugation at 1500 RCF for 10 min at 4°C, 200 μL of the supernatant was analyzed in duplicate for glucose concentration according to the hexokinase method using a commercially available kit (GLUC-HK; Randox Laboratories, Antrim, UK). Glycogen concentration is expressed as millimoles per kilogram of dry weight, and intra-assay coefficients of variation were <5%.

### RNA Isolation and Analysis

Muscle biopsy samples (~20 mg) were homogenized in 1 mL TRizol reagent (Thermo Fisher Scientific, Leicester, UK), and total RNA was isolated according to manufacturer’s guidelines. Concentrations and purity of RNA were assessed by UV spectroscopy at optical densities of 260 and 280 nm using a Nanodrop 3000 (Fisher, Roskilde, Denmark). Seventy nanograms of RNA was then used for each polymerase chain reaction (PCR). Samples were run in duplicate.

### Primers

The identification of primer sequences was enabled by Gene (NCBI, http://www.ncbi.nlm.nih.gov.gene), and primers were designed using Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast). Specificity was ensured using sequence homology searches so the primers only matched the experimental gene with no unintended targets identified for primer sequences. To prevent the amplification of gDNA, primers were ideally designed to yield products spanning exon–exon boundaries. Three or more GC bases in the last five bases at the 3-end and secondary structure interactions (hairpins, self-dimer, and cross dimer) within the primers were avoided so there would be no nonspecific amplification.

All primers were between 16 and 25 bp and amplified a product between 141 and 244 bp. All primers were purchased from Sigma (Suffolk, UK), and sequences for each gene are shown in parentheses: peroxisome proliferator-activated receptor coactivator (PGC-1) (fwd: TGCTAAGCAGCTCCGGA GAA, rev: TGCAAAGTTCCTCTCTGCT), tumor suppressor

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Changes in mRNA content were calculated using the comparative 
(all melt analysis presented single reproducible peaks for each 
aannealing/extension at 60°C steps, i.e., 40 cycles of denaturation at 95°C.

**BASIC SCIENCES**

Reverse Transcriptase Quantitative Real-Time PCR

Reverse transcriptase quantitative real-time PCR amplifications were performed using a Quantifast™ SYBR® Green RT-PCR one step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA). The following reverse transcriptase quantitative real-time PCR cycling parameters were used: hold 50°C for 10 min (reverse transcription/cDNA synthesis), initial denaturation and transcriptase inactivation at 95°C for 5 min, followed by PCR steps, i.e., 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 30 s. Upon completion, dissociation/melting curve analysis was performed to reveal and exclude nonspecific amplification or primer–dimer issues (all melt analysis presented single reproducible peaks for each target gene, suggesting the amplification of a single product).

Changes in mRNA content were calculated using the comparative Ct ([ΔΔCt] equation (34), where relative gene expression was calculated as $2^{-\Delta\Delta C_t}$ and where Ct represents the threshold cycle. GAPDH was used as a reference gene and did not change significantly between groups or time points studied (Ct = 24.2 ± 1); therefore, a pooled reference gene Ct was used in the relative gene expression equation. Furthermore, to enable calculation of expression values immediately post-exercise and 3-h postexercise, the calibrator condition in the ΔΔCt equation was assigned to the preexercise condition.

**Statistical Analysis**

All data were analyzed using Statistical Package for the Social Scientist (SPSS version 21, IBM, USA). Metabolic (i.e., blood metabolites, muscle glycogen, kinase activity, and mRNA data), physiological, and perceptual responses (i.e., HR, RPE, and oxidation rates) were analyzed using a two-way repeated-measures general linear model, where the within factors were time and condition (HCHO vs HFAT).

**Post hoc** LSD tests were used where significant main effects and interactions were observed to locate specific differences between time points and conditions. All data in text, figures, and tables are presented as mean ± SD, with P values ≤ 0.05 indicating statistical significance.

| TABLE 1. HR, RPE, and substrate oxidation responses during the HIT protocol in both the HCHO and the HFAT trials. |

<table>
<thead>
<tr>
<th>HIT (Bout No.)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tr>
<td>HR (bpm)</td>
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<tr>
<td>HCHO</td>
<td>177 ± 9</td>
<td>181 ± 9</td>
<td>184 ± 8</td>
<td>185 ± 6*</td>
<td>186 ± 6*</td>
<td>185 ± 5*</td>
<td>185 ± 5*</td>
<td>186 ± 7*</td>
</tr>
<tr>
<td>HFAT</td>
<td>173 ± 10</td>
<td>174 ± 8</td>
<td>180 ± 6</td>
<td>182 ± 7*</td>
<td>182 ± 7*</td>
<td>179 ± 6</td>
<td>182 ± 8*</td>
<td>184 ± 7*</td>
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<tr>
<td>RPE</td>
<td></td>
<td></td>
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<tr>
<td>HCHO</td>
<td>14 ± 1.4</td>
<td>15 ± 1.5*</td>
<td>16 ± 2.0*</td>
<td>17 ± 1.6*</td>
<td>18 ± 0.9*</td>
<td>18 ± 1.0*</td>
<td>19 ± 0.6*</td>
<td>19 ± 0.7*</td>
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<tr>
<td>HFAT</td>
<td>14 ± 1.6</td>
<td>15 ± 1.8*</td>
<td>16 ± 1.9*</td>
<td>17 ± 1.1*</td>
<td>18 ± 1.2*</td>
<td>18 ± 1.0*</td>
<td>19 ± 0.9*</td>
<td>19 ± 1.03*</td>
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<tr>
<td>CHO oxidation (g min⁻¹)</td>
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<tr>
<td>HCHO</td>
<td>5.5 ± 2.6</td>
<td>5.1 ± 1.4</td>
<td>4.9 ± 1.4</td>
<td>4.4 ± 1.4</td>
<td>3.9 ± 2.1*</td>
<td>3.7 ± 1.9*</td>
<td>3.5 ± 2.3*</td>
<td>3.8 ± 2.7*</td>
</tr>
<tr>
<td>HFAT</td>
<td>5.5 ± 2.7</td>
<td>4.8 ± 1.8</td>
<td>4.5 ± 1.6</td>
<td>4.4 ± 1.5</td>
<td>4.1 ± 2.2*</td>
<td>3.6 ± 1.9*</td>
<td>3.6 ± 2.4*</td>
<td>3.7 ± 2.7*</td>
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<tr>
<td>Fat oxidation (g min⁻¹)</td>
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<tr>
<td>HCHO</td>
<td>0.00 ± 0.29</td>
<td>0.00 ± 0.12</td>
<td>0.00 ± 0.14</td>
<td>0.06 ± 1.8</td>
<td>0.25 ± 2.2*</td>
<td>0.3 ± 2.5*</td>
<td>0.38 ± 3.1*</td>
<td>0.31 ± 3.5*</td>
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<tr>
<td>HFAT</td>
<td>0.00 ± 0.98</td>
<td>0.00 ± 0.11</td>
<td>0.00 ± 0.13</td>
<td>0.09 ± 1.8</td>
<td>0.19 ± 2.1*</td>
<td>0.3 ± 2.5*</td>
<td>0.37 ± 3.0*</td>
<td>0.34 ± 3.4*</td>
</tr>
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</table>

*Significant difference from HIT-1, P < 0.05.

**TABLE 2. HR, RPE, and substrate oxidation during the SS protocol in both the HCHO and HFAT trials.**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
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<tbody>
<tr>
<td>HR (bpm)</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HCHO</td>
<td>166 ± 12</td>
<td>169 ± 11</td>
<td>170 ± 12*</td>
<td>172 ± 12*</td>
</tr>
<tr>
<td>HFAT</td>
<td>161 ± 11</td>
<td>165 ± 12</td>
<td>166 ± 10*</td>
<td>168 ± 10*</td>
</tr>
<tr>
<td>RPE</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HCHO</td>
<td>13 ± 1.3</td>
<td>14 ± 1.5*</td>
<td>15 ± 1.8</td>
<td>15 ± 1.8*</td>
</tr>
<tr>
<td>HFAT</td>
<td>13 ± 1.4</td>
<td>14 ± 1.8*</td>
<td>15 ± 2.1</td>
<td>16 ± 1.4*</td>
</tr>
<tr>
<td>CHO oxidation (g min⁻¹)</td>
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</tr>
<tr>
<td>HCHO</td>
<td>3.4 ± 0.8</td>
<td>3.1 ± 1.2*</td>
<td>3.5 ± 0.9*</td>
<td>3.3 ± 0.6**</td>
</tr>
<tr>
<td>HFAT</td>
<td>2.8 ± 0.5</td>
<td>2.3 ± 0.4**</td>
<td>2.1 ± 0.5**</td>
<td>2.0 ± 0.6**</td>
</tr>
<tr>
<td>Fat oxidation (g min⁻¹)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>HCHO</td>
<td>0.00 ± 0.30</td>
<td>0.13 ± 0.30***</td>
<td>0.26 ± 0.31***</td>
<td>0.35 ± 0.31***</td>
</tr>
<tr>
<td>HFAT</td>
<td>0.32 ± 0.30</td>
<td>0.56 ± 0.29***</td>
<td>0.65 ± 0.31***</td>
<td>0.71 ± 0.40***</td>
</tr>
</tbody>
</table>

*Significant difference from 15 min, P < 0.05.
**Significant difference between conditions, P < 0.05.
RESULTS

Physiological responses and substrate use during exercise. Comparisons of subjects’ HR, RPE, and substrate oxidation during the HIT and SS protocols are displayed in Tables 1 and 2, respectively. HR, RPE, and lipid oxidation (all \( P < 0.01 \)) all displayed progressive increases during both HIT (see Table 1) and SS exercise (see Table 2), whereas CHO oxidation displayed a progressive decrease \( (P < 0.01) \) during both exercise protocols. In accordance with identical preexercise feeding in HIT, no significant differences were apparent in any of the aforementioned variables between HCHO and HFAT \( (P = 0.06, 0.19, 0.52, \) and 0.56, respectively). By contrast, however, during the SS exercise protocol, CHO oxidation was significantly greater in HFAT \( (P < 0.001) \), whereas fat oxidation was significantly greater during HFAT compared with HCHO \( (P < 0.001) \).

Plasma metabolite responses. Plasma glucose, lactate, NEFA, glycerol, and \( \beta \)-hydroxybutyrate all displayed significant changes \( (all \ P < 0.01) \) over the sampling period (see Table 3). However, in accordance with the provision of postexercise CHO feeding in the HCHO trial, plasma glucose was significantly higher compared with HFAT \( (P < 0.01) \), whereas postexercise high-fat feeding in HFAT induced significantly greater plasma NEFA, glycerol, and \( \beta \)-OHB \( (all \ P < 0.01) \) in HFAT compared with the HCHO trial.

Muscle glycogen and exercise-induced cell signaling. Exercise induced significant decreases \( (P < 0.01) \) in muscle glycogen immediately post-SS, although no differences were apparent between HCHO and HFAT at this time point (see Fig. 2A). However, in accordance with the provision of CHO after the SS exercise protocol in HCHO, muscle glycogen resynthesis was observed such that significant differences between HCHO and HFAT \( (P = 0.01) \) were observed at 3 and 15 h post-SS exercise. Neither exercise \( (P = 0.407) \) nor dietary condition \( (P = 0.124) \) affected AMPK-\( \alpha \)2 activity at any time point studied (see Fig. 2B). By contrast, p70S6K1 activity was significantly increased 3 h post-SS exercise \( (30 \text{ min postfeeding}) \) \( (P < 0.01) \), although this increase was suppressed \( (P = 0.08) \) in HFAT (see Fig. 2C). Furthermore, p70S6K1 activity was significantly reduced at 15 h post-SS exercise when participants were fasted compared with pre-HIT when they were high CHO and protein fed \( (P < 0.01) \).

Gene expression. Exercise increased the expression of PGC-1\( \alpha \) \( (P < 0.001) \), p53 \( (P = 0.032) \), CS \( (P = 0.05) \), Tffam \( (P = 0.05) \), PPAR \( (P < 0.01) \), and ERR\( \alpha \) \( (P = 0.01) \). However, there were no differences \( (all \ P > 0.05) \) between HFAT and HCHO trials (see Fig. 3A–F). By contrast, the exercise-induced increase \( (P = 0.001) \) in PDK4 mRNA was greater in HFAT versus HCHO \( (P = 0.003) \). Similarly, the mRNA expression of CD36 \( (P = 0.05) \) and CPT1 \( (P = 0.02) \) was significantly greater in HFAT in recovery from the SS exercise protocol (see Fig. 3). By contrast, neither exercise \( (P = 0.12) \) nor diet \( (P = 0.31) \) significantly affected GLUT expression (see Fig. 3).

DISCUSSION

The aim of the present study was to examine the effects of reduced CHO but high postexercise fat availability on the activation of key cell signaling kinases and expression of genes with putative roles in the regulation of mitochondrial biogenesis, lipid metabolism, and MPS. When compared with HCHO availability, we observed that postexercise high-fat feeding had no modulatory effect on AMPK-\( \alpha \)2 activity or the expression of those regulatory genes associated with mitochondrial biogenesis. Furthermore, although postexercise high-fat feeding augmented the expression of genes involved in lipid transport (i.e., FAT/CD36) and oxidation (i.e., CPT1), we also observed suppression of p70S6K1 activity despite sufficient post-exercise protein intake. This latter finding suggests that postexercise high-fat feeding may impair the regulation of MPS and skeletal muscle remodeling processes, thereby potentially causing maladaptive responses for training adaptation if performed long-term.

### Table 3. Plasma glucose, lactate, NEFA, glycerol, and \( \beta \)-OHB before and after the HIT and SS exercise protocols.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pre-HIT</th>
<th>Post-HIT</th>
<th>Pre-SS</th>
<th>Post-SS</th>
<th>3-h Post-SS</th>
<th>15-h Post-SS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>HCHO</td>
<td>5.2 ± 0.0863</td>
<td>7.6 ± 0.74**</td>
<td>4.7 ± 0.96***</td>
<td>8.7 ± 1.1***</td>
<td>5.9 ± 0.62**</td>
<td>5.5 ± 0.3*</td>
</tr>
<tr>
<td>HFAT</td>
<td>5.4 ± 0.84</td>
<td>7.6 ± 1.03**</td>
<td>5.3 ± 0.34***</td>
<td>5.6 ± 0.8**</td>
<td>5.7 ± 0.6**</td>
<td>5.1 ± 0.42*</td>
</tr>
<tr>
<td>Lactate (mmol L(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCHO</td>
<td>1.9 ± 0.42</td>
<td>7.8 ± 3.1***</td>
<td>1.5 ± 0.3***</td>
<td>1.6 ± 0.46**</td>
<td>1.5 ± 0.16**</td>
<td>1.1 ± 0.26**</td>
</tr>
<tr>
<td>HFAT</td>
<td>1.8 ± 0.41</td>
<td>7 ± 3.4**</td>
<td>1.1 ± 0.22***</td>
<td>1.3 ± 0.35**</td>
<td>0.8 ± 0.21**</td>
<td>0.9 ± 0.36**</td>
</tr>
<tr>
<td>NEFA (mmol L(^{-1}))</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>HCHO</td>
<td>0.01 ± 0.02</td>
<td>0.23 ± 0.27**</td>
<td>0.09 ± 0.13**</td>
<td>0.59 ± 0.56**</td>
<td>0.09 ± 0.17**</td>
<td>0.22 ± 0.22**</td>
</tr>
<tr>
<td>HFAT</td>
<td>0.05 ± 0.05</td>
<td>0.25 ± 0.28**</td>
<td>0.32 ± 0.21**</td>
<td>1.42 ± 0.74**</td>
<td>0.48 ± 0.27**</td>
<td>0.24 ± 0.14**</td>
</tr>
<tr>
<td>Glycerol (mmol L(^{-1}))</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCHO</td>
<td>14.8 ± 6.07</td>
<td>92.8 ± 27.1**</td>
<td>22.9 ± 12.3**</td>
<td>50.2 ± 37.4**</td>
<td>12.2 ± 4.28**</td>
<td>23.4 ± 20.1**</td>
</tr>
<tr>
<td>HFAT</td>
<td>12.9 ± 4.82</td>
<td>79.3 ± 25.4**</td>
<td>33 ± 7.3**</td>
<td>122.9 ± 57.3***</td>
<td>40.8 ± 12.4**</td>
<td>30.85 ± 12.6**</td>
</tr>
<tr>
<td>( \beta )-OHB (mmol L(^{-1}))</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>HCHO</td>
<td>0.07 ± 0.02</td>
<td>0.14 ± 0.04**</td>
<td>0.07 ± 0.02***</td>
<td>0.15 ± 0.08**</td>
<td>0.08 ± 0.02**</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>HFAT</td>
<td>0.07 ± 0.02</td>
<td>0.14 ± 0.04**</td>
<td>0.11 ± 0.05***</td>
<td>0.33 ± 0.21**</td>
<td>0.29 ± 0.2**</td>
<td>0.19 ± 0.017</td>
</tr>
</tbody>
</table>

*Significant difference between conditions, \( P < 0.05 \).

**Significant difference from pre-HIT, \( P < 0.05 \).
In accordance with the original train-low investigations examining cycling or knee extensor exercise (16,17,29,39), we also used a twice per day protocol, albeit consisting of morning HIT and afternoon SS running exercise protocol. This model is practically relevant given that many elite endurance athletes (including runners) train multiple times per day with limited recovery time between training sessions (14). Given that reduced CHO availability impairs high-intensity training capacity (17,39), we also chose to schedule the HIT session in the morning period after a standardized HCHO breakfast. As expected, no differences in cardiovascular strain, RPE, substrate use, and plasma metabolite responses were observed between the HCHO and the HFAT trials during the HIT session (see Tables 1 and 3). After completion of the HIT protocol, subjects then adhered to an HCHO or HFAT feeding.
protocol in the 3.5 h before commencing the afternoon SS exercise. Given that exogenous CHO feeding during exercise reduces oxidative adaptations even in the presence of reduced preexercise muscle glycogen (29), we also chose to feed exogenous CHO (at a rate of 60 g h⁻¹) during the afternoon SS protocol during the HCHO trial. Although we did not directly quantify muscle glycogen immediately before SS exercise, plasma metabolite and substrate use during SS exercise were clearly suggestive of differences in both endogenous and exogenous CHO availability between the HCHO and the HFAT trials. Indeed, plasma NEFA, glycerol, β-OHB, and whole body lipid oxidation were all greater during SS exercise undertaken in the HFAT trial compared with the HCHO trial (see Tables 2 and 3). On the basis of comparable muscle glycogen data post-SS exercise (see Fig. 2A) and greater whole body CHO oxidation during the HCHO trial (see Table 2), we also suggest that exercise-induced muscle glycogen use was greater during the SS exercise protocol when completed in the HCHO conditions (7).

Perhaps surprisingly, we observed that our SS exercise protocol did not increase AMPK-α2 activity in either the HCHO or HFAT trial. However, there are likely several physiologically valid reasons to explain the apparent lack of AMPK-mediated signaling. Indeed, exercise-induced AMPK activation is known to be intensity dependent, where >70% VO₂max is likely required to induce metabolic perturbations sufficient to mediate a signaling response (13). Furthermore, the AMPK response to exercise is attenuated with exercise training (8), an effect that is especially relevant for the present investigation given the trained status of our chosen population and the low plasma lactate observed (approximately 2 mmol L⁻¹) during SS exercise. Reduced absolute muscle fiber recruitment from the vastus lateralis, when compared with other lower extremity muscles recruited during walking and running (19), or when exercising at similar relative intensities during cycling (4) and where AMPK activation is typically reported (22), could also contribute, in part, to the lack of AMPK signaling observed here. Finally, although exercise-induced AMPK activity is also thought to be regulated, in part, via a glycogen-binding domain on β-subunit of the AMPK heterotrimer (26), it is possible that our runners did not exceed a potential “muscle glycogen threshold” that is required to fully activate the AMPK complex during prolonged endurance exercise (30). Indeed, previous data from our laboratory also using running exercise protocols (6,7) have typically only observed AMPK-related signaling when postexercise whole muscle homogenate glycogen is <200 mmol kg⁻¹ dry weight. Despite previous suggestions that train-low training sessions should be targeted to SS exercise protocols so as to not compromise training intensity (5), our data therefore suggest (at least for AMPK-mediated signaling) that perhaps it is the actual completion of a high-intensity stimulus per se (especially in trained athletes) that is really required to create a metabolic milieu that is conducive to augmentation of necessary signaling networks.

In contrast to Yeo et al. (38), we also observed no modulatory effect of postexercise high-fat availability on resting AMPK-α2 activity. Indeed, these authors observed that 5 d of a fat loading protocol increased resting AMPK-α2 activity as well as the exercise-induced phosphorylation of ACCSε²²¹. Such discrepancies between studies are likely due to the differences in duration of high-fat feeding in that we adopted an acute high-fat feeding protocol (<24 h), whereas the latter authors adopted a 5-d “fat adaptation” protocol that also increased resting IMTG stores. In this regard, it is noteworthy that the magnitude of change in resting AMPK-α2 activity was positively correlated with the elevations in IMTG storage (38).

In contrast to our hypothesis, we also observed comparable two- to threefold changes between trials in the mRNA expression of those genes with key regulatory roles associated with mitochondrial biogenesis. For example, the expression levels of PGC-1α, p53, Tfam, PPAR, and ERRα mRNA were all elevated with similar magnitude and time course in recovery from the SS protocol in both the HCHO and the HFAT trials. Such data conflict with previous observations from our laboratory (7) and others (32) where postexercise CHO restriction (i.e., keeping muscle glycogen low) augments the expression of many of the aforementioned genes. However, in our previous report, we simultaneously adopted a CHO but calorie restriction feeding protocol, whereas the present design incorporated a reduced CHO but isocaloric and protein-matched feeding protocol in our HFAT trial. The similarities in metabolic adaptation to both CHO and calorie restriction, such data raise the question whether the enhanced mitochondrial responses observed when “training low” are due to transient periods of CHO restriction, calorie restriction, or indeed a combination of both. This point is especially relevant from an applied perspective given that many endurance athletes present daily with transient periods of both CHO and calorie restriction because of multiple training sessions per day as well as longer-term periods of suboptimal energy availability (14).

In agreement with multiple studies demonstrating a role of both acute elevations in FFA availability (7,23) as well as high-fat feeding protocols (11), we also observed that the postexercise expression levels of PDK4, FAT/CD36, and CPT1 mRNA were elevated in the HFAT trial versus the HCHO trial. However, unlike Arkinstall et al. (4), we did not detect any suppressive effects of high-fat availability on GLUT4 mRNA expression, although a longer and more severe period of CHO restriction used by these investigators (i.e., 48 h of absolute CHO intake <1 g kg⁻¹ body mass resulting in muscle glycogen levels <150 mmol kg⁻¹ dry weight) may explain the discrepancy between studies. Nonetheless, the dietary protocol studied here clearly alters the expression of genes with potent regulatory roles in substrate use and, if performed long term, may increase the capacity to use lipids as a fuel but induce suppressive effects on CHO metabolism (through suppression of the PDH complex), thus potentially limiting high-intensity performance (35). Although we did not directly quantify the signaling mechanisms underpinning these responses (owing to a lack of a
muscle tissue), we suggest both p38MAPK and PPAR-mediated signaling are likely involved. Indeed, using a twice per day exercise model, Cochran et al. (12) also observed enhanced p38MAPK phosphorylation during the afternoon exercise protocol (despite similar preexercise muscle glycogen availability) that was associated with the enhanced circulating FFA availability during the afternoon exercise. Furthermore, pharmacological ablation of circulating FFA availability during exercise suppresses p38MAPK compared with control conditions (40). In addition, FFA-mediated signaling can also directly mediate PPAR binding to the CPT1 promoter thereby modulating CPT1 expression (31).

We also examined the effects of postexercise fat feeding on the regulation of p70S6K activity, a key signaling kinase associated with regulating MPS. In relation to the effects of endurance exercise per se, the majority of studies are typically limited to measures of phosphorylation status with some studies reporting increases (25) and others reporting no change. When examined quantitatively using the \( \left[ \gamma^{32P} \right] \) ATP kinase assay, our data agree with previous observations from Apro et al. (3), who also reported no change but conflict with recent data from our group where we observed an exercise-induced suppression of p70S6K activity (18). Nonetheless, the exhaustive (a fatiguing cycling HIT protocol) and muscle glycogen depleting (<100 mmol·kg\(^{-1}\) dry weight) nature of the latter exercise protocol versus the moderate-intensity nature of the afternoon SS running protocol studied here likely explains the discrepancy between studies.

In relation to the effects of postexercise feeding, we also provide novel data by demonstrating that postexercise high-fat feeding was associated with a suppression of p70S6K activity (albeit \( P = 0.08 \)) at 3 h postcompletion of the SS exercise protocol when compared with the elevated response observed in HCHO (when using both a mean difference and standard deviation of differences of 50 fmol·min\(^{-1}\)·mg\(^{-1}\)). we estimate a sample size of 12–13 would be required to achieve statistical significance with 90% power, as calculated using Minitab statistical software, version 17). Although we did not measure circulating insulin levels in this study, it is of course possible that the suppressed p70S6K response observed here may be due to reduced upstream insulin-mediated activation of protein kinase B (PKB). Indeed, we recently observed postexercise p70S6K activity to be suppressed in conditions of simultaneous carbohydrate and calorie restriction in a manner associated with reduced insulin and upstream signaling of PKB (18). Alternatively, the suppression of p70S6K observed here may be mediated through direct effects of postexercise high-fat feeding that are independent of CHO availability, energy availability, and insulin. Indeed, Stephens et al. (36) observed infusion of intralipid and heparin to elevate circulating FFA concentrations attenuates MPS in human skeletal muscle in response to ingesting 21 g amino acids under euglycemic hyperinsulenic clamp conditions. Furthermore, Kimball et al. (21) also reported that high-fat feeding impairs MPS in rat liver in a manner associated with reduced p70S6K phosphorylation, an effect that may be induced through sestrin 2 and sestrin 3-mediated impairment of mechanistic target of rapamycin complex signaling. Clearly, further research is required to examine the effects of high-fat feeding on direct measures (and associated regulatory sites) of MPS within the physiological context of the exercising human.

In summary, we provide novel data by concluding that postexercise high-fat feeding has no modulatory affect on AMPK-α2 activity or the expression of those genes associated with regulatory roles in mitochondrial biogenesis. Furthermore, although postexercise high-fat feeding augmented the expression of genes involved in lipid transport and oxidation, we also observed a suppression of p70S6K1 activity despite sufficient postexercise protein intake. This latter finding suggests that postexercise high-fat feeding may impair the regulation of MPS and postexercise muscle remodeling, thereby potentially causing maladaptive responses for training adaptation if performed long-term. Future studies should now examine the functional relevance of the signaling responses observed here, not only in terms of acute MPS but also in terms of chronic skeletal muscle and performance adaptations induced by long-term use of this feeding strategy.

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The authors report no conflict of interest. The results of the present study do not constitute endorsement by the American College of Sports Medicine.

**REFERENCES**


