Post-exercise carbohydrate and energy availability induce independent effects on skeletal muscle cell signalling and bone turnover: implications for training adaptation

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Key Points Summary

- Reduced carbohydrate (CHO) availability before and after exercise may augment endurance training-induced adaptations of human skeletal muscle, as mediated via modulation of cell signalling pathways.
- However, it is not known whether such responses are mediated by CHO restriction, energy restriction or a combination of both.
- In recovery from a twice per day training protocol where muscle glycogen concentration is maintained within 200-350 $mmol.kg^{-1}$ dw, we demonstrate acute post-exercise CHO and energy restriction (i.e. <24 hours) does not potentiate potent cell signalling pathways that regulate hallmark adaptations associated with endurance training.
- In contrast, consuming CHO before, during and after an acute training session attenuated markers of bone resorption, effects that are independent of energy availability.
- Whilst the enhanced muscle adaptations associated with CHO restriction may be regulated by absolute muscle glycogen concentration, the acute within day fluctuations in CHO availability inherent to twice per day training may have chronic implications for bone turnover.

Abstract

We examined the effects of post-exercise carbohydrate (CHO) and energy availability (EA) on potent skeletal muscle cell signalling pathways (regulating mitochondrial biogenesis and lipid metabolism) and indicators of bone metabolism. In a repeated measures design, nine males completed a morning (AM) and afternoon (PM) high-intensity interval (HIT) (8 x 5-min at 85% VO_{2peak}) running protocol (interspersed by 3.5 hours) under dietary conditions of 1) high CHO availability (HCHO: CHO ~12 $g.kg^{-1}$, EA~ 60 $kcal.kg^{-1}$ FFM), 2) reduced CHO but high fat availability (LCHF: CHO ~3 $g.kg^{-1}$, EA~ 60 $kcal.kg^{-1}$ FFM) or 3), reduced CHO and reduced energy availability (LCAL: CHO ~3 $g.kg^{-1}$, EA~ 20 $kcal.kg^{-1}$ FFM). Muscle glycogen was reduced to ~200 $mmol.kg^{-1}$dw in all trials immediately post PM-HIT ($P<0.01$) and remained lower at 17-h (171, 194 and 316 $mmol.kg^{-1}$dw) post PM-HIT in LCHF and LCAL ($P<0.001$) compared to HCHO. Exercise induced comparable p38MAPK phosphorylation ($P<0.05$) immediately-post PM-HIT and similar mRNA expression (all $P<0.05$) of PGC-1α, p53 and CPT1 mRNA in HCHO, LCHF and LCAL. Post-exercise circulating βCTX was lower in HCHO ($P<0.05$) compared to LCHF and LCAL, whereas exercise-induced increases in IL-6 were larger in LCAL ($P<0.05$) compared to LCHF and HCHO. In conditions where glycogen concentration is maintained within 200-350 $mmol.kg^{-1}$ dw, we conclude post-exercise CHO and energy restriction (i.e. <24 hours) does not potentiate cell signalling pathways that regulate hallmark adaptations associated with endurance training. In contrast, consuming CHO before, during and after HIT running attenuates bone resorption, effects that are independent of energy availability and circulating IL-6.

Keywords: βCTX, IL-6, caloric restriction, PGC-1α

Introduction

The concept of deliberately promoting reduced carbohydrate (CHO) availability to enhance endurance training induced adaptations of skeletal muscle (i.e. the 'train-low' paradigm) is gaining increased acceptance amongst athletic populations (Bartlett et al. 2015; Marquet et al. 2016; Burke et al. 2018; Stellingwerff et al. 2019). Over the last decade, a multitude of research designs examining the train-low approach (e.g. fasted training, twice per day training, sleep low-train low etc.) have consistently demonstrated that commencing and/or recovering from a training session with reduced CHO availability activates potent cell signalling pathways that regulate many of the hallmark muscle adaptations inherent to endurance training (Yeo *et al.* 2008; Bartlett *et al.* 2013; Psilander *et al.* 2013). Accordingly, the strategic periodisation of reductions in endogenous and/or exogenous CHO availability during 3-10 weeks of training increases mitochondrial enzyme activity and protein content (Morton *et al.* 2009; Yeo *et al.* 2008; Van Proeyen *et al.* 2011), whole body (Yeo *et al.* 2008) and intramuscular (Hulston *et al.* 2010) lipid metabolism, and may improve exercise capacity (Hansen *et al*. 2005) and performance (Marquet *et al*. 2016).

The mechanisms underpinning the apparent enhanced training adaptation associated with CHO periodisation is more difficult to interpret in those experimental designs in which CHO restriction is simultaneously accompanied by energy restriction. Indeed, the performance improvements observed by Marquet *et al*. (2016) were also associated with a 1 kg reduction in fat mass induced by the sleep-low model. Notwithstanding reductions in body mass, it is possible that many of the skeletal muscle adaptations associated with training-low are mediated by repeated and transient periods of energy restriction as opposed to CHO restriction *per se*. We recently observed that the post-exercise mRNA expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), p53, mitochondrial transcription factor A (Tfam) and peroxisome proliferator-activated receptor (PPAR) mRNA were increased with similar magnitude and time-course when a low CHO and high fat diet was consumed versus an isoenergetic high CHO feeding strategy (Hammond *et al.* 2016). Such data conflict with previous observations from our laboratory (Bartlett *et al*. 2013) where simultaneous post-exercise CHO and calorie restriction augments the mRNA expression of many of the aforementioned genes. Given the similarities in metabolic adaptation to both CHO

and calorie restriction (Meynet and Ricci, 2014), such data raise the question as to whether the acute and chronic muscle adaptations observed when training low are actually due to transient periods of calorie restriction (as mediated by a reduction in CHO intake) as opposed to CHO restriction per se. This point is especially relevant given that many endurance athletes present daily with transient periods of both CHO and calorie restriction due to multiple training sessions per day as well as longer term periods (i.e. weeks) of sub-optimal energy availability (Fudge *et al*. 2006; Vogt *et al*. 2005, Stellingwerff *et al.* 2019).

In addition to the potential effects of muscle glycogen concentration on skeletal muscle adaptations (Impey *et al*. 2018), it is also possible that the acute and transient within day fluctuations in both CHO and energy availability (especially in the twice per day training model) can exert regulatory effects on other physiological systems, many of which are associated with the Relative Energy Deficiency in Sport (RED-S) syndrome (Mountjoy *et al.* 2014). Therefore, whilst train-low and/or energy restriction may be beneficial for certain aspects of training adaptation, the conscious or unconscious (as a default of the athlete's habitual training structure and daily pattern of feeding) manipulation of CHO and energy availability may have negative implications for indicators of the RED-S syndrome. As such, the acute regulation of bone turnover (i.e. alterations in resorption and formation, Sale *et al*., 2015), immune function (Nieman, 2007), endocrine function (Loucks *et al.* 1998; Hagmar *et al.* 2013) and appetite regulation (Considine *et al.* 1996) may be negatively impacted, the result of which could increase injury risk and impair athletic performance if performed chronically.

With this in mind, the aim of the present study was to examine the effects of post-exercise CHO and caloric restriction on the modulation of skeletal muscle cell signalling pathways associated with the regulation of mitochondrial biogenesis and lipid metabolism as well as indicators of bone metabolism. We employed a repeated measures crossover design whereby male runners completed a twice per day exercise model consisting of a morning and afternoon high-intensity interval training session. Runners completed the training sessions under three dietary conditions comprising high CHO availability (HCHO) in the recovery period after both training sessions (i.e. usually considered as best practice recovery nutrition), reduced CHO but high fat availability (LCHF) or finally, reduced CHO and reduced energy intake (LCAL). In

this way, our experimental design allowed us to evaluate the effects of post-exercise CHO restriction in modulating adaptive responses of muscle and bone in conditions of both sufficient or reduced energy availability. We hypothesised that post-exercise CHO restriction only modulates cell signalling, gene expression and bone metabolism when energy intake is simultaneously reduced.

Methods

Ethical Approval: All participants gave written informed consent prior to participation after all experimental procedures and potential risks had been fully explained. The study was approved by the ethics committee of Liverpool John Moores University (Ethics No: 15 SPS 60) and conformed to the standards set by the latest revision of the *Declaration of Helsinki* (except for registration in a database).

Participants: Nine male runners volunteered to participate in the study (mean ± SD: age, 21 ± 1.9 years; body mass, 71.4 ± 7 kg; height, 1.78 ± 0.04 m; VO_{2peak}, 57 ± 5.1 $ml \cdot kg^{-1} \cdot min^{-1}$). None of the participants had any history of musculoskeletal or neurological disease, nor were they under any pharmacological treatment over the course of the testing period. Participants were instructed to refrain from any strenuous physical activity, alcohol and caffeine consumption in the 24 h prior to each experimental trial. On the basis of previous studies demonstrating that CHO availability can regulate skeletal muscle p38MAPK signalling (Cochran et al. 2012; mean paired differences of -2 fold change between conditions and SD of differences of 1.5-fold), post-exercise PGC-1α mRNA expression (Pilegaard et al. 2005; mean paired differences of -3 fold change between conditions and SD of differences of 2-fold) and post-exercise AUC for circulating plasma CTx concentrations (Sale et al. 2015; mean paired differences of 5% changes between conditions and SD of differences of 3%), it was estimated that sample sizes of 7-9 were deemed appropriate to detect statistical significance at an alpha level of 0.05 and 80% power. Sample size estimation calculations were undertaken using Minitab software, version 19.1.

Design**:** In a repeated measures, randomised, cross-over design separated by 7 days, participants completed a twice per day exercise model under three different dietary conditions, consisting of either high CHO availability (HCHO) in the recovery period

after both training sessions (i.e. best practice nutrition), reduced CHO but high fat availability (LCHF), or reduced CHO but reduced energy availability (LCAL). The twice per day exercise model comprised a morning (9-10 am) high-intensity interval (AM-HIT) training session (8 x 5-min at 85% VO_{2peak}) followed by an afternoon (130-230 pm) training session consisting of the same high-intensity interval exercise bout (PM-HIT). To promote training compliance during the AM-HIT protocol in all three trials, participants adhered to a standardised high CHO breakfast prior to this session. However, during the 3.5 h recovery between the HIT sessions and in the recovery period upon completion of the PM-HIT exercise protocol until the subsequent morning, participants adhered to either the HCHO, LCHF, or LCAL feeding protocol. Muscle biopsies were obtained from the *m. vastus lateralis* muscle immediately pre AM-HIT, immediately post PM-HIT and at 3 h and 17 h post PM-HIT. An overview of the experimental design and nutritional protocols are shown in Figure 1.

***Preliminary testing*:** At 7 days prior to the first main experimental trial, participants performed a combined running economy and maximal incremental running test to volitional fatigue on a motorised treadmill (h/p/Cosmos, Nussdorf-Traunstein, Germany). Both peak oxygen uptake and individual running speeds for the subsequent experimental trials were determined. Following a 10-minute warm up at a self-selected treadmill speed, the running economy test commenced, beginning with a 3-min stage at a treadmill speed of 10 $km.h^{-1}$. Running speed was then increased by 1 $km.h^{-1}$ every 3-min thereafter until participants reached >90% of their age predicted maximal heart rate. Participants were then given a 5-min rest period before commencing the maximal incremental test. This test began with a 2-min stage at a treadmill speed 2 $km.h^{-1}$ slower than the final speed of the running economy test. 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. VO_{2peak} was defined as the highest VO_2 value obtained during any 10-s period and was stated as being achieved by two of the following criteria: 1) heart rate was within 10 $beats.min^{-1}$ ($b.min^{-1}$) of age-predicted maximum, 2) respiratory exchange ratio > 1.1, and 3) plateau of oxygen consumption despite increased workload. These measurements were recorded via breath-by-breath gas analysis obtained continuously throughout both tests using a CPX Ultima series online gas

analysis system (Medgraphics, Minnesota, US), with heart rate (Polar, Kempele, Finland) also recorded continuously during exercise.

***Experimental protocols*:**

***AM HIT protocol*:** In the 24-h preceding each main experimental trial, participants consumed a standardised high CHO diet in accordance with typical nutritional recommendations (Thomas et al. 2016) for endurance athletes (8 $g.kg^{-1}$ CHO, 2 $g.kg^{-1}$ protein, and 1 $g.kg^{-1}$ fat) performing between 1-3 hours of exercise per day. On the morning of each experimental trial, participants reported to the laboratory between 7 and 7.15 am where a venous blood sample was collected from an antecubital vein in the anterior crease of the forearm. Participants were then given a standardised high-CHO breakfast (2 $g.kg^{-1}$ CHO, 0.3 $g.kg^{-1}$ protein, and 0.1 $g.kg^{-1}$ fat; comprising breakfast cereal, milk, orange juice, bread and jam), and at 2-h post-prandial, a muscle biopsy sample was obtained from the vastus lateralis muscle. Participants were then fitted with a heart rate monitor and nude body mass (SECA, Hamburg, Germany) was recorded before commencing the first high intensity interval running (AM-HIT) protocol which lasted ~1-h. The HIT protocol consisted of 8 x 5-min bouts running at a velocity corresponding to 85% VO_{2peak} interspersed with 1-min of recovery at walking pace. The intermittent protocol started and finished with a 10-min warm up and cool down at a velocity corresponding to 50% $VO_{2peak,}$ and a further venous blood sample was obtained immediately upon completion of the protocol. Water was consumed ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trials. Heart rate was measured continuously during exercise (Polar, Kempele, Finland) and ratings of perceived exertion (RPE, Borg, 1973) were obtained upon completion of each HIT bout. In order to determine substrate utilisation and energy expenditure during exercise (Jeukendrup and Wallis, 2005), expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, Medgraphics, Minnesota, US) for the final 2-mins of each 5-min interval.

***PM HIT protocol*:** During the 3.5 h recovery period between the AM-HIT and PM-HIT protocols, participants consumed either the HCHO (3.5 $g.kg^{-1}$ CHO, 0.8 $g.kg^{-1}$ Protein, 0.2 $g.kg^{-1}$ Fat), LCHF (0 $g.kg^{-1}$ CHO, 0.8 $g.kg^{-1}$ Protein, 1.2 $g.kg^{-1}$ Fat), or LCAL (0 $g.kg^{-1}$ CHO, 0.8 $g.kg^{-1}$ Protein, 0.1 $g.kg^{-1}$ Fat) feeding protocols (the pattern and frequency of feeding is shown in Figure 1). Following the recovery period, another venous blood sample was obtained immediately prior to commencing the afternoon HIT exercise protocol. After a 10-min warm up at a velocity corresponding to 50% VO_{2peak}, participants subsequently commenced the PM HIT protocol. During exercise, participants also consumed 60 $g.h^{-1}$ of CHO (SiS GO Isotonic Gels, Science in Sport, Blackburn, UK) in HCHO whereas no energy was consumed in the LCHF or LCAL trials. Water was consumed ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Expired gases were collected for the last 2-mins of each 5-min interval throughout the exercise trial (CPX Ultima, Medgraphics, Minnesota, US) and substrate utilisation and energy expenditure was determined according to the method of Jeukendrup and Wallis (2005). Heart rate was measured continuously during exercise (Polar, Kempele, Finland) and RPE (Borg, 1973) were obtained at the end of each HIT bout. Upon completion of the PM-HIT protocol until sleep, participants consumed either the HCHO (6.4 $g.kg^{-1}$ CHO, 1.3 $g.kg^{-1}$ Protein, 0.3 $g.kg^{-1}$ Fat), LCHF (0.8 $g.kg^{-1}$ CHO, 1.3 $g.kg^{-1}$ Protein, 2.3 $g.kg^{-1}$ Fat), or LCAL (0.8 $g.kg^{-1}$ CHO, 1.3 $g.kg^{-1}$ Protein, 0.1 $g.kg^{-1}$ 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 post- and at 3 h and 17 h post completion (i.e. 8 am and in a fasted state) of the PM-HIT exercise protocol. The total energy and estimated calcium intake across the whole trial period in HCHO was: ~12.6 $g.kg^{-1}$ CHO, ~2.3 $g.kg^{-1}$ Protein, ~0.6 $g.kg^{-1}$ Fat, 1051 mg calcium, in LCHF was: ~3 $g.kg^{-1}$ CHO, ~2.3 $g.kg^{-1}$ Protein, ~3 $g.kg^{-1}$ Fat , 1780 mg calcium (where both trials were matched for total energy intake), and in LCAL was ~3 $g.kg^{-1}$ CHO, ~2.3 $g.kg^{-1}$ Protein, ~0.4 $g.kg^{-1}$ Fat, 865 mg calcium. Typical energy sources in the HCHO trial consisted of rice, bananas, sports drinks (e.g. Lucozade Original, UK; GO Energy and REGO, Science in Sport, UK), sports gels (GO Isotonic Energy Gels, Science in Sport, UK) and confectionary products (e.g. Jaffa Cakes, McVities, UK) whereas increased dietary fat intake in the LCHF trial was reflective of increased intake of oily fish, avocado, butter, coconut oil, walnuts and cheese. In the LCAL trial, CHO intakes were limited

to smaller absolute portions of rice and none of the aforementioned sources of dietary fat were consumed.

Energy Availability: Energy availability was estimated for each experimental trial as calculated from: energy intake minus energy expenditure during AM-HIT and PM-HIT exercise / fat free mass (FFM) (Mountjoy *et al.*, 2014). Whilst FFM was not directly measured, we based estimates of FFM on the assumption that all participants were ~15% body fat (as based on unpublished data from our laboratory examining male participants of similar body mass, training history and maximal oxygen uptake, Impey *et al.*). The total energy expenditure from both AM-HIT and PM-HIT in HCHO was 1474 ± 107 kcal, in LCHF was 1429 ± 142 kcal and in LCAL was 1455 ± 190 kcal. Estimated energy availability across the whole trial period in HCHO was 58 ± 5.3 $kcal.kg^{-1}$ FFM, in LCHF was 59 ± 6.4 $kcal.kg^{-1}$ FFM, and in LCAL was 20 ± 4.1 $kcal.kg^{-1}$ FFM.

Blood sampling and analysis: Venous blood samples were collected into vacutainers containing EDTA or lithium heparin and stored on ice until centrifugation at 1500 xg for 15-mins at 4ºC. Following centrifugation, aliquots of plasma were stored in a freezer at -80ºC for subsequent analysis. Samples were later analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA), glycerol, and β-hydroxybutyrate using commercially available enzymatic spectrophotometric assays (RX Daytona Analyser, Randox, Co. Antrim, UK) as per the manufacturers' instructions. Samples were also analysed using commercially available solid phase enzyme-linked immunosorbent assays (ELISA) for insulin (DIAsource, Belguim), leptin (R&D systems, Minnesota, USA) ghrelin (Invitrogen, Thermo Fisher Scientific, UK) and adiponectin (Abcam, Cambridge, UK) as per the manufacturers instructions. Plasma βCTX and P1NP were measured by ECLIA on a fully automated COBAS e601 system (Roche Diagnostics, Mannheim, Germany). These markers were selected for use as they are the preferred markers of use as reference analytes for bone turnover markers in clinical studies (Vasikaran *et al.*, 2011). The inter-assay CV for βCTX was ≤3% between 0.2 – 1.5 μg.L-1, with a sensitivity of 0.01 $\mu g.L^{-1}$, and the inter-assay CV for P1NP was ≤3% between 20 – 600 $\mu g.L^{-1}$, with a sensitivity of 8 $\mu g.L^{-1}$. The assays used a sandwich test principle involving two incubations. Flow cytometric detection of IL-6 was performed using a dual-laser FACSCalibur flow cytometer (BD biosciences, USA) calibrated for 2-colour analysis. Cytokine concentrations were

measured using a cytometric bead array (CBA, BD Biosciences, San Diego, USA) according to the manufacturers instructions. Bead populations with distinct fluorescence intensities coated with phycoerythrin (PE)-conjugated capture antibodies specific for IL-6 were used. Following acquisition of sample data using the flow cytometer, the sample results were generated in graphical and tabular format using the BDA CBA Analysis Software.

***Muscle biopsies*:** Muscle biopsy samples (~60 mg) were obtained from the lateral portion of the vastus lateralis muscle using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge x 10 cm length, (Bard Biopsy Systems, Tempe, AZ, USA). Consecutive biopsies were taken from alternate legs. Samples collected from the same limb were obtained from separate incision sites 2-3 cm apart under local anaesthesia (0.5% Marcaine) and immediately frozen in liquid nitrogen and stored at -80ºC for later analysis.

Analysis of muscle glycogen: Muscle glycogen concentration was determined according to the methods described by Van Loon et al (2000). Approximately 3-5 mg of freeze-dried muscle was powdered and all visible blood and connective tissue removed. The freeze-dried sample was then hydrolysed by incubation in 500 µl of 1M HCl for 3 hours at 100˚C. After cooling to room temperature for ~20-min, samples were neutralized by the addition of 250µl 0.12 mol.L^{-1} Tris/2.1 mol.L^{-1} KOH saturated with KCl. Following centrifugation at 1500 RCF for 10-mins at 4ºC, 200 µl of the supernatant was analysed 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 mmol.kg^{-1} dry weight.

Analysis of phosphorylated p38 MAPK $^{T180/Y182}$ phosphorylation: Flow cytometric detection of phosphoproteins was performed using a dual-laser BD C6 accuri flow cytometer (BD biosciences, USA). Phospho-p38 concentrations were measured using a cytometric bead array (CBA, BD Biosciences, San Diego, USA) according to the manufacturers instructions. Bead populations with distinct fluorescence intensities coated with phycoerythrin (PE)-conjugated capture antibodies specific for phospho-p38 were used. Following acquisition of sample data using the flow cytometer, the sample results were generated in graphical and tabular format using the BDA CBA

Analysis Software (Hungary Software Ltd., for BD Biosciences, San Jose, CA, USA) where median values were used. Briefly, muscle samples (~20 mg) were homogenised in 200µl of denaturation buffer (BD biosciences, UK) in beaded tubes (Roche, UK) using a Roche MagnaLyser instrument. Samples were homogenized for 45 seconds at 6000 rpm x 3 with 5-mins on ice between runs. Protein concentrations were determined using a BCA protein assay, and samples added to the assay diluent provided (100µg/sample). Standards were prepared by serial dilution of a stock protein in the BD CBA Cell Signalling Flex Set. All samples and standards were incubated with the capture beads conjugated to the p38MAPK $^{T180/Y182}$ antibody for 3-h then with the Phycoerythrin (PE) detection reagent for 1-h. Samples were then washed using the wash buffer provided and centrifuged at 300g for 5-mins before supernatant was removed and 300µl of wash buffer was added for re-suspension of the capture beads. All samples were analysed on the flow cytometer with 300 events captured per sample.

RNA isolation and analysis: Muscle biopsy samples (~20 mg) were homogenized in 1ml TRIzol reagent (Thermo Fisher Scientific, UK) in beaded tubes (Roche, UK) using a Roche MagnaLyser instrument. Samples were homogenized for 45 seconds at 6000 rpm x 3 with 5-mins on ice between runs. Total RNA was then isolated according to manufacturer's guidelines. High quality RNA was assessed by UV spectroscopy at ODs of 260 and 280 nm (mean±SD was 1.99±0.05 for 260/280 nm ratios) using a Nanodrop 3000 (Fisher, Rosklide, Denmark). 70 ng RNA was then used for each PCR reaction. Samples were analysed in duplicate.

Primers: Identification of primer sequences was enabled by Gene (NCBI, http://www.ncbi.nlm.nih.gov.gene) and primers 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. In order to prevent amplification of gDNA, primers were ideally designed to yield products spanning exon-exon boundaries. 3 or more GC bases in the last 5 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 non-specific amplification. All primers were between 16 and 25bp, and amplified a product between 141 – 244bp.

All primers were purchased from Sigma (Suffolk, UK) and sequences for each gene are shown in Table 1 for: peroxisome proliferator-activated γ receptor coactivator **(**PGC-1), tumour suppressor protein (p53), mitochondrial transcription factor A (Tfam), pyruvate dehydrogenase kinase isozyme 4 (PDK4), carnitine palmitoyltransferase (CPT1), fatty acid translocase (FAT/CD36), Sirtuin 1 (SIRT1), Parkin, and GAPDH.

Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR): rt-qRT-PCR amplifications were performed using a QuantiFast^{TM} SYBR$^{®}$ Green RT-PCR one step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA, USA). The following rt-qTR-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: 40 cycles of denaturation at 95°C for 10s, and annealing/extension at 60°C for 30s. Upon completion, dissociation/melting curve analysis were performed to reveal and exclude non-specific amplification or primer-dimer issues (all melt analysis presented single reproducible peaks for each target gene suggesting amplification of a single product). Changes in mRNA content were calculated using the comparative C_t ($^{\Delta\Delta}C_t$) equation (Schmittgen and Livak, 2008) where relative mRNA expression was calculated as $2^{-\Delta\Delta ct}$ and where represents the threshold cycle. GAPDH was used as a reference gene and did not change significantly between groups or time points studied (C_t = 24.2±1), therefore a pooled reference gene C_t was used in the relative mRNA expression equation above. Furthermore, to enable calculation of mRNA expression values immediately post and 3-h post exercise, the calibrator condition in the delta delta C_t equation was assigned to the pre-exercise condition.

Statistical analysis: All data were analysed using Statistical Package for the Social Scientist (SPSS version 21, IBM, USA). Metabolic responses (i.e. blood metabolites, muscle glycogen, kinase activity, mRNA data), physiological and perceptual responses (i.e. HR, RPE, and oxidation rates) were analysed using a two-way repeated-measures general linear model, where the within factors were time and condition. Post hoc LSD tests were used where significant main effects and interactions were observed in order 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.

Results

Physiological responses and substrate utilisation during exercise. Comparisons of subject's heart rate, RPE and substrate oxidation during both the AM-HIT and PM-HIT protocols are displayed in Figure 2. Heart rate, RPE and lipid oxidation (all $P<0.001$) progressively increased during both AM-HIT and PM-HIT exercise whereas CHO oxidation progressively decreased ($P<0.001$) during both exercise protocols (see Figure 2). In accordance with identical pre-exercise feeding in all trials prior to AM-HIT, no significant differences were apparent in any of the aforementioned variables between HCHO, LCHF, and LCAL (all $P>0.05$). In contrast, CHO oxidation was significantly greater during PM-HIT in HCHO compared to LCHF ($P=0.001$) and LCAL ($P=0.004$) whereas lipid oxidation was significantly greater during LCHF ($P=0.014$) and LCAL ($P=0.011$) compared to HCHO. No differences were observed in either CHO ($P=0.595$) or lipid ($P=0.403$) oxidation between LCAL and LCHF during PM HIT.

Plasma metabolite responses. Plasma glucose, lactate, NEFA, glycerol and β-hydroxybutyrate all displayed significant changes (all $P<0.001$) over the sampling period (Figure 3). However, in accordance with the provision of CHO in the HCHO trial, plasma glucose was significantly higher compared with LCHF ($P<0.001$) and LCAL ($P=0.003$) whereas restricting CHO and energy intake post-exercise induced significantly greater plasma NEFA and β-OHB in LCHF ($P=0.001$ and 0.001) and LCAL ($P=0.007$ and 0.001) compared with the HCHO trial. There was a tendency for higher circulating NEFA in LCHF compared with LCAL ($P=0.06$) and β-OHB was also different ($P=0.024$) between LCHF and LCAL. Plasma glycerol significantly increased in response to both AM-HIT ($P=0.001$) and PM-HIT ($P<0.001$), although this response was not different between trials ($P=0.362$). Similarly, plasma lactate concentrations were significantly increased in response to AM-HIT ($P<0.001$) and PM-HIT ($P=0.004$), with no differences between feeding conditions ($P=0.383$). Consistent with CHO feeding before and after PM-HIT, plasma insulin concentration was significantly higher (all $P<0.01$) immediately post, 3-h post, and 17-h post-

exercise in HCHO compared with LCHF and LCAL (Figure 3). Additionally, insulin concentration significantly changed over time (P<0.001) such that significant differences from Pre- AM HIT were evident immediately post (P<0.001) and 3-h after completion of the PM HIT session (P=0.024).

Muscle glycogen. Exercise significantly decreased (P<0.01) muscle glycogen immediately post-PM HIT, though no differences were apparent between HCHO, LCHF, and LCAL at this time-point (see Figure 4). However, in accordance with the provision of CHO after the PM HIT exercise protocol in HCHO, muscle glycogen re-synthesis was observed such that glycogen concentration was higher in HCHO compared with both LCHF (P=0.028) and LCAL (P=0.002) at 3-h and 17-h post exercise.

p38MAPK$^{T180/Y182}$ Phosphorylation. Exercise induced significant increases in p38MAPK phosphorylation immediately post PM-HIT (P=0.037) though no differences (P=0.755) were observed between trials (Figure 5). At 17-h post PM-HIT, p38MAPK phosphorylation had returned to basal levels in all three experimental trials.

Gene expression. Exercise increased the expression of PGC-1α (P<0.001), p53 (P=0.01), and CPT1 mRNA (P<0.001) although there were no differences (all P>0.05) between trials (Figure 6). In contrast, the exercise-induced increase (P=0.001) in PDK4 mRNA was greater in LCHF (P=0.002) and LCAL (P=0.034) versus HCHO at 3-h post exercise (Figure 6). Similarly, mRNA expression of SIRT1 was significantly greater in LCAL compared to HCHO (P=0.013) at 17 h -post exercise (Figure 6). In contrast, neither exercise nor dietary condition significantly affected the mRNA expression of CD36 (P=0.901 and P=0.206, respectively), Parkin (P=0.25 and 0.74, respectively) or Tfam (P= 0.17 and P=0.38 respectively).

Bone metabolism. Exercise significantly increased P1NP (P<0.01) following both AM-HIT (P=0.011) and PM-HIT (P=0.005) with concentrations subsequently decreasing at 3-h (P=0.041) and 17-h post-exercise (P=0.002) (Figure 7). No differences were apparent between trials (P=0.633). Exercise significantly reduced βCTX concentration following AM-HIT (P<0.001) whilst PM-HIT significantly increased βCTX (P<0.001) (Figure 6). βCTX concentration was significantly lower

immediately pre-, post- and 3-h post-PM HIT in HCHO compared to LCHF (P=0.032) and LCAL (P=0.035). No differences were apparent in the response of βCTX between the LCHF and LCAL trials (P=0.178).

Appetite hormones. Whilst exercise did not affect circulating leptin concentration (P=0.72), leptin was significantly lower (P=0.041) in LCAL compared to HCHO in the post exercise sampling period (Figure 7). There was a tendency for differences in leptin concentration between HCHO and LCHF (P=0.057) though no differences were apparent between LCAL and LCHF (P=0.27). Exercise significantly reduced ghrelin concentration to comparable levels in all three trials following both AM-HIT (P = 0.001) and PM-HIT (P = 0.025), with no significant differences between conditions (P=0.408) (Figure 7). Ghrelin concentration returned to baseline levels at 17-h post PM-HIT in all conditions (P = 0.392). Neither exercise (P=0.524) nor dietary condition (P=0.156) significantly affected adiponectin concentration throughout the sampling period (Figure 7).

Interleukin-6 (IL-6) responses. Exercise significantly increased IL-6 concentration immediately post PM-HIT (P=0.016) and at 3-h post PM-HIT (P=0.009), the magnitude of which was higher in LCAL compared to LCHF (P=0.021) and HCHO (P=0.016) (Figure 7). There were no differences in post-exercise elevations in IL-6 concentration between LCHF and HCHO (P=0.145). Plasma IL-6 concentration returned to baseline levels at 17-h post-PM-HIT in all three experimental trials.

Discussion

In contrast to our hypothesis, we provide novel data by demonstrating that simultaneous post-exercise CHO and energy restriction did not augment acute cell signalling (i.e. p38MAPK-PGC-1α pathway) that is associated with mitochondrial biogenesis and lipid metabolism, as compared with conditions of high energy availability. We also report for the first time that consuming CHO before, during and after HIT running attenuates circulating βCTX concentrations in the hours after exercise, effects that are independent of energy availability. When taken together, our data suggest that in recovery conditions where muscle glycogen concentration remains within the range of 200-350 mmol.kg^{-1} dw, acute post-exercise CHO and

energy restriction (i.e. <24 hours) does not potentiate the activation of key cell signalling responses that are associated with the regulation of hallmark adaptations to endurance training. Nonetheless, the acute within day fluctuation in CHO availability that is inherent to twice per day training protocols modulates bone resorption, though the chronic implications of these acute alterations remain to be determined.

Similar to the original train-low investigations (Hansen *et al*. 2005; Yeo *et al.* 2008; Morton *et al*. 2009), we employed a twice per day exercise protocol (considered representative of real-world practices often adopted by elite endurance athletes), whereby multiple training sessions are completed on the same day with limited recovery time between sessions (Fudge *et al.* 2006). Having adhered to a high CHO diet during the day prior to all three experimental trials and after consuming a standardised high CHO breakfast, we observed no differences in cardiovascular strain, RPE, substrate availability or substrate oxidation during the AM-HIT session. In accordance with the provision of CHO intake between training sessions during the HCHO trial, we subsequently observed higher rates of CHO oxidation during PM-HIT when compared with both the LCHF and LCAL trials, likely reflective of the higher pre-exercise muscle glycogen availability and the provision of exogenous CHO during exercise (Bartlett *et al.* 2013). Nonetheless, despite higher circulating NEFA levels in LCHF compared with the LCAL trial, we observed comparable lipid oxidation rates between the LHCF and LCAL trials, both of which were significantly higher than HCHO. These data are in agreement with classical studies demonstrating that in high-intensity exercise conditions, elevations in circulating free fatty acid (FFA) availability (i.e. >1 mmol.L^{-1}) does not readily translate to increased rates of whole body lipid oxidation (Romijn *et al*. 1995), owing to limitations in FFA uptake across the mitochondrial membrane during conditions of high glycolytic flux (Sidossis *et al*. 1997).

It is, of course, difficult to draw accurate conclusions on rates of lipolysis based on measurements of circulating glycerol and NEFA per se. Nonetheless, on the basis of statistical differences in plasma NEFA concentrations between trials immediately post- PM-HIT and consistent with the well-documented effects of CHO feeding (Horowitz *et al.* 1997), it is assumed that lipolysis was suppressed in the HCHO trial when compared with both the LCAL and LCHF trials. The fact that plasma glycerol did also not display statistical differences between trials may be potentially reflective

of greater glycerol uptake by the liver in both the LCAL and LCHF trials in order to provide a gluconeogenic substrate to maintain plasma glucose concentration (Ahlborg *et al.* 1974; Jensen *et al.* 2001). Interestingly, the return of β-OHB towards basal levels at +17 h in the LCHF trial is consistent with our previous publication utilising an almost identical dietary and exercise protocol (Hammond *et al.* 2016), whilst the progressive increase in β-OHB at this time-point in the LCAL trial is consistent with the well-documented effects of post-exercise calorie restriction on ketosis (Impey *et al.* 2016). In relation to the former, it is possible that that the provision of dietary fat intake in the evening meal (i.e. 9 pm) may result in an increase in liver uptake of NEFA in an attempt to preferentially stimulate gluconeogenesis (Chen *et al.* 1999), the result of which may actually suppress ketogenesis. We acknowledge, however, that further studies are now required to directly test this hypothesis.

In relation to upstream skeletal muscle cell signalling, the phosphorylation of p38MAPK$^{T180/Y182}$ immediately post PM-HIT was comparable between all trials. Despite the potentially increased pre-exercise muscle glycogen availability and utilisation during HCHO (owing to the consumption of CHO between sessions), such a comparable signalling response is not unexpected given that the absolute post-exercise glycogen concentration (i.e. approximately 200 mmol.kg^{-1} dw) was similar between trials. In accordance with comparable upstream signalling (Wright et al. 2007), we subsequently observed similar magnitudes of mRNA expression of PGC-1α, p53, and CPT1 in the first 3 hours of recovery from PM-HIT. Furthermore, despite the sustained CHO and energy restriction for the subsequent 12-hour recovery period, we observed that neither energy nor CHO availability had no further modulatory effect on mRNA expression (with the exception of both PDK4 and SIRT1). Such data contrast with those of Pilegaard *et al*. (2005) who observed that 8 hours of post-exercise CHO restriction (0.5 g.kg^{-1} of CHO) enhanced PGC-1α, CPT1 and CD36 mRNA when compared with recovery conditions where 5 g.kg^{-1} of CHO was consumed in the initial 8 h post-exercise period. Such discrepancies between studies may be explained by the absolute glycogen concentrations achieved by the chosen feeding protocol. Indeed, whereas we achieved absolute muscle glycogen concentrations of approximately 350 (in the HCHO trial) versus 200 mmol.kg^{-1} dw (in the LCHF and LCAL trials) at 17 hours post-completion of the PM-HIT session, the feeding protocol adopted by Pilegaard *et al*. (2005) achieved glycogen concentrations

of 550 versus 350 mmol.kg^{-1} dw in their conditions of high and low CHO availability, respectively. When considered this way, our data may lend further support for the glycogen threshold hypothesis (Impey *et al*. 2018) surmising that the acute cell signalling (Gejl *et al.* 2017) and chronic training adaptations (Hansen *et al.* 2005; Morton *et al.* 2009; Yeo *et al.* 2008) associated with "train-low" protocols typically only present when absolute glycogen concentration is depleted to <300 mmol.kg^{-1} dw. As such, it is possible that the acute manipulation of both CHO and energy availability in relation to the exercise protocols and participant training status studied here were likely not sufficient to elicit post-exercise metabolic conditions that could be considered indicative of "true" train-low conditions. Indeed, in a previous study from our laboratory where we utilised a sleep low manipulation of muscle glycogen (as achieved via evening glycogen depleting exercise followed by overnight CHO and energy restriction), we observed that differences in muscle glycogen availability of 400 versus 100 mmol.kg^{-1} dw induced marked differences in expression of PGC-1α, CPT1 and Tfam mRNA (Bartlett *et al.* 2013). Future studies should therefore adopt experimental designs and feeding protocols that more readily achieve distinct differences in muscle glycogen availability.

Exercise training is largely considered anabolic to bone, as mediated by the mechanical signalling pathways inherent to the pattern of loading (Ozcivici *et al.* 2010; Thompson *et al.* 2012; Pagnotti et al. 2019). Nonetheless, the extent of increased bone formation and decreased bone resorption that may occur in response to exercise training (Fujimura *et al.* 1997) is, of course, dependent on genetics, age, exercise modality (i.e. loading pattern) and energy availability. In this way, bone mineral density (BMD) may differ markedly amongst elite athletic populations such as soccer players, swimmers, cyclists, runners and jockeys (Olmedillas *et al*. 2012; Gomez-Bruton *et al*. 2013; Fredericson *et al.* 2007; Scofield and Hecht, 2012; Wilson *et al.* 2018). In relation to the acute markers of bone metabolism studied here, we observed comparable responses of P1NP and βCTX following the AM-HIT training session, likely due to the identical CHO intake in both the 24 h control diet and the pre-training breakfast. In contrast, subsequent ingestion of CHO in the 3-h recovery period prior to PM-HIT caused a greater suppression in βCTX compared with those conditions where energy was restricted (LCAL) or matched by consuming the isocaloric high fat diet (LCHF). Similarly, when CHO was ingested during PM-HIT

and in the subsequent 3-h recovery period, there was a greater suppression in βCTX immediately post- and 3-h post-exercise. The apparent suppression of βCTX with CHO feeding agrees with previously reported observations, albeit under resting conditions, where a reduction in βCTX in response to an oral glucose tolerance test has been reported (Bjarnason *et al.* 2002; Bergmann *et al.* 2019). The finding that CHO availability attenuates post-exercise circulating βCTX is also similar to previous researchers who reported that consuming an 8% CHO solution before and during a prolonged bout of running (120-mins at 70% of VO_{2max}) induced a greater reduction in βCTX compared with a placebo solution (Sale *et al.* 2015). Furthermore, de Sousa et al. (2014) also observed a reduction in the bone resorption response (as determined by βCTX) to an acute exercise bout following an 8 day 'overload training program' in individuals fed a higher CHO diet during training. The CHO group consumed an additional 1 g of maltodextrin·kg^{-1} body mass per hour of running during the 8 days of training compared with the control group. When specifically examining the effects of feeding during the post-exercise recovery period, Townsend *et al.* (2017) also demonstrated that consuming a mixed CHO and protein recovery drink immediately following an exhaustive run significantly suppressed βCTX when compared to a placebo solution. When taken together, the present data therefore confirms but extends the previous observations given that we observed no differences in βCTX between the LCHF and LCAL trials, thus demonstrating that it is the energy from CHO, rather than overall energy availability, that is important in attenuating the bone resorption response to an acute training session. In contrast to βCTX, however, we observed no affect of dietary condition on markers of bone formation (i.e. P1NP). Such data contrast with those of Sale *et al.* (2015) who reported that CHO feeding before and during exercise also reduces post-exercise P1NP concentrations. Our data therefore suggest that the influence of CHO availability in the current study was confined (at least within the study timeframe) to bone resorption. We acknowledge, however, the limitation of solely measuring βCTX and P1NP and suggest that future studies may benefit from measurement of additional markers of bone formation and resorption such as that associated with Wnt-β-catenin signalling and the RANKL/RANK/OPG pathway (Ozcivici *et al.* 2010). The assessment of renal function may also prove beneficial given the possibility that changes in renal function as a result of high-intensity exercise and energy restriction may influence the excretion rate of βCTX and hence, its relative concentration.

The potential mechanisms for acute suppression of bone resorption by CHO feeding are still not fully understood, although it has been suggested that the enteric hormones might play a part in mediating the effects of glucose on bone metabolism (Bjarnson *et al*., 2002; Henriksen *et al*., 2003). Indeed, when enteric hormone secretion was limited by somatostatin administration, the reduction in bone resorption following oral glucose ingestion was negated (Clowes et al., 2003). Accordingly, Bergmann et al. (2019) have also recently demonstrated that the gut derived incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), could be involved in regulation of bone resorption during glucose administration, as evidenced by a suppression in βCTX in response to glucose infusion when both hormones are also simultaneously infused. Additionally, specific regulatory effects of insulin (Clowes *et al,* 2002), leptin (Thomas & Burguera, 2002; Holloway *et al.* 2002) and/or IL-6 (Sale *et al*. 2015) signalling have also been postulated. Support for leptin related signalling is also provided by the observation that we also observed higher circulating leptin concentrations in the HCHO trial when compared with both the LCAL (P=0.041) and LCHF trials (albeit P=0.057), an effect that is likely secondary to the hyperinsulinemia induced by CHO feeding (Kolaczynski *et al.* 1996; Gonzalez *et al.* 2019). In relation to IL-6, Sale et al. (2015) reported a strong correlation between changes in IL-6 and βCTX in the post-exercise period and therefore postulated that IL-6 may regulate bone turnover via stimulating osteoclastogenesis and bone resorption. It is noteworthy, however, that although we observed differences in insulin and leptin between trials, circulating IL-6 was not apparently affected by CHO availability. Indeed, the repeated bout of high intensity running in PM-HIT significantly increased circulating IL-6 in all three experimental conditions, the magnitude of which was greater in LCAL compared to LCHF and HCHO. Whilst the effects of muscle glycogen (Steensberg *et al.* 2001; Keller *et al.* 2001; Chan *et al.* 2004) and exogenous (Nieman *et al.*, 1998; Nieman *et al.* 2003; Febbraio *et al.* 2003) CHO availability on the regulation of exercise-induced IL-6 production is well documented, the present data suggest that energy availability plays a more dominant regulatory role in mediating IL-6 responses, as compared with CHO availability *per se*. In contrast, an alternative interpretation is that high fat availability in the LCHF trial (despite also being low in absolute CHO content) may have suppressed IL-6 production to levels that were comparable to the HCHO trial

(Badenhorst *et al*. 2016; Phillips *et al.* 2003; DiLorenzo *et al.* 2014). When taken together, the present data suggest that energy availability (but not CHO availability) is the more dominant factor in modulating components of cytokine production when training under conditions representative of the twice per day train low model.

Whilst the clinical implications of such acute and transient alterations in bone resorption remain to be determined, it would seem sensible for athletes to avoid prolonged periods of low CHO availability in order in to reduce the potentially detrimental effects on bone mass (Ihle & Loucks, 2004) and/or bone injury risk. The findings of the current study might also lend some further support to the requirement for post-exercise feeding (particularly of CHO) in order to attenuate the bone resorption response to intense exercise or high training volumes. That said, this must be carefully considered over time, given that these alterations in bone metabolism are also likely to be an important part of the bone adaptive response, which remains an area requiring significant future research. Given the acute nature of this study, we were, of course, not able to confirm whether the longer-term adherence to the training and dietary regimen studied here would result in morphological changes to bone. Indeed, as discussed previously, we were limited to the use of blood borne markers of bone metabolism and selected the preferred markers of use as reference analytes for bone turnover markers in clinical studies (Vasikaran *et al.*, 2011). Nonetheless, we acknowledge that future studies would benefit from the use of scanning techniques (e.g., DXA, pQCT or HR-pQCT) or bone biopsy analyses to determine whether our chosen exercise and dietary approach does indeed manifest as long-term morphological changes to bone.

In summary, we provide novel data demonstrating that in conditions where post-exercise muscle glycogen concentration is maintained within the range of 200-350 mmol·kg^{-1} dw, short-term periods of acute CHO and energy restriction (*i.e.*, <24 hours) do not potentiate potent skeletal muscle signalling pathways associated with the regulation of mitochondrial biogenesis and lipid metabolism. In addition, promotion of high CHO availability before, during and in recovery from exercise may be of greater importance for the acute regulation on markers of bone metabolism when compared with energy intake *per se.* In relation to skeletal muscle adaptation, future studies should now examine the potential presence of a muscle glycogen threshold as an important regulator of adaptations to endurance training. Additionally,

the long-term implications (in relation to other physiological systems) of the acute within day fluctuations in both CHO and energy availability inherent to chronic training twice per day should also be examined, which would also allow the determination of any effects on bone mass and structure using bone scanning techniques.

Additional Information Section:

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Competing Interests

JPM is a consultant for Science in Sport (SiS). SiS, GlaxoSmithKline (GSK) and Lucozade Ribena Suntory (LRS) have funded his previous research on glycogen metabolism and exercise.

Author Contributions

KMH, CS, CSt, APS, JPM: Substantial contributions to the conception or design of the work; All authors: the acquisition, analysis, or interpretation of data for the work; KMH, CS, CSt, APS and JPM: Drafting the work or revising it critically for important intellectual content; All authors: Final approval of the version to be published; All authors: Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved**.**

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Table 1 - Primer sequences.

Figure 1 – Overview of the experimental protocol employed in each trial. HIT = 8 x 5-mins running at a workload equal to 85% VO_{2peak} interspersed by 1-min recovery.

Figure 2 Heart rate (A&B), CHO oxidation (C&D), lipid oxidation (E&F), and RPE (G&H) responses during each HIT bout in the HCHO, LCHF, and LCAL trials. * denotes significant difference from HIT bout 1 ($P<0.05$), # denotes significant difference between HCHO and LCHF ($P<0.05$), $ denotes significant difference between HCHO and LCAL ($P<0.05$).

Figure 3 Plasma glucose (A), lactate (B), NEFA (C), Glycerol (D), β-OHB (E), and insulin (F) before and after the AM-HIT and PM-HIT protocols. * denotes significant difference from pre-exercise ($P<0.05$), # denotes significant difference between HCHO and LCHF ($P<0.05$), $ denotes significant difference between HCHO and LCAL ($P<0.05$).

Figure 4 Skeletal muscle glycogen concentration before the AM-HIT and after the PM-HIT exercise protocols. * denotes significant difference from pre-exercise ($P<0.05$), # denotes significant difference from HCHO ($P<0.05$).

Figure 5 Skeletal muscle phosphorylated p38MAPK$^{T180/Y182}$ before the AM-HIT and after the PM-HIT exercise protocols. * denotes significant difference from pre-exercise ($P<0.05$).

Figure 6 (A) PGC-1 α, (B) p53, (C) SIRT1, (D) Tfam, (E) CPT1, (F) CD36, (G) PDK4, and (H) Parkin mRNA before AM-HIT exercise and after PM-HIT exercise. * denotes significant difference from pre-exercise ($P<0.05$), # denotes significant difference from HCHO and LCHF ($P<0.05$), $ denotes significant difference from HCHO ($P<0.05$).

Figure 7 Plasma (A) P1NP, (B) βCTX, (C) leptin, (D) ghrelin, (E) IL-6, and (F) adiponectin before and after the AM-HIT and PM-HIT protocols. * denotes significant difference from pre-exercise ($P<0.05$), # denotes significant difference between HCHO and LCHF ($P<0.05$), $ denotes significant difference between HCHO and LCAL ($P<0.05$), and **a** denotes significant difference between LCHF and LCAL ($P<0.05$).