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RESEARCH ARTICLE

Graded reductions in preexercise muscle glycogen impair exercise capacity but do not augment skeletal muscle cell signaling: implications for CHO periodization

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Hearris MA, Hammond KM, Seaborne RA, Stocks B, Shepherd SO, Philp A, Sharples AP, Morton JP, Louis JB. Graded reductions in preexercise muscle glycogen impair exercise capacity but do not augment skeletal muscle cell signaling: implications for CHO periodization. *J Appl Physiol* 126: 1587–1597, 2019. First published May 2, 2019; doi:10.1152/japplphysiol.00913.2018.—We examined the effects of graded muscle glycogen on exercise capacity and modulation of skeletal muscle signaling pathways associated with the regulation of mitochondrial biogenesis. In a repeated-measures design, eight men completed a sleep-low, train-low model comprising an evening glycogen-depleting cycling protocol followed by an exhaustive exercise capacity test [8 × 3 min at 80% peak power output (PPO), followed by 1-min efforts at 80% PPO until exhaustion] the subsequent morning. After glycogen-depleting exercise, subjects ingested a total of 0 g/kg (L-CHO), 3.6 g/kg (M-CHO), or 7.6 g/kg (H-CHO) of carbohydrate (CHO) during a 6-h period before sleeping, such that exercise was commenced the next morning with graded ($P < 0.05$) muscle glycogen concentrations (means ± SD: L-CHO: 88 ± 43, M-CHO: 185 ± 62, H-CHO: 278 ± 47 mmol/kg dry wt). Despite differences ($P < 0.05$) in exercise capacity at 80% PPO between trials (L-CHO: 18 ± 7, M-CHO: 36 ± 3, H-CHO: 44 ± 9 min), exercise induced comparable $AMPK^{Thr172}$ phosphorylation (~4-fold) and PGC-1α mRNA expression (~5-fold) after exercise and 3 h after exercise, respectively. In contrast, neither exercise nor CHO availability affected the phosphorylation of $p38MAPK^{Thr180/Tyr182}$ or $CaMKII^{Thr268}$ or mRNA expression of p53, Tfam, CPT-1, CD36, or PDK4. Data demonstrate that when exercise is commenced with muscle glycogen < 300 mmol/kg dry wt, further graded reductions of 100 mmol/kg dry weight impair exercise capacity but do not augment skeletal muscle cell signaling.

NEW & NOTEWORTHY We provide novel data demonstrating that when exercise is commenced with muscle glycogen below 300 mmol/kg dry wt (as achieved with the sleep-low, train-low model) further graded reductions in preexercise muscle glycogen of 100 mmol/kg dry wt reduce exercise capacity at 80% peak power output by 20–50% but do not augment skeletal muscle cell signaling.

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exercise capacity; mitochondrial biogenesis; muscle glycogen; train low, sleep low

INTRODUCTION

Skeletal muscle glycogen is recognized as the predominant energy substrate used during endurance exercise (14) and plays an important role in regulating the capacity to sustain exercise at a given workload (4, 13, 16). Additionally, muscle glycogen acts as a regulatory molecule (32) that is able to modulate cell signaling and transcriptional responses to exercise and subsequently augment selected skeletal muscle markers of training adaptation [e.g., succinate dehydrogenase (29), citrate synthase (12), and β-hydroxyacyl-CoA dehydrogenase (18) enzyme activity and cytochrome-*c* oxidase subunit IV content (47)]. Most notably, exercise commenced with reduced muscle glycogen [as defined as a "train-low" session (40)] augments the AMP-activated protein kinase (AMPK)-peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) signaling axis (3, 35, 39, 46) and results in the augmented expression of target genes with putative roles in the regulation of mitochondrial biogenesis and substrate utilization (3, 25, 33). Although a multitude of research designs have been used to study the physiological and molecular responses to train-low exercise, the recently developed "sleep-low, train-low" model [which requires athletes to perform an evening training session, restrict carbohydrate (CHO) during overnight recovery, and then complete a fasted training session the subsequent morning] provides a potent strategy to augment mitochondria-related cell signaling (3, 5, 25). Furthermore, repeated bouts of sleep-low, train-low is the only train-low model shown to enhance performance in trained endurance athletes (27, 28).

Given that the enhanced training response associated with the sleep-low, train-low model is potentially regulated by muscle glycogen availability, it is prudent to consider the absolute glycogen concentrations required to facilitate this response. In this regard, examination of available data demonstrates that the augmented signaling and transcriptional responses associated with train-low models are particularly apparent when absolute preexercise muscle glycogen concentra-

tions are ≤300 mmol/kg dry wt (20). Such data suggest the presence of a muscle glycogen threshold, whereby a critical absolute level of glycogen must be surpassed to induce the augmented cell signaling responses associated with the train-low model (33). In accordance with data derived from acute exercise protocols, the notion of a glycogen threshold is also apparent when investigating selected skeletal muscle markers of training adaptation (20). For example, train-low sessions commenced with glycogen concentrations < 300 mmol/kg dry wt (12, 29, 47) result in augmented oxidative enzyme activity and/or content after 3–10 wk of training. In contrast, when train-low sessions are commenced with markedly higher preexercise muscle glycogen concentrations (400–500 mmol/kg dry wt) skeletal muscle markers of training adaptation are not augmented (11). Nonetheless, although training with low muscle glycogen augments selected signaling events, absolute training volume (19) and/or intensity (18, 24, 47) may be reduced because of a lack of muscle substrate and/or an impairment in the contractile apparatus of skeletal muscle (10, 30). The challenge that exists is to therefore facilitate the prosignaling environment while simultaneously maintaining the ability to complete the desired workload and intensity in order to promote training adaptation.

With this in mind, the aim of the present study was to examine the effects of graded preexercise glycogen concentrations on both exercise capacity and the modulation of selected skeletal muscle signaling pathways with putative roles in the regulation of mitochondrial biogenesis. Our model of graded preexercise muscle glycogen was achieved through a sleep-low, train-low model that adopted CHO intakes considered practically viable (within the time course of sleep-low designs) and representative of real-world refeeding strategies. Although the use of such sleep-low, train-low models is primarily designed for athletic populations, the use of recreational populations allows for a greater understanding of the molecular events that occur in response to such train-low designs, given the difficulties of collecting muscle biopsies from elite athletes. We hypothesized that the activation of skeletal muscle signaling pathways would be proportionally dependent on preexercise muscle glycogen concentrations.

METHODS

Participants

Eight recreationally active men (means ± SD: age 22 ± 3 yr; body mass 76.0 ± 12.7 kg; height 177.9 ± 5.7 cm) took part in this study. Mean peak oxygen consumption ($\dot{V}O_{2peak}$) and peak power output (PPO) for the cohort were 48.9 ± 7.0 ml·kg^{-1}·min^{-1} and 273 ± 21 W, respectively. None of the subjects had any history of musculoskeletal or neurological disease or was under any pharmacological treatment during the course of the testing period. All subjects provided written informed consent, and all procedures conformed to the standards set by the Declaration of Helsinki (2008). The study was reviewed and approved by the local Research Ethics Committee of Liverpool John Moores University.

Experimental Design

With a sleep-low, train-low model and a repeated-measures design, with each experimental trial separated by a minimum of 7 days, subjects undertook an evening bout of glycogen depletion exercise followed by the consumption of graded quantities of CHO [low CHO (L-CHO): 0 g/kg; medium CHO (M-CHO): 3.6 g/kg; high CHO (H-CHO): 7.6 g/kg] across a 6-h period, so that exhaustive exercise was commenced the next morning with three different levels of preexercise muscle glycogen concentrations. Skeletal muscle biopsies were obtained from the vastus lateralis immediately before, after, and 3 h after exercise. During the H-CHO and M-CHO trials, an additional muscle biopsy was obtained at a matched time point corresponding to the point of exhaustion in the L-CHO trial, allowing for work-matched comparison between trials. Consequently, all subjects completed the L-CHO trial first, whereas the subsequent M-CHO and H-CHO trials were completed in a randomized and counterbalanced order. An overview of the experimental protocol is shown in Fig. 1.

Assessment of Peak Oxygen Uptake

At least 7 days before experimental trials, all subjects were assessed for $\dot{V}O_{2peak}$ and PPO on an electronically braked cycle ergometer (Excalibur Sport; Lode, Groningen, The Netherlands). After completion of a 10-min warm-up at 75 W, the test began at 100 W and consisted of 2-min stages with 30-W increments in resistance until volitional exhaustion. $\dot{V}O_{2peak}$ was considered as being achieved by the following end-point criteria: *1*) heart rate (HR) within 10 beats/min of age-predicted maximum, *2*) respiratory exchange ratio > 1.1, and *3*) plateau of oxygen consumption despite increased workload. Peak aerobic power was taken as the final stage completed during the incremental test.

Overview of Sleep-Low, Train-Low Model

Phase 1: Glycogen depletion exercise. In the 24 h preceding glycogen-depleting exercise (i.e., from 12 PM the day prior), subjects consumed a standardized high-CHO diet (8 g/kg CHO, 2 g/kg protein, and 1 g/kg fat), having refrained from alcohol and vigorous physical exercise for the previous 48 h. The standardized diet consisted of three main meals and three CHO-rich snacks, with subjects required to stop eating 3 h before commencing glycogen-depleting exercise. On the day of glycogen-depleting exercise, subjects reported to the laboratory at ~3 PM to perform a bout of intermittent glycogen-depleting cycling, as previously completed in our laboratory (19, 43). The pattern of exercise and total time to exhaustion in the subject's initial trial were recorded and replicated in all subsequent trials. Subjects were permitted to consume water ad libitum during exercise, with the pattern of ingestion replicated during subsequent trials.

Phase 2: Carbohydrate refeeding strategy. To facilitate our overnight sleep-low model, subjects were fed 30 g of whey protein isolate (Science in Sport, Nelson, UK) mixed with 500 ml of water immediately after the cessation of glycogen-depleting exercise to reflect real-world practice as per current nutritional guidelines (44). Subjects in the L-CHO trial then refrained from eating for the remainder of the evening, whereas subjects in the M-CHO and H-CHO trials were provided with a mixture of CHO drinks (maltodextrin; Science in Sport) and gels (GO isotonic energy gel; Science in Sport) to be consumed at hourly intervals. In the M-CHO trial subjects were provided with CHO at a rate of 1.2 g·kg^{-1}·h^{-1} for 3 h, whereas in the H-CHO trial subjects were provided with 1.2 g·kg^{-1}·h^{-1} for 3 h followed by a high-CHO meal (4 g/kg CHO, 51 ± 1 g protein, and 17 ± 1 g fat) consisting of bread, soup, rice, fresh juice, rice pudding, and jam after 4 h of recovery. In this way, total CHO intakes in the L-CHO, M-CHO, and H-CHO trials equated to 0, 3.6 g/kg, and 7.6 g/kg, respectively, with fluid intake allowed ad libitum.

Phase 3: High-intensity interval cycling and exercise capacity test. To facilitate our train-low exercise session, subjects arrived the subsequent morning between 8 and 9 AM in a fasted state, and a venous blood sample was collected from the antecubital vein and a muscle biopsy taken from the vastus lateralis. Subjects then completed the high-intensity interval (HIIT) cycling protocol, consisting of 8 × 3-min intervals at 80% PPO, interspersed with 1-min rest. During exercise, HR was continuously measured and the final HR for each

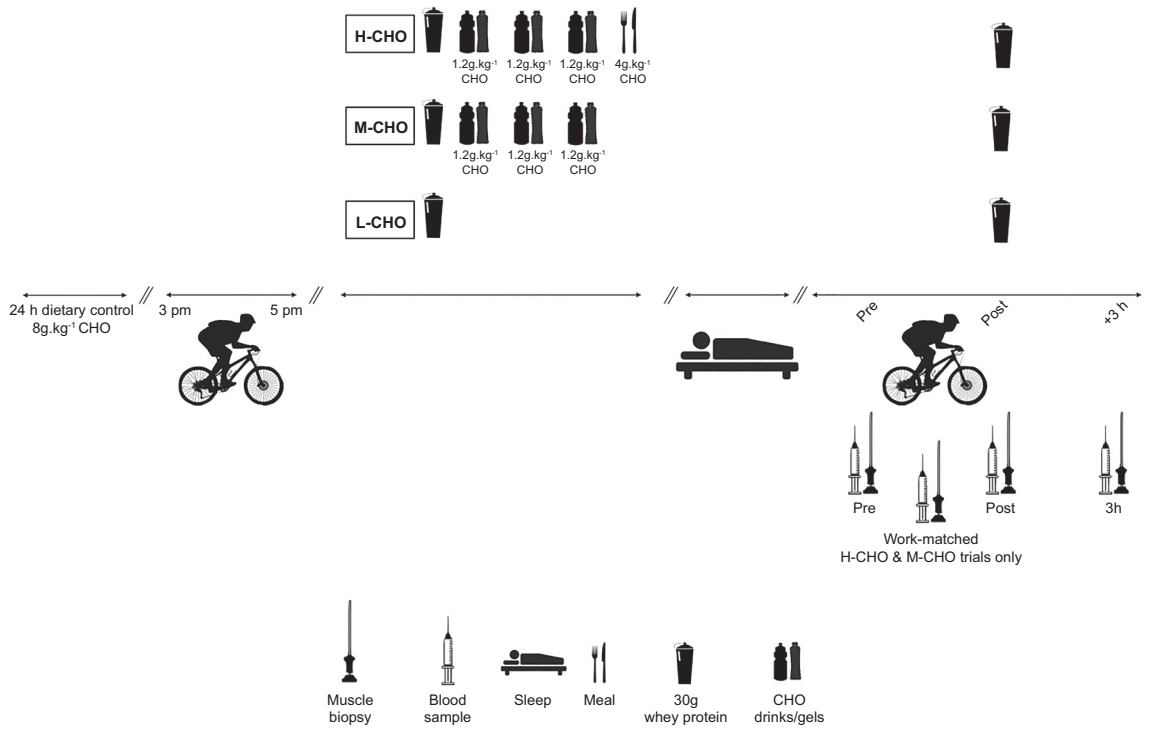

Fig. 1. Schematic overview of the experimental sleep-low, train-low protocol. After 24 h of standardized dietary conditions, subjects completed an evening bout of glycogen-depleting cycling exercise. Upon completion, subjects received 3 graded levels of carbohydrate [high (H-CHO), medium (M-CHO), low (L-CHO)] in order to manipulate preexercise muscle glycogen the subsequent morning. After an overnight fast, subjects completed an exhaustive bout of cycling exercise. Muscle biopsies were obtained before exercise (Pre), at the point of exhaustion [after exercise (Post)], and 3 h after exercise (3 h). During H-CHO and M-CHO trials, an additional muscle biopsy was obtained at a time point corresponding to the point of exhaustion in the L-CHO trial, allowing for work-matched comparison between trials.

3-min interval was recorded, whereas ratings of perceived exertion (RPE) were recorded upon completion of each interval. Expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima; Medgraphics) for the final 1.5 min of each interval, and substrate utilization was assessed with the equations of Jeukendrup and Wallis (22) given the validity of indirect calorimetry for the assessment of substrate utilization at exercise intensities up to 80–85% maximum oxygen consumption ($\dot{V}O_{2max}$) (37). Upon completion of the high-intensity cycling protocol, subjects were provided with 5 min of active recovery before commencing an exercise capacity test consisting of intermittent "1-min efforts" corresponding to 80% PPO interspersed with 1-min recovery periods at 40% PPO. This intermittent protocol was followed until the subjects reached volitional exhaustion and has been utilized previously in our laboratory (19). After the completion of the exercise capacity test and collection of the postexercise biopsy, subjects were fed 30 g of whey protein (Science in Sport) mixed with 500 ml of water.

Blood Analysis

Venous blood samples were collected in vacutainers containing K_2EDTA, lithium heparin, or serum separation tubes and stored on ice or at room temperature until centrifugation at 1,500 *g* for 15 min at 4°C. Samples were collected immediately before exercise, at the point of exhaustion (postexercise) and 3 h after exercise, and an additional sample was obtained at a time point during the M-CHO and H-CHO trials corresponding to the point of exhaustion in the L-CHO trial. Plasma was divided into aliquots and stored at −80°C until analysis. Samples were later analyzed for plasma glucose, lactate, nonesterified fatty acids (NEFA), and glycerol with commercially available enzymatic spectrophotometric assays (RX daytona analyzer; Randox Laboratories, Crumlin, UK) per manufacturer's instructions.

Muscle Biopsies

Skeletal muscle biopsies (~20 mg) were obtained from the vastus lateralis immediately before exercise, at the point of exhaustion (postexercise), and 3 h after exercise. During the M-CHO and H-CHO trials, an additional muscle biopsy was obtained at a time point corresponding to the point of exhaustion in the L-CHO trial, thereby allowing for "work-matched" comparison between trials. For the work-matched biopsy, subjects dismounted the cycle ergometer and were moved to the adjacent biopsy suite. After collection of the biopsy sample (~5 min), subjects recommenced cycling exercise. Muscle biopsies were obtained from separate incision sites 2–3 cm apart with a Bard Monopty Disposable Core Biopsy Instrument (12 gauge × 10-cm length; Bard Biopsy Systems, Tempe, AZ) under local anesthesia (0.5% Marcaine), immediately frozen in liquid nitrogen, and stored at −80°C for later analysis.

Muscle Glycogen Concentration

Muscle glycogen concentrations were determined by the acid hydrolysis method described by van Loon et al. (26). Approximately 2–5 mg of freeze-dried tissue was powdered, dissected of all visible blood and connective tissue, and subsequently hydrolyzed by incubation in 500 μl of 1 M HCl for 3 h at 95°C. After cooling to room temperature, samples were neutralized by the addition of 250 μl of 0.12 mol/l Tris-2.1 mol/l KOH saturated with KCl. After centrifugation, 200 μl of supernatant was analyzed in duplicate for glucose concentration by the hexokinase method with a commercially available kit (GLUC-HK; Randox Laboratories). Glycogen concentration is expressed as millimoles per kilogram of dry weight, and intraassay coefficients of variation were <5%.

RNA Isolation and Analysis

Muscle samples (~20 mg) were homogenized in 1 ml of TRIzol reagent (Thermo Fisher Scientific), and total RNA was isolated according to the manufacturer's guidelines. Concentrations and purity of RNA were assessed by UV spectroscopy at optical densities of 260 and 280 nm with a Nanodrop 3000 (Fisher, Roskilde, Denmark) with an average 260 nm-to-280 nm ratio of 1.9 ± 0.1. A quantity of 70 ng of RNA was used for each 20-μl PCR reaction.

Reverse Transcriptase Quantitative Real-Time Polymerase Chain Reaction

Reverse transcriptase quantitative real-time polymerase chain reaction (RT-PCR) amplifications were performed with a QuantiFast SYBR Green RT-PCR one-step kit on a Rotogene 300Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA). The primers used are listed in Table 1. RT-PCR was performed as follows: hold at 50°C for 10 min (reverse transcription/cDNA synthesis), 95°C for 5 min (transcriptase inactivation and initial denaturation step), and PCR steps of 40 cycles of 95°C for 10 s (denaturation), 60°C for 30 s (annealing and extension). Upon completion, dissociation/melting curve analyses were performed to reveal and exclude nonspecific amplification or primer-dimer issues (all melt analysis in this study presented single reproducible peaks for each target gene suggesting amplification of a single product). After initial screening of suitable reference genes, GAPDH showed the most stable threshold cycle (C_t) values across all RT-PCR runs and subjects, regardless of experimental condition (25.3 ± 1.0) and was therefore selected as the reference gene in all RT-PCR assays. The average PCR efficiency for all RT-PCR runs (90 ± 2%) was similar for all genes across all time points and experimental conditions. As such, the relative gene expression levels were calculated with the comparative C_t ($\Delta\Delta C_t$) equation (38), where relative expression was calculated as $2^{-\Delta\Delta C_t}$. mRNA expression for all target genes was calculated relative to the reference gene (GAPDH) within the same subject and condition and relative to the preexercise value in the H-CHO condition.

SDS-PAGE and Western Blotting

Muscle samples (~20 mg) were powdered on dry ice and homogenized (FastPrep-24 5G Instrument) for 2 × 40 s at 6 m/s in a 10-fold mass of ice-cold lysis homogenization buffer [in mM: 20 Na-pyrophosphate, 150 NaCl, 50 HEPES (pH 7.5), 20 β-glycerophosphate, 10 NaF, 1 EDTA (pH 8.8), 1 EGTA (pH 8.8), 3 benzamidine, and 1 1,4-dithiothreitol, with 10% glycerol, 1% NP-40, 1% Phosphatase Inhibitor Cocktail 2 (Sigma), 1% Phosphatase Inhibitor Cocktail 3 (Sigma), and 4.8% complete Mini Protease Inhibitor Cocktail (Roche)]. The resulting homogenate was centrifuged at 4°C for 10 min at 8,000 *g*, and the supernatant was used for the determination of protein concentrations with the DC protein assay (Bio-Rad). Samples were resuspended in 4× Laemmli buffer, boiled for 5 min, and separated by SDS-PAGE before being transferred to nitrocellulose membranes (Pall Life Sciences, Pensacola, FL). After transfer, membranes were stained for protein with Ponceau S (Sigma-Aldrich, Gillingham, UK), blocked in TBS-Tween containing 3% nonfat milk for 1 h, and incubated overnight in primary antibodies [AMPKα (no. 2603), phospho (p)-AMPK^{Thr172} (no. 2531), acetyl-CoA carboxylase (ACC; no. 3676), p-ACC^{Ser79} (no. 3661), p38MAPK (no. 9212), p-p38MAPK$^{Thr180/Tyr182}$ (no. 4511), CaMKII (no. 3362), and p-CaMKII^{Thr268} (no. 12716) from Cell Signaling Technologies] before incubation in relevant secondary antibodies [anti-rabbit (no. 7074) from Cell Signaling Technologies] for 1 h at room temperature. Proteins were detected via chemiluminescence (Millipore, Watford, UK) and quantified by densitometry with GeneTools software (Syngene, Cambridge, UK). Sufficient muscle was available for Western blot analysis for seven subjects. Data are reported as the phosphorylated protein of interest normalized to total protein, and each time point is reported relative to the preexercise value in the H-CHO condition.

Statistical Analysis

All statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS version 24). Comparisons of average physiological responses and exercise capacity were analyzed with a one-way repeated-measures general linear model, whereas changes in physiological and molecular responses between conditions (i.e., muscle glycogen, mRNA expression, and activity of signaling molecules) were analyzed with a two-way repeated-measures general linear model, where the within factors were time and condition. Here the postexercise sampling point in the L-CHO trial was also used as the work-matched sampling point, as this corresponded to the same sampling point and allowed for comparison between trials. Where a significant main effect was observed, pairwise comparisons were analyzed according to Bonferroni post hoc tests to locate specific differences. All data in text, figures, and table are presented as means ± SD, with P values ≤ 0.05 indicating statistical significance.

RESULTS

Skeletal Muscle Glycogen and Exercise Capacity

The exercise and nutritional strategy employed was successful in achieving graded levels of preexercise muscle glycogen (H-CHO 278 ± 47, M-CHO 185 ± 62, L-CHO 88 ± 43 mmol/kg dry wt) such that exercise was commenced with three distinct levels of muscle glycogen ($P = 0.016$) (Fig. 2*A*). Exhaustive exercise significantly reduced ($P < 0.001$) muscle glycogen concentration to comparable levels (<100 mmol/kg dry wt), with no difference between conditions ($P = 0.11$). In accordance with the observed differences in preexercise muscle glycogen concentration, total exercise time spent at 80% PPO in the H-CHO trial (44 ± 9 min) was significantly greater than that in both M-CHO (36 ± 3 min) ($P = 0.037$) and L-CHO (18 ± 6 min) ($P < 0.001$) trials, while that in the M-CHO trial was significantly greater than in the L-CHO trial ($P < 0.001$) (Fig. 2*B*). Given the low preexercise muscle glycogen concentration of subjects in the L-CHO trial, six of the eight subjects were unable to complete the prescribed HIIT

Table 1. *Primers used for real-time RT-PCR*

Gene	Forward Primer	Reverse Primer
PGC-1	TGCTAAACGACTCCGAGAA	TGCAAAGTTCCCTCTCTGCT
p53	ACCTATGGAAACTACTTCCTGAAA	CTGGCATTCTGGGAGCTTCA
Tfam	TGGCAAGTTGTCCAAAGAAACCTGT	GTTCCCTCCAACGCTGGGCA
CD36	AGGACTTTCCTGCAGAATACCA	ACAAGCTCTGGTTCTTATTCACA
PDK4	TGGTCCAAGATGCCTTTGAGT	GTTGCCCGCATTGCATTCTT
CPT1	GACAATACCTCGGAGCCTCA	AATAGGCCTGACGACACCTG
GAPDH	AAGACCTTGGGCTGGGACTG	TGGCTCGGCTGGCGAC

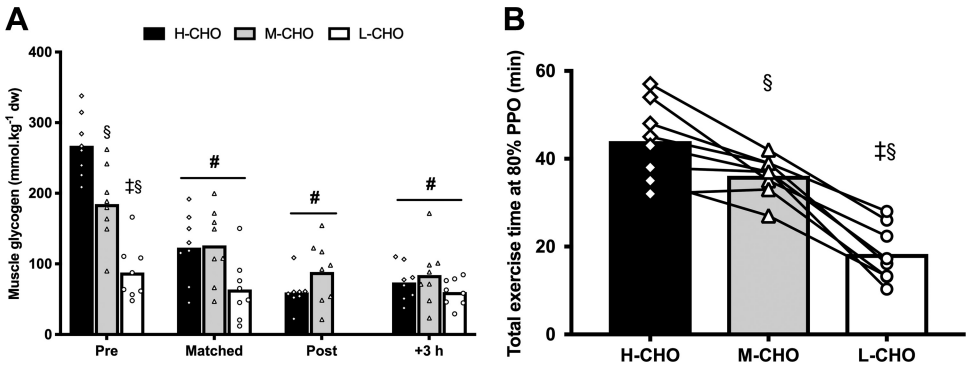

Fig. 2. *A*: skeletal muscle glycogen concentration. dw, Dry weight. *B*: exercise capacity at 80% peak power output (PPO) (reflective of set work protocol plus time to exhaustion). H-CHO, high carbohydrate; M-CHO, medium carbohydrate; L-CHO, low carbohydrate; Pre, preexercise; Matched, work-matched time points; Post, point of exhaustion; +3 h, 3 h after exercise. Data are presented as means, and individual data points represent individual subjects ($n = 8$). $^{\#}P < 0.05$, significantly different from Pre; §$P < 0.05$, significantly different from H-CHO; ‡$P < 0.05$, significantly different from M-CHO.

protocol. Therefore, exercise capacity data are presented as the total amount of time spent at 80% PPO, are inclusive of the exercise performed during the prescribed HIIT protocol and subsequent capacity test, and exclude all time spent at rest/recovery.

Physiological and Metabolic Responses to Exercise

Subject s average HR (Fig. 3*A*) across the HIIT session, when matched for work done (H-CHO 163 ± 16, M-CHO 167 ± 15, L-CHO 171 ± 17 beats/min), was significantly higher in the L-CHO trial compared with H-CHO ($P = 0.031$) only. Similarly, subjects average RPE (Fig. 3*B*) across the HIIT session (H-CHO 13 ± 1, M-CHO 14 ± 1, L-CHO 16 ± 1 arbitrary units) was significantly higher in the L-CHO trial compared with both M-CHO ($P = 0.041$) and H-CHO ($P = 0.012$) trials. Exhaustive exercise resulted in a significant reduction in plasma glucose ($P = 0.036$), where plasma glucose was significantly lower in the L-CHO trial compared with the H-CHO trial only ($P = 0.015$) (Fig. 3*C*). Exhaustive exercise resulted in a significant increase in plasma lactate ($P = 0.001$), NEFA ($P < 0.001$), and glycerol ($P = 0.012$) but did not display any significant differences between trials (Fig. 3, *D–F*, respectively). However, when matched for work done, plasma NEFA ($P = 0.01$) and plasma glycerol ($P = 0.017$) were increased to a significantly greater extent in the L-CHO trial compared with the H-CHO trial only. In addition, subjects in the L-CHO trial oxidized significantly less CHO ($P = 0.048$) and greater amounts of lipid ($P = 0.004$) compared with the H-CHO trial only (Fig. 3, *G* and *H*, respectively).

Regulation of Mitochondrial Biogenesis-Related Cell Signaling

Exhaustive exercise induced significant increases in $AMPK^{Thr172}$ phosphorylation ($P = 0.017$) but did not display any significant differences between trials ($P = 0.548$) (Fig. 4*A*). Similarly, exhaustive exercise induced significant increases in ACC^{Ser79} phosphorylation ($P = 0.005$), although phosphorylation was higher in the M-CHO trial compared with the L-CHO trial only ($P = 0.021$) (Fig. 4*B*). When exercise duration was matched to the postexercise sampling point in the L-CHO group, the increase in $AMPK^{Thr172}$ phosphorylation remained comparable between groups ($P = 0.269$) and the increase in ACC^{Ser79} phosphorylation still remained higher in the M-CHO trial compared with the L-CHO trial ($P = 0.021$). In contrast, exhaustive exercise did not induce phosphorylation of $p38MAPK^{Thr180/Tyr182}$ ($P = 0.656$) (Fig. 4*C*) or $CaMKII^{Thr286}$ ($P = 0.707$) (Fig. 4*D*). Representative Western blots are shown in Fig. 4*E*. With regard to exercise-induced gene expression, exhaustive exercise induced a significant increase in PGC-1α mRNA expression at 3 h after exercise ($P = 0.001$) but did not display any significant differences between trials (Fig. 5*A*). In contrast, p53, transcription factor A, mitochondrial (Tfam), carnitine palmitoyltransferase 1 (CPT-1), CD36, and pyruvate dehydrogenase kinase 4 (PDK4) mRNA expression (Fig. 5, *B–F*, respectively) was unaffected by either glycogen availability or the exhaustive exercise protocol ($P > 0.05$).

DISCUSSION

Using a sleep-low, train-low model, we examined the effects of three distinct levels of preexercise muscle glycogen on exercise capacity and the modulation of selected skeletal muscle signaling pathways with putative roles in mitochondrial biogenesis. We provide novel data by demonstrating that *1*) graded reductions in preexercise muscle glycogen of 100 mmol/kg dry wt reduce exercise capacity at 80% PPO by ~20–50% and *2*) despite significant differences in preexercise muscle glycogen availability, we observed comparable increases in $AMPK^{Thr172}$ phosphorylation and PGC-1α mRNA. In contrast to our hypothesis, these data suggest that graded levels of muscle glycogen < 300 mmol/kg dry wt do not augment skeletal muscle cell signaling, a finding that may be related to the fact that commencing exercise with <300 mmol/kg dry wt is already a critical level of absolute glycogen [as suggested by Impey et al. (20)] that is required to induce a metabolic milieu conducive to cell signaling. In relation to the goal of promoting cell signaling, our data therefore suggest that reducing preexercise glycogen concentrations below 300 mmol/kg dry wt does not confer any additional benefit within the context of the sleep-low, train-low model.

To achieve our intended model of graded glycogen concentrations, we adopted a sleep-low, train-low design in which subjects performed an evening bout of glycogen-depleting exercise and subsequently ingested three graded quantities of CHO that were practically viable within the time course of the sleep-low model. This strategy was effective in achieving graded differences in preexercise muscle glycogen concentration (278 vs. 185 vs. 88 mmol/kg dry wt in H-CHO, M-CHO, and L-CHO, respectively) and represents muscle glycogen resynthesis rates (~30 $mmol \cdot kg^{-1} \cdot h^{-1}$) commonly observed with CHO feeding rates of 1–1.2 $g \cdot kg^{-1} \cdot h^{-1}$ (21). A novel aspect of our chosen study design was that we employed a

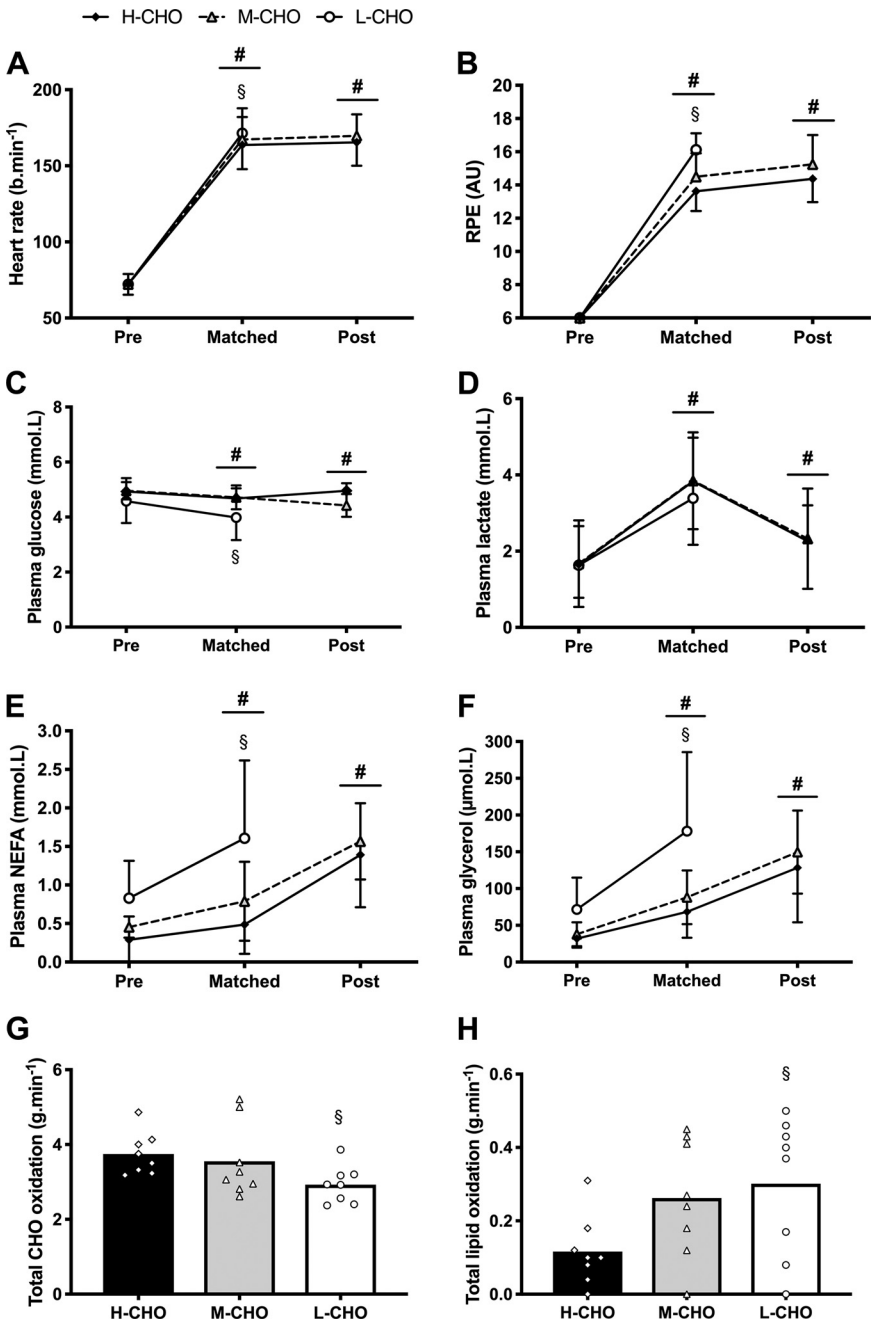

Fig. 3. *A–F*: heart rate (*A*), rating of perceived exertion [RPE, arbitrary units (AU); *B*], and plasma glucose (*C*), lactate (*D*), nonesterified fatty acids (NEFA; *E*), and glycerol (*F*) before exercise (Pre), at work-matched time points (Matched), and after exercise (Post). *G* and *H*: average carbohydrate (CHO; *G*) and lipid (*H*) oxidation during exercise. H-CHO, high CHO; M-CHO, medium CHO; L-CHO, low CHO. $^{\#}P < 0.05$, significantly different from Pre; §$P < 0.05$, significantly different from H-CHO. Data are presented as means ± SD (*A–F*) and as means and individual data points representing individual subjects ($n = 8$) (*G* and *H*).

sampling point in both the H-CHO and M-CHO trials that was matched to the point of exhaustion in the L-CHO trial, thus allowing for the assessment of mitochondria-related signaling events at both work-matched and exhaustive exercise time points while also allowing for the assessment of exercise capacity. In accordance with differences in muscle glycogen, both NEFA availability and lipid oxidation were greater in the L-CHO trial compared with the H-CHO trial at the work-matched sampling point. However, at the point of exhaustion, plasma NEFA and glycerol were comparable between all conditions, which is likely reflective of the postexercise muscle glycogen concentrations in all three conditions given the well-documented effects of muscle glycogen (2) on substrate utilization during exercise.

Consistent with the well-documented effects of muscle glycogen on exercise capacity (4, 16) we observed that even small differences in preexercise muscle glycogen concentration (~100 mmol/kg dry wt) can induce changes in exercise capacity at 80% PPO of between ~20% and 50% (8–18 min). Although we acknowledge that the lack of blinding to each experimental condition may have influenced exercise capacity (despite subjects receiving no feedback during exercise), it is unclear whether prior knowledge of CHO intake alone would enhance exercise performance (17). Nonetheless, these data are consistent with previous data (1, 6) that suggest that differences in muscle glycogen of 100–120 mmol/kg dry wt enhance exercise capacity at 70% $\dot{V}O_{2max}$ by 5–12 min. Therefore, the 8-min difference in exercise capacity between M-CHO and

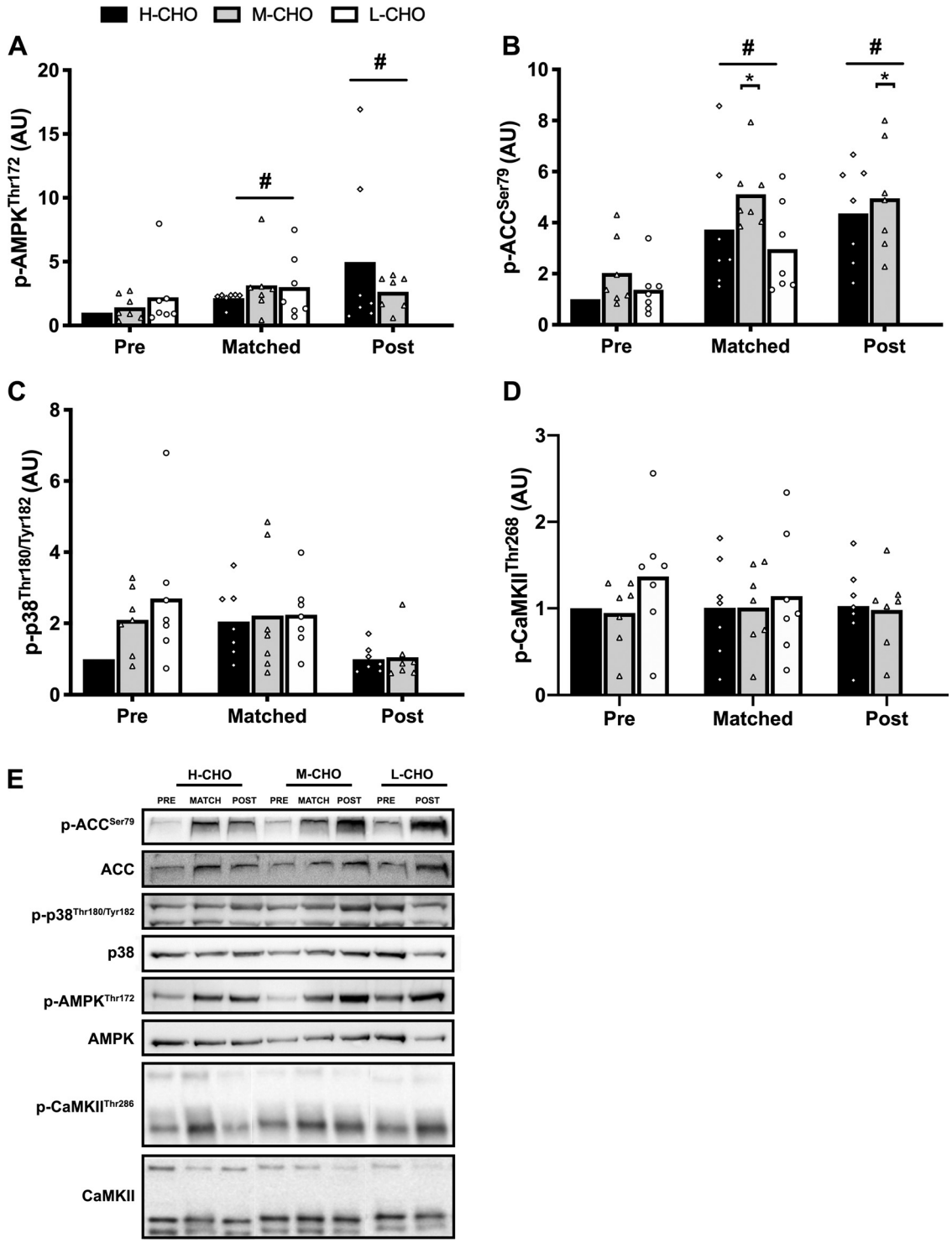

Fig. 4. AMP-activated protein kinase (AMPK)^{Thr172} phosphorylation (*A*), acetyl-CoA carboxylase (ACC)^{Ser79} phosphorylation (*B*), p38$^{Thr180/Tyr182}$ phosphorylation (*C*), CaMKII^{Thr286} phosphorylation (*D*), and representative Western blot images (*E*) before exercise (Pre), at work-matched time point (Matched), and after exercise (Post). AU, arbitrary units; H-CHO, high carbohydrate; M-CHO, medium carbohydrate; L-CHO, low carbohydrate; p, phospho. $^{\#}P < 0.05$, significantly different from Pre; $^{*}P < 0.05$, significantly different from L-CHO. Data are presented as means, and individual data points represent individual subjects ($n = 7$).

H-CHO trials is likely more representative of changes in muscle glycogen concentration. Although we consider that the present data may help to characterize what is considered a worthwhile change in absolute muscle glycogen concentration in determining exercise capacity, we acknowledge that these changes should be considered in the context of each individual, given the interindividual variability between subjects in the present study. Furthermore, as the capacity for glycogen stor-

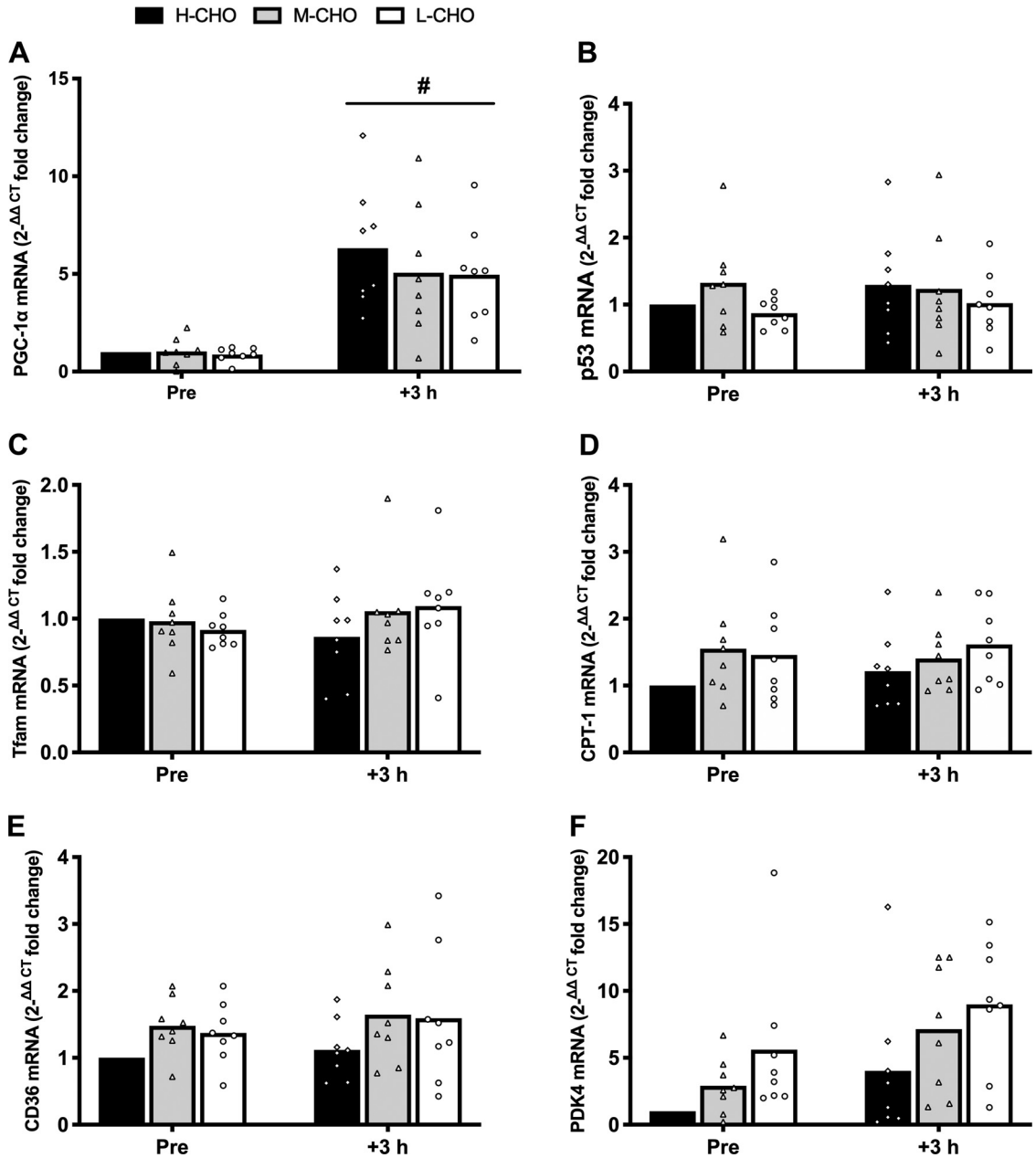

Fig. 5. Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α; *A*), p53 (*B*), transcription factor A, mitochondrial (Tfam; *C*), carnitine palmitoyltransferase 1 (CPT-1; *D*), CD36 (*E*), and pyruvate dehydrogenase kinase 4 (PDK4; *F*) mRNA expression (calculated as $2^{-\Delta\Delta C_t}$, where C_t is threshold cycle) before (Pre) and 3 h after (+3 h) exercise. $^{\#}P < 0.05$, significantly different from Pre. Data are presented as means, and individual data points represent individual subjects ($n = 8$).

age is enhanced and its utilization during exercise reduced among well-trained populations (1, 15, 23), such small differences in muscle glycogen (as observed within the present study) may allow for extended exercise times among well-trained individuals.

In relation to postexercise mitochondria-related signaling, it is widely accepted that commencing work-matched exercise protocols with reduced muscle glycogen induces greater skeletal muscle signaling (20). For example, $AMPK^{Thr172}$ phosphorylation (46), AMPK-α2 activity (45), and nuclear abundance (39) are all augmented when acute exercise is commenced with reduced preexercise muscle glycogen. In contrast, we observed no enhancement in $AMPK^{Thr172}$ or ACC^{Ser79} phosphorylation at our work-matched time point (i.e., after the completion of ~20-min high-intensity cycling) despite graded reductions in preexercise muscle glycogen concentrations. This apparent lack of augmented cell signaling may be explained by subjects already commencing exercise with preexercise glycogen concentrations < 300 mmol/kg dry wt, an absolute concentration that was previously suggested to facilitate the enhanced cell signaling responses associated with low glycogen availability (20). Indeed, our range of preexercise muscle glycogen concentrations is distinctly lower than previous work that reports greater skeletal muscle signaling following work-

matched exercise protocols. For example, high-glycogen trials are commonly commenced with muscle glycogen concentrations between 400 and 600 mmol/kg dry wt (3, 36) and remain above 300 mmol/kg dry wt after exercise (3, 36, 45). In such instances, these researchers observed attenuated (45) or abolished (3, 36) activation of cell signaling pathways. Interestingly, despite the completion of significantly more work in both the M-CHO and H-CHO trials, no further increases in $AMPK^{Thr172}$ phosphorylation were observed after exhaustive exercise. Although both AMPK activity and ACC phosphorylation are known to be regulated by exercise duration (41), these responses appear to be closely linked to changes in muscle glycogen concentrations (9, 41). With this in mind, the lack of augmented signaling in response to further exercise in the present study may be explained by the relatively small changes in muscle glycogen from the work-matched time point to exhaustion.

In contrast to AMPK and ACC, we did not observe any change in the phosphorylation status of $p38MAPK^{Thr180/Tyr182}$ or $CAMKII^{Thr286}$ in response to either exercise or muscle glycogen concentration, although we note the large interindividual variability and recommend the use of larger sample sizes in the future. These data are in agreement with previous work that demonstrates no change in p38MAPK or CAMKII phosphorylation with a variety of train-low methodologies, including sleep-low, train-low (3, 25), twice-per-day training (46), and fasted training (42). Although augmented p38MAPK phosphorylation has been observed when preexercise muscle glycogen is reduced (163 vs. 375 mmol/kg dry wt), this is apparent only within the nucleus and not the cytoplasm (7). Therefore, further work should utilize cellular fractionation methodologies to investigate the cellular localization of such exercise-inducible kinases.

Despite the observed augmented mRNA expression of PGC-1α within the postexercise recovery period, exhaustive exercise did not augment the mRNA expression of other mitochondria (p53 or Tfam)- or substrate utilization (PDK4, CPT1, or CD36)-related genes. Although the time course of mRNA expression for these genes is not well understood, the lack of change in mRNA expression in the present study may be explained by our chosen sampling points in accordance with our sleep-low, train-low exercise model. Indeed, given that our preexercise biopsy was sampled within ~14 h of glycogen-depleting exercise, it is difficult to determine whether mRNA expression was already elevated at preexercise. For instance, time course studies have revealed that the mRNA expression of Tfam (31), PDK4 and CPT1 (34) is enhanced for up to 24 h after exercise, which coincides with our preexercise sampling time point (~14 h between the 2 exercise bouts). However, given the time course of phosphorylation of our chosen protein targets (8), it is highly unlikely that any of these proteins would be phosphorylated at preexercise as a result of the previous evening's glycogen depletion exercise.

Practically, these data suggest that in the context of the sleep-low, train-low model, where muscle glycogen is depleted to very low levels (~100 mmol/kg dry wt), insufficient time is available to restore muscle glycogen to normal levels. As such, individuals undertaking sleep-low, train-low models, which reduce muscle glycogen to very low levels, should consume CHO in accordance with the energetic requirements of the subsequent morning session, given that withholding CHO intake overnight appears to confer no additional benefit in relation to cell signaling but impairs exercise capacity. In contrast, it appears that when muscle glycogen is not depleted to such low levels (>300 mmol/kg dry wt), withholding CHO intake in the postexercise period may prolong the acute cell signaling and gene expression responses (25, 34). With this in mind, it should be noted that driving glycogen depletion below 300 mmol/kg dry wt would likely be more difficult and require considerably more work in well-trained individuals (11), given that they display an enhanced capacity for glycogen storage and reduced utilization during exercise (1, 15, 23). In practice, it appears that careful consideration of the individual's training status and the metabolic demands of each training session is required to ensure appropriate day-to-day periodization of CHO in order to ensure that absolute training intensity is not compromised while also creating a metabolic milieu conducive to facilitating the metabolic adaptations associated with 'train low.'

In summary, we provide novel data by demonstrating that graded reductions in preexercise muscle glycogen below 300 mmol/kg dry wt (as achieved with a sleep-low, train-low model) impair exercise capacity but do not augment skeletal muscle cell signaling responses. Practically, our data suggest that, within the context of the sleep-low, train-low model (when muscle glycogen is depleted to very low levels), overnight CHO restriction is not required to augment skeletal muscle cell signaling and thus CHO should be consumed in accordance with the metabolic demands of the subsequent morning session. Future studies should investigate stepwise reductions in preexercise muscle glycogen, within a wider range (i.e., 100–600 mmol/kg dry wt), in order to investigate the existence of a potential glycogen threshold (20) and allow for a better definition of its potential upper and lower limits.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.A.H., J.P.M., and J.B.L. conceived and designed research; M.A.H., K.M.H., R.A.S., B.S., S.O.S., A.P., J.P.M., and J.B.L. performed experiments; M.A.H., B.S., A.P., A.P.S., J.P.M., and J.B.L. analyzed data; M.A.H., J.P.M., and J.B.L. interpreted results of experiments; M.A.H., J.P.M., and J.B.L. prepared figures; M.A.H., J.P.M., and J.B.L. drafted manuscript; M.A.H., K.M.H., R.A.S., B.S., S.O.S., A.P., A.P.S., J.P.M., and J.B.L. edited and revised manuscript; M.A.H., K.M.H., R.A.S., B.S., S.O.S., A.P., A.P.S., J.P.M., and J.B.L. approved final version of manuscript.

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