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RESEARCH PAPER

Graded reductions in pre-exercise glycogen concentration do not augment exercise-induced nuclear AMPK and PGC-1 α protein content in human muscle

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Abstract

We examined the effects of graded muscle glycogen on the subcellular location and protein content of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) and mRNA expression of genes associated with the regulation of mitochondrial biogenesis and substrate utilisation in human skeletal muscle. In a repeated measures design, eight trained male cyclists completed acute high-intensity interval (HIT) cycling (8 \times 5 min at 80% peak power output) with graded concentrations of pre-exercise muscle glycogen. Following initial glycogen-depleting exercise, subjects ingested 2 g kg⁻¹ (L-CHO), 6 g kg⁻¹ (M-CHO) or 14 g kg⁻¹ (H-CHO) of carbohydrate during a 36 h period, such that exercise was commenced with graded ($P < 0.05$) muscle glycogen concentrations (mmol (kg dw)⁻¹: H-CHO, 531 \pm 83; M-CHO, 332 \pm 88; L-CHO, 208 \pm 79). Exercise depleted muscle glycogen to < 300 mmol (kg dw)⁻¹ in all trials (mmol (kg dw)⁻¹: H-CHO, 270 \pm 88; M-CHO, 173 \pm 74; L-CHO, 100 \pm 42) and induced comparable increases in nuclear AMPK protein content (~ 2 -fold) and PGC-1 α (~ 5 -fold), p53 (~ 1.5 -fold) and carnitine palmitoyltransferase 1 (~ 2 -fold) mRNA between trials (all $P < 0.05$). The magnitude of increase in PGC-1 α mRNA was also positively correlated with post-exercise glycogen concentration ($P < 0.05$). In contrast, neither exercise nor carbohydrate availability affected the subcellular location of PGC-1 α protein or PPAR, SCO2, SIRT1, DRP1, MFN2 or CD36 mRNA. Using a sleep-low, train-low model with a high-intensity endurance exercise stimulus, we conclude that pre-exercise muscle glycogen does not modulate skeletal muscle cell signalling.

KEYWORDS

CHO restriction, train-low, vastus lateralis

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1 | INTRODUCTION

The concept of deliberately commencing endurance exercise with reduced muscle glycogen (i.e. the train-low paradigm, Burke et al., 2018) is now recognised as a potent nutritional strategy that is able to modulate acute skeletal muscle cell signalling (Bartlett et al., 2013; Wojtaszewski et al., 2003; Yeo et al., 2010) and transcriptional responses (Bartlett et al., 2013; Pilegaard et al., 2002; Psilander, Frank, Flockhart, & Sahlin, 2013). Furthermore, repeated bouts of train-low exercise can subsequently augment many hallmark muscle adaptations inherent to the endurance phenotype. Indeed, the strategic periodisation of dietary carbohydrate (CHO) in order to commence exercise with low muscle glycogen (during 3–10 weeks of training) enhances mitochondrial enzyme activity and protein content (Hansen et al., 2005; Morton et al., 2009; Yeo et al., 2008) and whole body and intra-muscular lipid metabolism (Hulston et al., 2010), and, in some instances, improves exercise capacity (Hansen et al., 2005) and performance (Marquet et al., 2016a, 2016b). As such, the train-low paradigm and wider CHO periodisation strategies have subsequently gained increased recognition amongst athletic populations (Burke et al., 2018; Impey et al., 2018; Stellingwerff, 2012).

Skeletal muscle glycogen appears to exert its regulatory effects primarily through the AMP-activated protein kinase (AMPK)–peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) signalling axis, whereby exercise-induced AMPK α 2 activity (Wojtaszewski et al., 2003), phosphorylation (Yeo et al., 2010) and nuclear abundance (Steinberg et al., 2006) are all augmented under conditions of reduced pre-exercise muscle glycogen. These effects may be partly mediated through the glycogen binding domain present on the β subunit of AMPK (McBride & Hardie, 2009; McBride, Ghilagaber, Nikolaev, & Hardie, 2009). This allows for the physical tethering of AMPK to the glycogen granule and may subsequently inhibit its translocation to the nucleus and its activation of transcriptional regulatory proteins. In this way, the subcellular localisation of AMPK may play an important role in the signal transduction pathway regulating train-low responses, whereby its translocation to the nucleus could allow for interaction with transcriptional regulatory proteins, such as PGC-1 α , to control gene expression (McGee et al., 2003). Indeed, endurance exercise also appears to increase the nuclear abundance of PGC-1 α (Little, Safdar, Cermak, Tarnopolsky, & Gibala, 2010; Little, Safdar, Bishop, Tarnopolsky, & Gibala, 2011), and may constitute the initial phase of exercise-induced adaptive responses.

Despite the potential regulatory role of muscle glycogen availability in exercise-induced cell signalling, the absolute concentrations of muscle glycogen required to facilitate such responses are currently unknown (Impey et al., 2018). Recent suggestions propose the potential existence of a muscle glycogen threshold, a metabolic window of absolute muscle glycogen concentration whereby the augmented signalling and transcriptional responses associated with train-low models are particularly evident (Impey et al., 2018). However, using a sleep-low, train-low model, we recently demonstrated that commencing exercise with stepwise reductions in pre-exercise muscle glycogen concentrations below 300 mmol (kg dw)⁻¹ (within the range

New Findings

- **What is the central question of this study?**

What is the absolute level of pre-exercise glycogen concentration required to augment the exercise-induced signalling response regulating mitochondrial biogenesis?

- **What is the main finding and its importance?**

Commencing high-intensity endurance exercise with reduced pre-exercise muscle glycogen concentrations confers no additional benefit to the early signalling responses that regulate mitochondrial biogenesis.

of 300–100 mmol (kg dw)⁻¹) does not augment skeletal muscle cell signalling in response to exhaustive exercise (Hearris et al., 2019). Such findings may be related to the fact that commencing exercise with <300 mmol (kg dw)⁻¹ is already a critical level of absolute glycogen (as suggested by Impey et al., 2018) that is required to induce a metabolic milieu conducive to cell signalling. We therefore suggested that future research should investigate stepwise reductions in pre-exercise muscle glycogen within a wider range of pre-exercise muscle glycogen concentration (i.e. 600–200 mmol (kg dw)⁻¹) in order to investigate the existence of a potential glycogen threshold and allow for a better definition of its potential upper and lower limits.

With this in mind, the aim of the present study was to test the hypothesis that graded pre-exercise muscle glycogen concentrations modulate the exercise-induced nuclear abundance of AMPK and PGC-1 α protein content as well as the transcription of genes with putative roles in the regulation of mitochondrial biogenesis. To achieve our model of graded pre-exercise muscle glycogen concentrations, we utilised an experimental protocol previously studied in our laboratory (Impey et al., 2016) consisting of an amalgamation of train-low protocols whereby participants perform a glycogen depletion protocol on the evening of Day 1, consume a modified CHO intake throughout Day 2 and then perform fasted exercise on the morning of Day 3. In this way, we studied trained male cyclists who commenced an acute bout of work-matched non-exhaustive high-intensity interval cycling (HIT) with low (~200 mmol (kg dw)⁻¹), moderate (~350 mmol (kg dw)⁻¹) or high (~550 mmol (kg dw)⁻¹) pre-exercise muscle glycogen concentrations.

2 | METHODS

2.1 | Ethical approval

All subjects provided written informed consent and all procedures conformed to the standards set by the *Declaration of Helsinki* (2008).

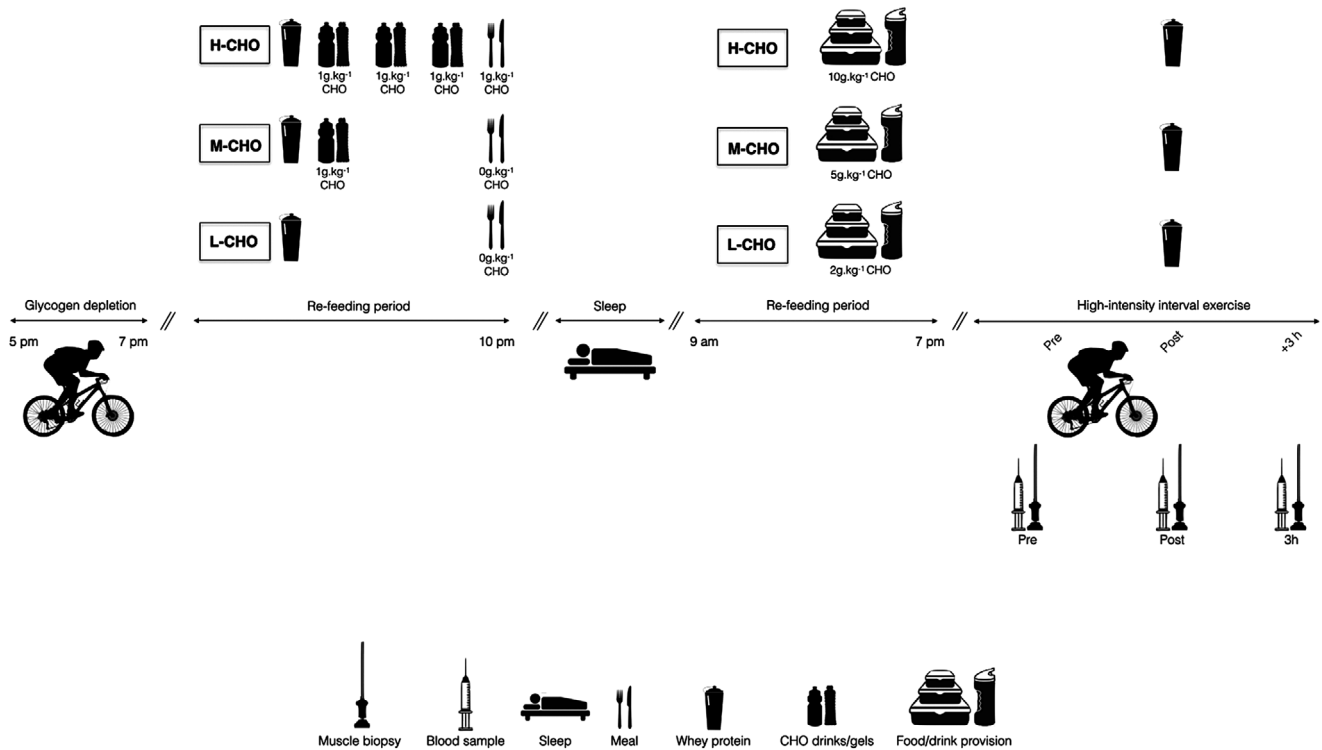


FIGURE 1 Schematic overview of the experimental protocol. Following the completion of an evening bout of glycogen-depleting cycling exercise subjects received three graded levels of CHO in order to manipulate pre-exercise muscle glycogen on the day of the main experimental trial. Following an overnight fast, subjects completed a high-intensity intermittent cycling exercise (8×5 min at 80% peak power output). Muscle biopsies were obtained pre-, post- and 3 h post-exercise

The study was approved by the local Research Ethics Committee of Liverpool John Moores University (ref. no. HM17/SPS/029)

2.2 | Participants

Eight endurance-trained amateur male cyclists (mean \pm SD: age, 30 ± 10 years; body mass 72.6 ± 9.4 kg; height, 177.0 ± 8.9 cm) took part in this study. Mean peak oxygen consumption ($\dot{V}_{O_{2peak}}$) and peak power output (PPO) for the cohort were 60.4 ± 7.7 ml kg^{-1} min^{-1} and 338 ± 45 W, respectively. Cyclists were classed as trained according to guidelines for subject classification in research (De Pauw et al., 2013; Jeukendrup, Craig, & Hawley, 2000). A priori sample size determination was performed, assuming an effect of muscle glycogen on PGC-1 α mRNA of 1.34 (Bartlett et al., 2013), an α -value of 0.05 and a power of 0.80. None of the subjects had any history of musculoskeletal or neurological disease, nor were they under any pharmacological treatment during the course of the testing period.

2.3 | Experimental design

In 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 (L-CHO: 2 g kg^{-1} , M-CHO: 6 g kg^{-1} , H-CHO: 14 g kg^{-1} for low, medium and high groups, respectively) across a ~ 36 h period so as to manipulate pre-exercise muscle glycogen prior to a bout of high-intensity interval exercise (8×5 min at 80% PPO). All trials were performed in a randomised and counterbalanced order. Skeletal muscle biopsies were obtained from the vastus lateralis immediately before, post- and 3 h post-exercise. An overview of the experimental protocol is shown in Figure 1.

2.4 | Assessment of peak oxygen uptake

At least 7 days prior to experimental trials, all subjects were assessed for $\dot{V}_{O_{2peak}}$ and PPO on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). Following the 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. During the test, gas exchange measurements were made using an online gas analysis system (CPX Ultima, MGC Diagnostics, St Paul, MN, USA). The highest 30 s average of O_2 uptake was considered to represent $\dot{V}_{O_{2peak}}$, which was stated as being achieved by the following end-point criteria: (1) heart rate within 10 beats min^{-1} of age-predicted maximum, (2) respiratory exchange ratio >1.1 , and (3) plateau of oxygen consumption despite increased workload (Howley, Bassett, & Welch, 1995).

PPO was taken as the final stage completed during the incremental test.

2.5 | Overview of sleep-low, train-low model

2.5.1 | Phase 1: glycogen depletion exercise

In the 24 h preceding glycogen-depleting exercise, subjects recorded all food and drink consumed and were asked to replicate this for all subsequent trials, and also refrained from alcohol and vigorous physical exercise for the previous 48 h. On the day of glycogen-depleting exercise (Day 1), subjects reported to the laboratory at approximately 17.00 h to perform a bout of intermittent glycogen-depleting cycling, as previously completed in our laboratory (Hearris et al., 2019; Impey et al., 2016; Taylor et al., 2013). The pattern of exercise and total time to exhaustion in the subject's initial trial was 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.

2.5.2 | Phase 2: carbohydrate re-feeding strategy

To facilitate the goal of achieving graded differences in muscle glycogen concentrations between trials, subjects were provided with varying amounts of CHO during the ~36 h recovery period prior to the subsequent bout of high-intensity interval exercise. Within the immediate ~4 h recovery period following glycogen-depleting exercise, subjects in the H-CHO trial were provided with CHO at a rate of $1 \text{ g kg}^{-1} \text{ h}^{-1}$ for 3 h from a mixture of CHO drinks and gels (Science in Sport, Nelson, UK) followed by a high carbohydrate meal providing a further 1 g kg^{-1} CHO. Subjects in the M-CHO trial consumed 1 g kg^{-1} CHO immediately following exercise whilst subjects in the L-CHO trial refrained from CHO intake throughout the remainder of the evening. Across all trials, subjects also consumed 30 g of whey protein isolate (Science in Sport) mixed with 500 ml of water immediately following the cessation of glycogen-depleting exercise to reflect real-world practice as per current nutritional guidelines (Thomas et al., 2016). Over the course of the following day (Day 2), subjects consumed either 10 g kg^{-1} (H-CHO), or 5 g kg^{-1} (M-CHO) or 2 g kg^{-1} (L-CHO) carbohydrate (i.e. between 09.00 and 21.00 h). In this way, total CHO intakes in the H-CHO, M-CHO and L-CHO trials equated to 14, 6 and 2 g kg^{-1} CHO, respectively, over the course of the ~36 h following glycogen depletion. In all trials, subjects also consumed 2 g kg^{-1} protein and 1 g kg^{-1} fat with fluid intake allowed *ad libitum*. Total energy intake over the course of the ~36 h equated to 5306 ± 687 , 2978 ± 386 and 1816 ± 235 kcal in the H-CHO, M-CHO and L-CHO trials, respectively.

2.5.3 | Phase 3: high intensity interval cycling

Subjects arrived at the laboratory between 08.00 and 09.00 h on the morning of Day 3 (in a fasted state), where 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 (HIT) cycling protocol, consisting of 8×5 min intervals at 80% PPO, interspersed with 1 min rest. During exercise, heart rate (HR) was continuously measured and the final HR for each 5 min interval was recorded whilst ratings of perceived exertion (RPE) were recorded upon completion of each interval (Borg, 1982). Expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, MGC Diagnostics, St Paul, MN, USA) for the final 2.5 min of every alternative interval (i.e. intervals 2, 4, 6 and 8) and substrate utilisation was assessed using the equations of Jeukendrup & Wallis (2005) given the validity of indirect calorimetry for the assessment of substrate utilisation at exercise intensities up to 80–85% $\dot{V}_{O_{2\max}}$ (Romijn, Coyle, Hibbert, & Wolfe, 1992). Following the completion of the exercise bout and collection of the post-exercise biopsy, subjects were fed 30 g whey protein (Science in Sport) mixed with 500 ml of water as previously described (Hearris et al., 2019), which we have previously demonstrated does not influence post-exercise signalling responses (Taylor et al., 2013).

2.6 | Blood analysis

Venous blood samples were collected in vacutainers containing K_2 EDTA, lithium heparin or serum separation tubes and stored on ice or at room temperature until centrifugation at 1500 g for 15 min at 4°C. Samples were collected immediately prior to and after exercise. Plasma was aliquoted and stored at -80°C until analysis. Samples were later analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA) and glycerol using commercially available enzymatic spectrophotometric assays (RX Daytona Analyser, Randox Laboratories, Crumlin, UK) as per manufacturer instructions.

2.7 | Muscle biopsies

Skeletal muscle biopsies (~60 mg) were obtained from the vastus lateralis immediately prior to exercise, immediately upon completion of the exercise bout and at 3 h post-exercise. Muscle biopsies were obtained from separate incision sites 2–3 cm apart using a Bard Monoply Disposable Core Biopsy Instrument (12-gauge \times 10 cm length, Bard Biopsy Systems, Tempe, AZ, USA) under local anaesthesia (0.5% Marcaine) and immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

2.8 | Muscle glycogen concentration

Muscle glycogen concentrations were determined according to the acid hydrolysis method described by Van Loon et al. (van Loon, Saris, Kruijshoop, & Wagenmakers, 2000). Approximately 2–5 mg of freeze-dried tissue was powdered, dissected of all visible blood and connective

TABLE 1 Primers used for RT-qPCR

Gene name	Gene symbol	Forward primer	Reverse primer	Product length (bp)
Peroxisome proliferator-activated receptor γ co-activator 1- α	PPARGC1A	TGCTAACGACTCCGAGAA	TGCAAAGTCCCTCTCTGCT	67
Tumour protein p53	P53	ACCTATGGAACTACTTCTGAAA	CTGGCATTCTGGGAGCTTCA	141
Peroxisome proliferator-activated receptor δ	PPAR δ	ATGGAGCAGCCACAGGAGGAAGCC	GCATGAGGCCCGTCACAGC	201
Synthesis of cytochrome c oxidase	SCO2	CTTCACTCACTGCCCTGACA	CGGTCAGACCCAACAGCTT	191
Sirtuin 1	SIRT1	GAGCCATGAAGTATGACAAAGATGA	GGCACTTCATGGGGTATGGAA	109
Dynamic-related protein 1	DRP1	CACCCGGAGACCTCTCATT	CCCCATTCTTCTGCTTCCAC	99
Mitofusin 2	MFN2	CCCCCTTGCTTTATGCTGATGTT	TTTTGGGAGAGGTGTGCTTATTTTC	168
Carnitine palmitoyltransferase 1 (CPT-1)	CPT-1	GACAATACCTCGGAGCCTCA	AATAGGCCTGACGACACCTG	94
Cluster of differentiation 36	CD36	AGGACTTTCCTGCAGAATACCA	ACAAGCTCTGGTTCTTATTCACA	199
β_2 microglobulin	B2M	TGCTGTCTCCATGTTTGTATGTATCT	TCTCGCTCCCCACCTCTAAGT	85

tissue and subsequently hydrolysed by incubation in 500 μ l of 1 M HCl for 3 h at 95°C. After cooling to room temperature, samples were neutralised by the addition of 250 μ l 0.12 mol l⁻¹ Tris–2.1 mol l⁻¹ KOH saturated with KCl. Following centrifugation, 200 μ l of supernatant was analysed in duplicate for glucose concentration according to the hexokinase method using a commercially available kit (GLUC-HK; Randox Laboratories). Glycogen concentrations are expressed as mmol (kg dw)⁻¹ and intra-assay coefficients of variation were <5%.

2.9 | RNA isolation and analysis

Muscle samples (~20 mg) were homogenised in 1 ml TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and total RNA isolated by phenol–chloroform extraction. Concentrations and purity of RNA were assessed by UV spectroscopy at optical densities of 260 and 280 nm, using a NanoDrop 3000 (Thermo Fisher Scientific, Roskilde, Denmark) with an average 260/280 ratio of 1.93 \pm 0.08. A quantity of 50 ng total RNA was used for each 20 μ l PCR reaction.

2.10 | Reverse transcription–quantitative real-time polymerase chain reaction

Reverse transcription–quantitative real-time polymerase chain reaction (RT–qPCR) amplifications were performed using QuantiFast™ SYBR® Green RT-PCR one-step kit on a Rotorgene 300Q (Qiagen, Crawley, UK) supported by Rotorgene Qiagen software (Hercules, CA, USA). RT–qPCR was performed as follows: 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: 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 non-specific 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). Following initial screening of suitable reference genes, β_2 microglobulin (B2M) showed the most stable C_t values across all RT-PCR runs and subjects, regardless of experimental condition (25.3 \pm 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 \pm 2%) was similar for all genes across all time points and experimental conditions. As such, the relative gene expression levels were calculated using the comparative C_t (2^{- $\Delta\Delta$ C_t}) equation (Schmittgen & Livak, 2008) where the relative expression was calculated as 2^{- $\Delta\Delta$ C_t}, where C_t represents the threshold cycle. mRNA expression for all target genes was calculated relative to the reference gene (B2M) within the same subject and condition and relative to the pre-exercise value in the H-CHO condition. The primers used are shown in Table 1.

2.11 | Subcellular fractionation

Isolation of nuclear and cytosolic fractions was performed using the method described by Dimauro, Pearson, Caporossi, & Jackson (2012). Protein concentrations were determined for both fractions via a BCA assay (Thermo Fisher Scientific, UK) and validation of the enrichment of nuclear and cytosolic fractions was confirmed by blotting the separate fractions against the nuclear protein histone H3 (Cell Signaling Technology (CST), Danvers, MA, USA: no. 9715, 1:500) and the cytosolic protein lactate dehydrogenase (CST: no. 2012, 1:1000) to demonstrate the absence of major contamination. Sufficient muscle was available for western blot analysis for seven subjects.

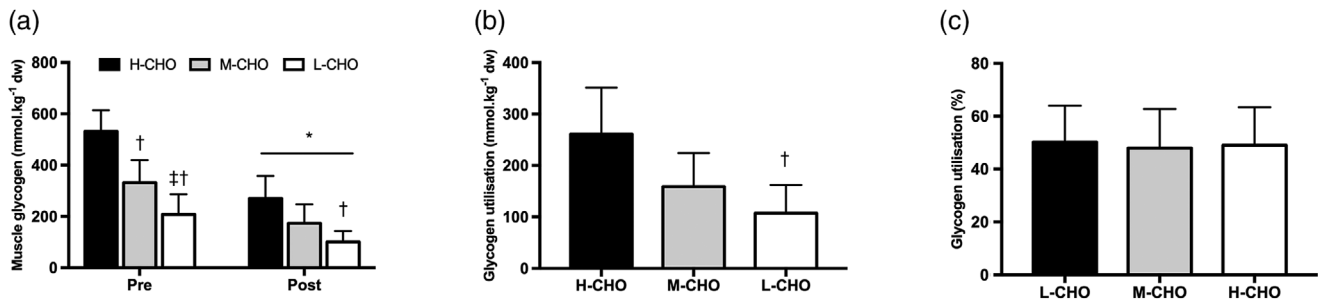


FIGURE 2 Skeletal muscle glycogen concentration (a) and utilisation during exercise (b,c). * $P < 0.05$, significantly different from pre-exercise; † $P < 0.05$, significantly different from H-CHO; †† $P < 0.05$, significantly different from M-CHO

2.12 | SDS page and western blotting

Following the determination of protein concentration within each fraction, samples were resuspended in 4× Laemlli buffer, denatured by boiling for 5 min at 95°C and separated by SDS-PAGE using 12% Mini-Protean TGX Stain-Free Gels (Bio-Rad, Watford, UK). After electrophoresis, stain-free gels were activated according to manufacturer instructions and semi-dry transferred to nitrocellulose membranes (TransBlot Turbo, Bio-Rad). Following transfer, a stain-free image was obtained for total protein loading normalisation. Membranes were subsequently blocked in Tris-buffered saline–Tween containing 5% non-fat milk for 1 h and incubated overnight in primary antibodies for AMPK α (CST: no. 2603, 1:1000) and PGC-1 α (Sigma-Aldrich, St-Louis, MO, USA: st-1202, 1:1000) before incubation in relevant secondary antibody (anti-rabbit: CST: no. 7074, 1:2000) for 1 h at room temperature the following morning. After washing, membranes were incubated in a chemiluminescence substrate (Thermo Fisher Scientific, UK) and visualised using a Chemi-doc MP system (Bio-Rad, Watford, UK) with band densities quantified using Image Lab software (Bio-Rad, Watford, UK). The volume density of each target band was normalised to the total amount of protein loaded into each lane using Stain-Free technology.

2.13 | Statistical analysis

All statistical analyses were performed using SPSS Statistics Version 24 (IBM Corp., Armonk, NY, USA). Comparisons of average physiological responses (e.g. total substrate oxidation) were analysed using 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 signalling molecules) were analysed using a two-way repeated measures general linear model, where the within factors were time and condition. Where a significant main effect was observed, pairwise comparisons were analysed according to the Bonferroni *post hoc* test in order to locate specific differences. Pearson's correlation was used to assess any associations between variables. All data in text, figures and tables are presented as means \pm SD with P -values ≤ 0.05 indicating statistical significance.

3 | RESULTS

3.1 | Skeletal muscle glycogen

Muscle glycogen displayed main effects for condition ($P < 0.001$), time ($P < 0.001$) and a treatment \times time interaction effect ($P = 0.001$). As such, the exercise and nutritional strategy employed was successful in achieving graded levels of pre-exercise muscle glycogen (mmol (kg dw)⁻¹: H-CHO, 531 \pm 83; M-CHO, 332 \pm 88; L-CHO, 208 \pm 79) such that HIT exercise was commenced with three distinct levels of muscle glycogen (L-CHO vs. M-CHO, $P = 0.024$; L-CHO vs. H-CHO, $P = 0.001$; M-CHO vs. H-CHO, $P = 0.022$) (Figure 2a). HIT exercise significantly reduced muscle glycogen concentrations (main effect of time, $P < 0.001$), with post-exercise muscle glycogen concentrations (mmol (kg dw)⁻¹: H-CHO, 270 \pm 88; M-CHO, 173 \pm 74; L-CHO, 100 \pm 42) remaining higher in the H-CHO trial when compared with the L-CHO trial only ($P = 0.013$). Furthermore, muscle glycogen utilisation during HIT exercise displayed a significant main effect ($P = 0.001$), with glycogen utilisation being significantly higher in the H-CHO trial (260 \pm 90 mmol (kg dw)⁻¹) when compared with the L-CHO trial (107 \pm 55 mmol (kg dw)⁻¹) only ($P = 0.011$) with no significant differences observed between M-CHO (159 \pm 65 mmol (kg dw)⁻¹) and either H-CHO or L-CHO trials ($P > 0.05$) (Figure 2b). However, the relative utilisation of muscle glycogen during HIT exercise was similar between all trials (H-CHO, 49.0 \pm 14.4%; M-CHO, 47.9 \pm 14.9%; L-CHO, 50.1 \pm 13.8%; $P = 0.928$) (Figure 2c).

3.2 | Physiological and metabolic responses to exercise

Average exercise intensity across the HIT session equated to 85 \pm 8%, 85 \pm 8% and 89 \pm 9% $\dot{V}_{O_{2peak}}$ for H-CHO, M-CHO and L-CHO trials, respectively. Subjects' average heart rate (Figure 3a) (beats min⁻¹: H-CHO, 173 \pm 9; M-CHO, 174 \pm 8; L-CHO, 176 \pm 7) and RPE (Figure 3b) (arbitrary units: H-CHO, 15 \pm 1; M-CHO, 16 \pm 2; L-CHO, 16 \pm 2) across the HIT session was similar between trials ($P > 0.05$). Plasma glucose displayed a significant main effect for condition ($P = 0.001$) but no time or condition \times time main effect ($P > 0.05$). Plasma lactate was significantly increased by HIT exercise (main effect of time, $P = 0.001$)

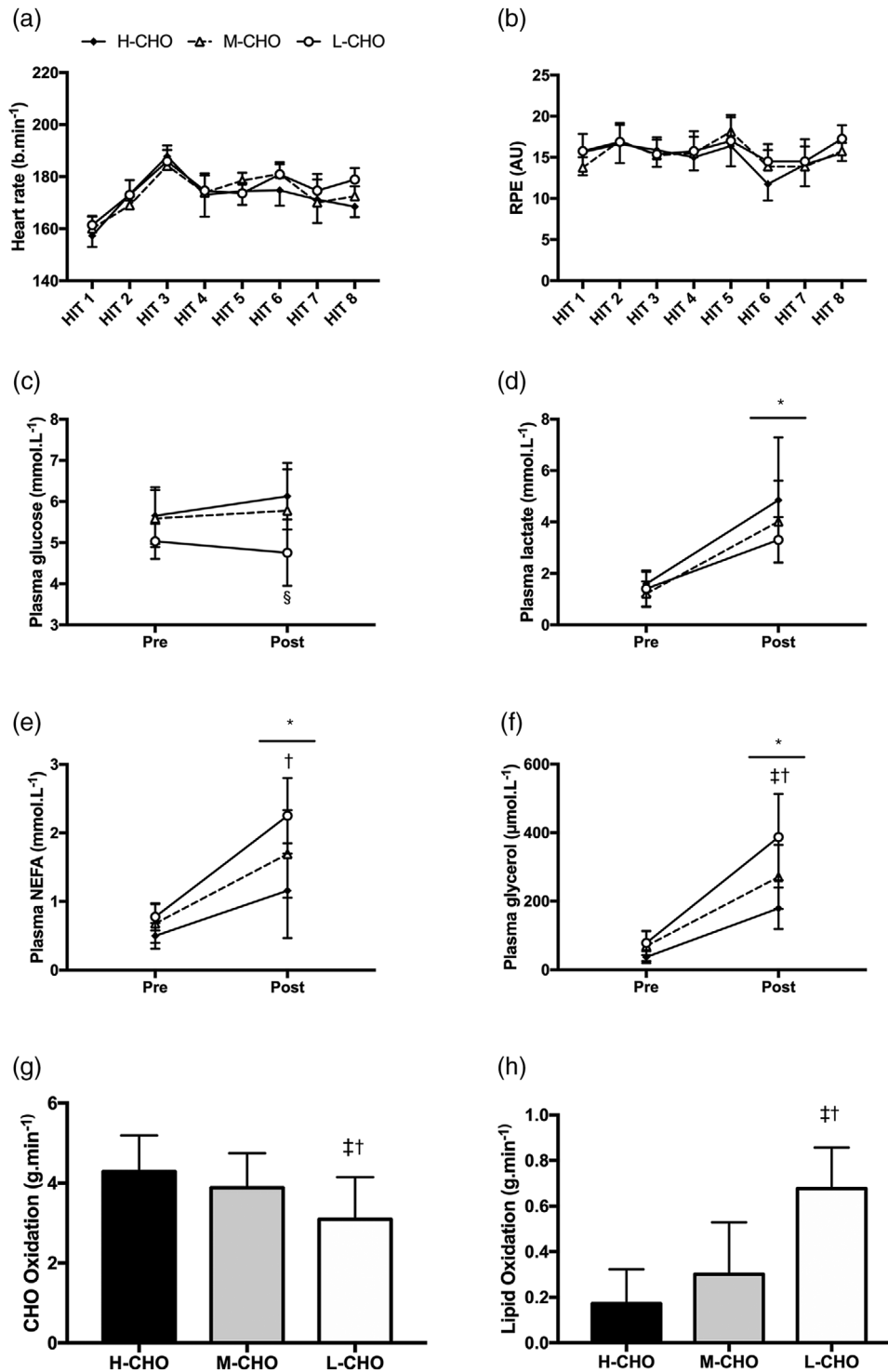


FIGURE 3 (a–f) Heart rate (a), RPE and plasma (b), glucose (c), lactate (d), NEFA (e), and glycerol (f) pre- and post-exercise. (g,h) Average CHO (g) and lipid (h) oxidation during exercise. * $P < 0.05$, significantly different from pre-exercise; † $P < 0.05$, significantly different from H-CHO; ‡ $P < 0.05$, significantly different from M-CHO

but did not display any main effect for condition or condition \times time ($P > 0.05$). Plasma NEFA displayed main effects for condition ($P < 0.001$), time ($P < 0.001$) and a condition \times time interaction effect ($P = 0.001$) with plasma NEFA concentrations being significantly higher in the L-CHO trial post-exercise when compared with the H-CHO trial

only ($P < 0.001$). Plasma glycerol displayed main effects for condition ($P < 0.001$), time ($P < 0.001$) and a condition \times time interaction effect ($P = 0.001$). Following exercise, plasma glycerol concentrations were significantly higher in the L-CHO trial when compared with both M-CHO ($P = 0.02$) and H-CHO ($P < 0.001$) trials. Post-exercise plasma

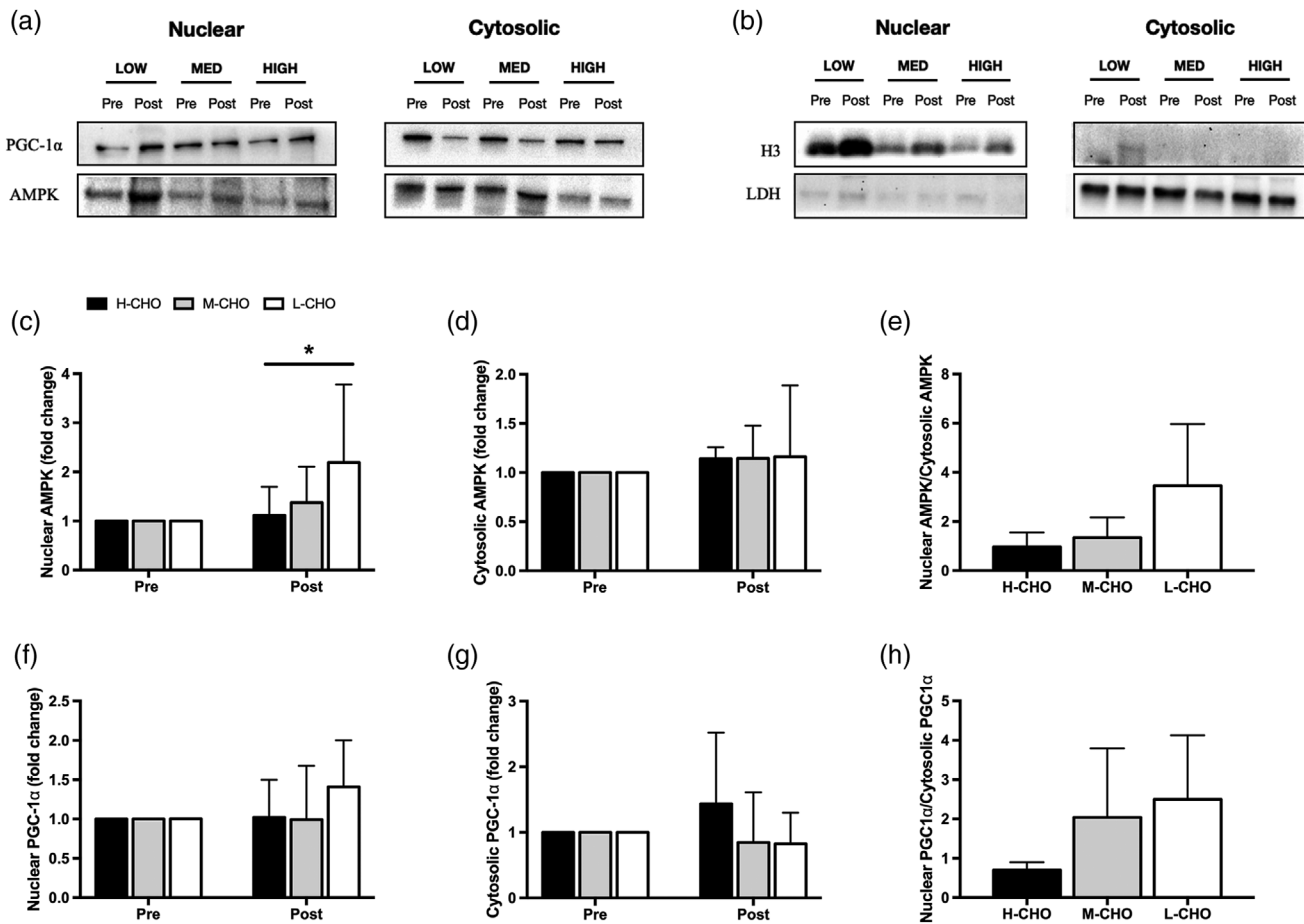


FIGURE 4 (a,b) Representative western blots for AMPK and PGC-1 α (a) and histone H3 and LDH (b), used as indicators of nuclear and cytosolic enrichment, in both nuclear and cytosolic fractions pre- and post-exercise. (c–e) Protein content of nuclear (c) and cytosolic (d) AMPK and the ratio between nuclear and cytosolic AMPK (e) post-exercise. (f–h) Protein content of nuclear (f) and cytosolic PGC-1 α (g) and the ratio between nuclear and cytosolic PGC-1 α (h) post-exercise. * $P < 0.05$, significantly different from pre-exercise

glycerol concentrations were also significantly higher in the M-CHO trial when compared with the H-CHO trial ($P = 0.029$). Similarly, subjects in the L-CHO trial achieved significantly lower rates of CHO oxidation and greater rates of lipid oxidation when compared with both M-CHO and H-CHO trials ($P < 0.005$) although no significant differences were observed between M-CHO and H-CHO trials for either CHO ($P = 0.427$) or lipid oxidation ($P = 0.687$) (Figures 3g and h, respectively).

3.3 | Regulation of mitochondrial biogenesis related cell signalling

The fractionation protocol was successful in achieving enriched fractions of both nuclear and cytosolic proteins (Figure 4b). HIT exercise induced an increase in the content of AMPK protein (main effect of time; $P = 0.025$) within the nucleus with no significant change within the cytosol (no main effect of time; $P = 0.20$). Despite this, there was no significant effect of pre-exercise muscle glycogen concentrations on AMPK localisation (no main effect of condition;

$P = 0.207$). In contrast, HIT exercise did not induce any change in the content of PGC-1 α protein in either nuclear or cytosolic (no main effect of time or condition, $P > 0.05$) fractions (Figure 4c–h). With regard to exercise induced gene expression, HIT exercise induced a significant increase in PGC-1 α , p53 and carnitine palmitoyltransferase 1 (CPT-1) mRNA expression at 3 h post-exercise (main effect of time, $P < 0.05$) but did not display any significant main effect for condition or condition \times time ($P > 0.05$) (Figure 5a,b,i). However, PPAR, SCO2, SIRT1, DRP1, MFN2 and CD36 mRNA expression (Figure 5c–h) was unaffected by either glycogen availability or the HIT exercise protocol (no main effect of time or condition, $P > 0.05$).

3.4 | Correlation between muscle glycogen and cell signalling responses

The absolute concentration of muscle glycogen immediately post-exercise demonstrated a significant correlation with PGC-1 α mRNA expression 3 h post-exercise ($P = 0.048$, $r = -0.416$; Figure 6). In contrast, no correlation was observed between post-exercise muscle

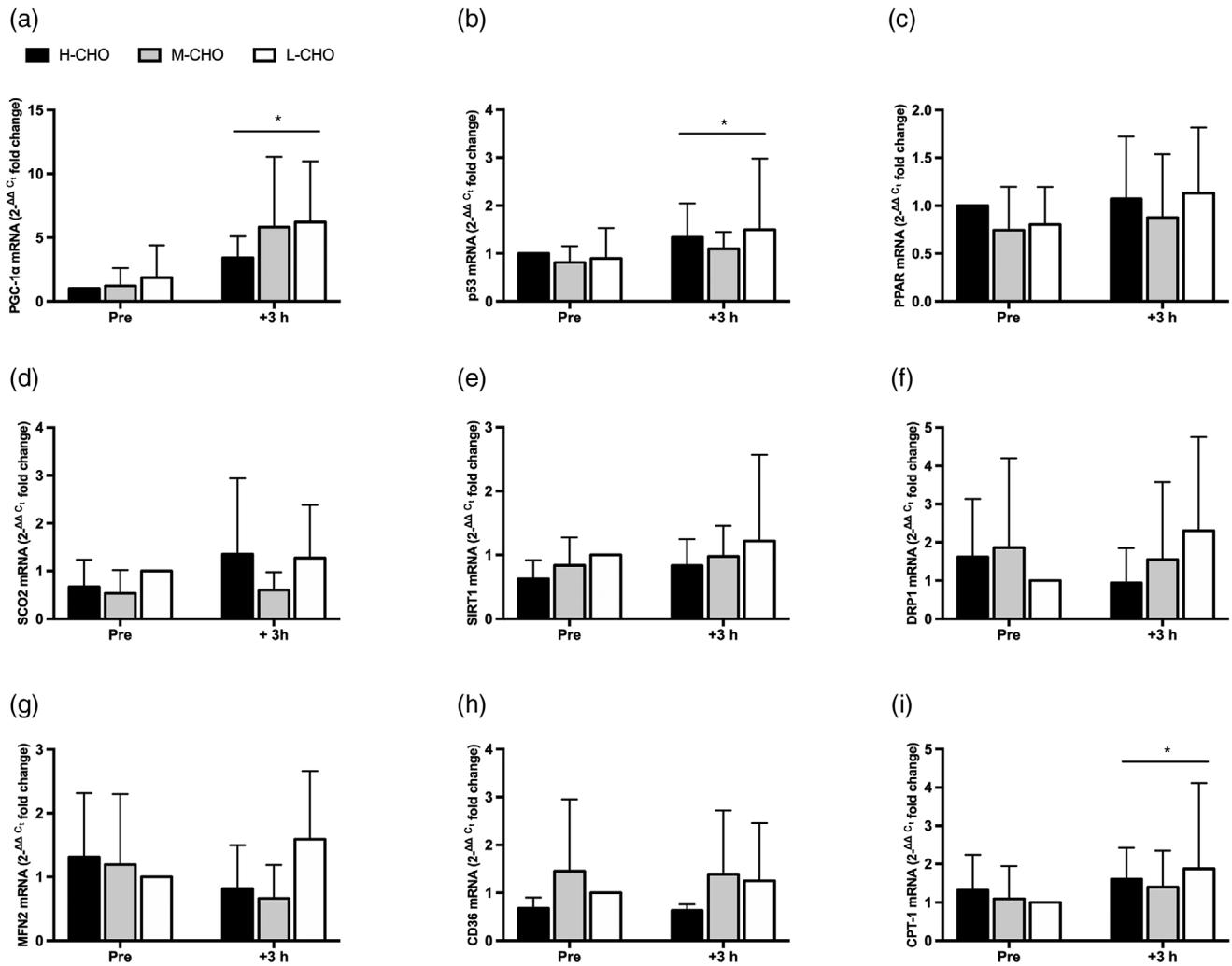


FIGURE 5 PGC-1 α (a), p53 (b), PPAR (c), SCO2 (d), SIRT1 (e), DRP1 (f), MFN2 (g), CD36 (h) and CPT-1 (i) mRNA expression pre- and 3 h post-exercise. * $P < 0.05$, significantly different from pre-exercise

glycogen concentration and mRNA expression of other target genes or the nuclear abundance of either AMPK or PGC-1 α proteins ($P > 0.05$).

4 | DISCUSSION

The aim of the present study was to test the hypothesis that graded pre-exercise muscle glycogen concentrations modulate the exercise-induced nuclear abundance of AMPK and PGC-1 α protein content as well as the transcription of genes with putative roles in the regulation of mitochondrial biogenesis. Using trained male cyclists, we demonstrate that commencing an acute bout of work-matched and non-exhaustive HIT cycling with graded pre-exercise muscle glycogen (within a range of 600–200 mmol (kg dw)⁻¹) does not modulate such early signalling responses. In the context of manipulating CHO availability around training, our data suggest that the metabolic stress of HIT exercise may override any potential effect of pre-exercise muscle glycogen and induce negligible modulatory effects on skeletal

muscle that is already subjected to the local metabolic challenge of high-intensity exercise.

To address our aim, we utilised an experimental protocol consisting of a purposeful amalgamation of previous train-low protocols whereby participants perform a glycogen depletion protocol on the evening of Day 1, consume a modified CHO intake throughout Day 2 and then perform fasted exercise on the morning of Day 3. This approach has been studied previously in our laboratory (Impey et al., 2016) and provides an extended recovery period following glycogen-depleting exercise (~36 h) during which larger amounts of CHO can be consumed when compared to the 12 h period associated with the traditional sleep-low, train-low protocol (Hearris et al., 2019; Lane et al., 2015). Indeed, this extended feeding period circumvents the inability to fully restore muscle glycogen to normative resting concentrations after ~12 h (Hearris et al., 2019), thus allowing for the investigation of a wider range of pre-exercise muscle glycogen concentrations (i.e. 600–200 mmol (kg dw)⁻¹). This strategy was effective in achieving graded differences in pre-exercise muscle glycogen concentrations (mmol (kg dw)⁻¹: H-CHO, 531 \pm 83; M-CHO, 332 \pm 88; L-CHO,

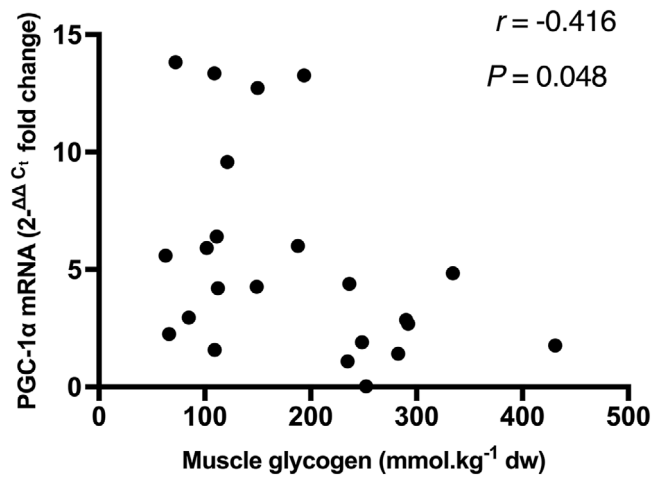


FIGURE 6 Correlation of PGC-1 α mRNA (3 h post-exercise) and post-exercise muscle glycogen concentrations

208 \pm 79) and is consistent with what is considered high, normal and low resting muscle glycogen concentrations in trained males (Areta & Hopkins, 2018). Whilst we acknowledge that the manipulation of CHO intake also results in considerable differences in total energy intake, such short-term differences in energy intake do not appear to influence the molecular response to exercise (Hammond et al., 2019). In contrast to our previous work (Hearris et al., 2019), we also employed a work-matched, non-exhaustive HIT exercise protocol in an attempt to prevent the depletion of muscle glycogen to the absolute concentrations normally associated with exhaustion (i.e. <100 mmol (kg dw)⁻¹; Hearris et al., 2019; Impey et al., 2016; Taylor et al., 2013). Despite differences in absolute glycogen utilisation between trials, we also observed a comparable relative utilisation (~50%) between trials, a magnitude of use that is consistent with that previously observed using this exercise protocol (Stepito, Martin, Fallon, & Hawley, 2001). As such, participants in all trials finished the HIT protocol with absolute glycogen concentrations <300 mmol (kg dw)⁻¹.

In relation to post-exercise signalling, the exercise-induced increase in nuclear AMPK protein content was not augmented in response to stepwise reductions in pre-exercise muscle glycogen concentrations, which is in contrast to previous reports of enhanced nuclear AMPK content when exercise is commenced with low pre-exercise muscle glycogen (Steinberg et al., 2006). Given that nuclear AMPK translocation in response to exercise may be partly regulated by absolute glycogen concentrations (due to the physical tethering of AMPK to the glycogen granule; Steinberg et al., 2006) the magnitude of muscle glycogen utilisation during exercise may also be important for AMPK's disassociation from the granule and subsequent translocation to other subcellular regions. With this in mind, the similar rates of glycogen utilisation (~50%) between trials, leading to absolute post-exercise glycogen concentrations <300 mmol (kg dw)⁻¹, may explain the comparable increases in nuclear AMPK content across all trials and subsequent discrepancies with previous work. Whilst the absolute concentrations of muscle glycogen required to facilitate the subcellular shuttling of AMPK are relatively unknown, post-

exercise glycogen concentrations in the present study are markedly higher (mmol (kg dw)⁻¹: H-CHO, 270 \pm 88; M-CHO, 173 \pm 74; L-CHO, 100 \pm 42) when compared with that of the low glycogen trial in previous work (17 \pm 6 mmol (kg dw)⁻¹) (Steinberg et al., 2006). In fact, post-exercise muscle glycogen concentrations in the present L-CHO trial are comparable to the control condition (111 \pm 35 mmol (kg dw)⁻¹) of previous work (Steinberg et al., 2006) and may suggest that the augmentation of nuclear AMPK may only occur at extremely low absolute glycogen concentrations normally associated with exhaustion.

Irrespective of muscle glycogen, the use of high-intensity endurance exercise within the present study is known to induce significantly greater metabolic stress and augments the phosphorylation and activation of AMPK when compared with low-intensity exercise models (Combes et al., 2015; Egan et al., 2010; Fiorenza et al., 2018; Wojtaszewski, Nielsen, Hansen, Richter, & Kiens, 2000). Given that such metabolic fluctuations ultimately regulate signalling kinase activity, the completion of high-intensity endurance exercise may provide a sufficient local metabolic challenge to skeletal muscle whereby reducing pre-exercise muscle glycogen induces negligible further modulatory effects on skeletal muscle. Although augmented signalling in response to high-intensity exercise commenced with low muscle glycogen has been previously observed (Yeo et al., 2010), recent data suggest this may be explained by the metabolic stress of training twice per day, as opposed to low pre-exercise glycogen concentrations *per se* (Andrade-Souza et al., 2019). Furthermore, as the exercise-induced activation of AMPK is reduced in trained individuals (McConnell, Wadley, Le plastrier, & Linden, 2020), the use of trained cyclists within the present study may offer further explanation for the discrepancy in results when compared with those of Steinberg et al. (2006).

Despite exercise-induced increases in nuclear AMPK, we failed to observe concomitant increases in nuclear PGC-1 α protein content. These data are in contrast to most (Andrade-Souza et al., 2019; Little et al., 2010, 2011) but not all (Tachtsis, Smiles, Lane, Hawley, & Camera, 2016) reports of enhanced nuclear PGC-1 α in response to acute endurance exercise. Our data are, however, in support of previous findings that demonstrate exercise-induced increases in nuclear PGC-1 α are unaffected by muscle glycogen availability (Andrade-Souza et al., 2019). The lack of change in nuclear PGC-1 α in the present study may be explained by our chosen sampling time point, given that, in some cases, increases in nuclear PGC-1 α have only been observed 3 h post-exercise (Little et al., 2011). Indeed, previous data suggest that the exercise-induced increase in PGC-1 α may follow a coordinated, temporal response later in recovery following its activation via upstream kinases AMPK and p38 mitogen-activated protein kinase MAPK within the cytosol immediately post-exercise (Little et al., 2011). Although not measured in the present study, we have previously demonstrated increases in AMPK phosphorylation following a similar bout of high-intensity endurance exercise (Hearris et al., 2019) irrespective of pre-exercise muscle glycogen concentrations. However, as PGC-1 α is regulated by numerous post-translational modifications, it is unclear whether an increase in its nuclear protein

content would have been observed later in recovery within the present study.

In relation to exercise-induced gene expression, we demonstrate that the expression of genes with putative roles in mitochondrial biogenesis and substrate utilisation is not augmented under conditions of reduced muscle glycogen concentrations. These candidate genes were chosen upon the basis of their reported time course of expression and to allow comparison with previously studied train-low investigations (Hammond et al., 2019; Impey et al., 2016; Lane et al., 2015; Psilander et al., 2013). The exercise-induced increase in PGC-1 α , p53 and CPT-1 are consistent with previous reports following a similar bout of high-intensity running (Hammond et al., 2019). However, the lack of change in other mitochondria-related genes is unclear given they display increased expression within the chosen time course across a range of exercise modalities (Fiorenza et al., 2018; Impey et al., 2016). In contrast to the previously reported effects of muscle glycogen concentrations on the regulation of gene expression (Pilegaard et al., 2002, 2005; Bartlett et al., 2013; Psilander et al., 2013), these data support our previous findings (Hearris et al., 2019) and collectively demonstrate that commencing high-intensity endurance exercise with reduced muscle glycogen concentrations confers no additional benefit to the expression of genes involved in mitochondrial biogenesis. The discrepancy in these data may be explained by the attainment of post-exercise muscle glycogen concentrations (i.e. <300 mmol (kg dw)⁻¹) associated with augmented gene expression responses to exercise (Impey et al., 2018) across all conditions. This raises the possibility that absolute post-exercise muscle glycogen concentrations (as opposed to pre-exercise) may also play an important role in regulating mitochondria-related skeletal muscle signalling responses to exercise given the observed significant correlation between post-exercise muscle glycogen concentrations and PGC-1 α mRNA. In line with this rationale, our laboratory has previously demonstrated comparable increases in the mRNA expression of PGC-1 α with the depletion of muscle glycogen to similar concentrations (~100 mmol (kg dw)⁻¹), despite marked differences in pre-exercise concentrations (600 vs. 300 mmol (kg dw)⁻¹) and exercise duration (Impey et al., 2016). Furthermore, these findings are supported by data that demonstrate post-exercise muscle glycogen concentrations regulate exercise-induced PGC-1 α mRNA expression (Fiorenza et al., 2018; Pilegaard et al., 2005; Psilander, 2014) and total protein abundance (Mathai, Bonen, Benton, Robinson, & Graham, 2008).

In summary, we provide novel data by demonstrating that graded pre-exercise muscle glycogen (within a range of 600–200 mmol (kg dw)⁻¹) does not modulate the exercise-induced nuclear abundance of AMPK or PGC-1 α nor does it affect the expression of genes with regulatory roles in mitochondrial biogenesis and substrate utilization. Practically, these data suggest that the additional stress of low pre-exercise muscle glycogen may not be required when performing high-intensity exercise that already subjects skeletal muscle to a sufficient metabolic challenge and may be better suited during conditions that do not elicit such cellular perturbations (e.g. prolonged low-intensity exercise completed below

lactate threshold). Restriction of CHO availability for the latter training sessions would also circumvent the impairment in self-selected training intensity observed when high-intensity exercise is performed with reduced muscle glycogen (Lane et al., 2013; Yeo et al., 2008). Given that post-exercise muscle glycogen concentrations were reduced to low levels across all trials (i.e. 100–250 mmol (kg dw)⁻¹), our data raise the possibility that the absolute post-exercise muscle glycogen concentration may also be an important factor in regulating exercise-induced skeletal muscle signalling responses associated with mitochondrial biogenesis, and further work is now required.

COMPETING INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

M.A.H., J.P.M. and J.B.L. designed the study. M.A.H., D.J.O., S.O.S., J.A.S. and J.B.L. performed experiments, M.A.H. and D.J.O. analysed data and M.A.H., D.J.O., A.P.S., J.P.M. and J.B.L. interpreted results of experiments. M.A.H., D.J.O., J.P.M. and J.B.L. drafted the manuscript and S.O.S., J.A.S. and A.P.S. edited and revised the manuscript. All authors approved the final version and agree 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. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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