Reduced carbohydrate availability enhances exercise-induced p53 signaling in human skeletal muscle: implications for mitochondrial biogenesis

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Bartlett JD, Louhelainen J, Iqbal Z, Cochran AJ, Gibala MJ, Gregson W, Close GL, Drust B, Morton JP. Reduced carbohydrate availability enhances exercise-induced p53 signaling in human skeletal muscle: implications for mitochondrial biogenesis. Am J Physiol Regul Integr Comp Physiol 304: R450–R458, 2013. First published January 30, 2013; doi:10.1152/ajpregu.00498.2012.—The mechanisms that regulate the enhanced skeletal muscle oxidative capacity observed when training with reduced carbohydrate (CHO) availability are currently unknown. The aim of the present study was to test the hypothesis that reduced CHO availability enhances p53 signaling and expression of genes associated with regulation of mitochondrial biogenesis and substrate utilization in human skeletal muscle. In a repeated-measures design, muscle biopsies (vastus lateralis) were obtained from eight active males before and after performing an acute bout of high-intensity interval running with either high (HIGH) or low CHO availability (LOW). Resting muscle glycogen (HIGH, 467 ± 19; LOW, 103 ± 9 mmol/kg dry wt) was greater in HIGH compared with LOW (P < 0.05). Phosphorylation (P-) of ACCSer79 (HIGH, 1.4 ± 0.4; LOW, 2.9 ± 0.9) and p53Ser15 (HIGH, 0.9 ± 0.4; LOW, 2.6 ± 0.8) was higher in LOW immediately postexercise and 3 h postexercise, respectively (P < 0.05). Before and 3 h postexercise, mRNA content of pyruvate dehydrogenase kinase 4, mitochondrial transcription factor A, cytochrome-c oxidase IV, and PGC-1α were greater in LOW compared with HIGH (P < 0.05), whereas carnitine palmitoyltransferase-1 showed a trend toward significance (P = 0.09). However, only PGC-1α expression was increased by exercise (P < 0.05), where three-fold increases occurred independently of CHO availability. We conclude that the exercise-induced increase in p53 phosphorylation is enhanced in conditions of reduced CHO availability, which may be related to upstream signaling through AMPK. Given the emergence of p53 as a molecular regulator of mitochondrial biogenesis, such nutritional modulation of contraction-induced p53 activation has implications for both athletic and clinical populations.

AMPK; PGC-1α; glycogen; high-intensity interval running

SKELETAL MUSCLE MITOCHONDRIAL biogenesis is one of the most prominent adaptations induced by endurance exercise training (20). At a molecular level, mitochondrial adaptations to exercise are thought to be due to the cumulative effects of the transient increases in the transcripts of mRNA that encode the upregulation of mitochondrial proteins (37). In considering possible contractile induced stressors for activating the acute cell signaling pathways associated with regulation of mitochondrial biogenesis, reductions in carbohydrate (CHO) availability is now emerging as one of the most potent signals (41). For example, in healthy subjects, the acute exercise-induced activation of the signaling kinases AMPK (60, 62) and p38MAPK (8, 12) are greater when preexercise glycogen availability is low. Transcription of several metabolic related genes such as PDK4, CPT-1, CD36, GLUT-4, UCP-3, and HSP72 are also enhanced when exercise is completed with reduced CHO availability before and/or during exercise (9, 11, 13, 43). Furthermore, we (32) and others (15, 21, 54, 63) have demonstrated that chronic exercise (i.e., endurance training) deliberately commenced with reduced endogenous and exogenous carbohydrate availability results in enhanced skeletal muscle oxidative capacity. Despite the emergence of this novel training paradigm, the precise molecular mechanisms underpinning these augmented training adaptations remain elusive. Although both AMPK and p38MAPK signaling has been shown to be increased with CHO restriction (12, 60, 62), their subsequent effects on downstream regulation of PGC-1α remain equivocal. For example, Pilegaard et al. (44) demonstrated that restricting CHO in the recovery phase following acute exercise (so as to minimize muscle glycogen resynthesis) augments expression of PGC-1α compared with provision of CHO to promote muscle glycogen resynthesis. In contrast, restricting CHO before, during, and after exercise (11, 12, 45) did not enhance PGC-1α mRNA expression compared with provision of CHO feeding. Furthermore, we observed that the training-induced increases in PGC-1α content in both the vastus lateralis and gastrocnemius of human skeletal muscle occurs independently of both endogenous and exogenous CHO availability (32). Taken together, these data suggest that acute transcriptional regulation of PGC-1α (as the proposed master regulator of mitochondrial biogenesis) may not be a central regulator of the enhanced training response that is associated with deliberately restricting CHO availability.

In this regard, an emerging regulator of mitochondrial biogenesis, which may be sensitive to CHO availability, is the tumor suppressor protein, p53 (48). Indeed, Saleem et al. (47) demonstrated that p53 knockout mice display reduced mitochondrial function and aerobic capacity. We also recently observed for the first time in human skeletal muscle that p53 phosphorylation increases in a signaling time course that appears related to upstream signaling through AMPK and P38MAPK (5). Given that both of these kinases have been shown to directly phosphorylate p53 in cell culture models (23, 49) and also considering they are upregulated during training with reduced CHO availability (12, 60), it is, therefore, tempting to speculate that p53 is sensitive to reduced CHO availability in contracting human skeletal muscle. Such a hypothesis...
REduced carbohydrate availability and p53 signaling

is particularly attractive as a potential signaling pathway regulating the enhanced training adaptations associated with reduced CHO availability given that p53 is a potent regulator of synthesis of cytochrome-c oxidase 2 (SCO2) and cytochrome-c oxidase (COX) subunit II (30), dynamin-related protein 1 (26), mitofusion 2 (57), mitochondrial transcription factor A (Tfam), and mtDNA content (34, 64).

The aim of the present study was to, therefore, test the hypothesis that reduced CHO availability enhances p53 signaling and expression of metabolic genes associated with regulation of mitochondrial biogenesis and substrate utilization in human skeletal muscle. To this end, we used a repeated-measures design, whereby healthy male subjects performed acute high-intensity interval running (HIT) in conditions of high or low CHO availability. Our chosen experimental design effectively allowed us to examine two extremes of CHO availability, one in which subjects commenced exercise with reduced muscle glycogen (after having performed glycogen-depleting exercise the evening prior) but also refrained from CHO intake before, during, and after HIT exercise. As such, this approach represents an exercise and nutritional intervention in which subjects effectively sleep, train, and recover with low CHO availability. In contrast, the high CHO trial conformed to traditional nutritional athletic guidelines (1), whereby exercise was performed with high CHO before, during, and after exercise. Although our data may initially be considered in the context of enhancing endurance-training adaptations and exercise performance, the potential of p53 as a signaling axis regulating mitochondrial biogenesis also has obvious health implications, given the function of the mitochondrion and the emerging role of p53 in the pathology of cancer, aging, and insulin resistance (55, 56).

METHODS

Subjects. Eight recreationally active men volunteered to participate in the study (mean ± SD: age, 25 ± 5 yr; body mass, 78 ± 8 kg; height, 1.77 ± 0.04 m; V\textsubscript{O2max}, 55 ± 6 ml·kg\textsuperscript{-1}·min\textsuperscript{-1}). The experimental procedures and potential risks associated with the study were explained, and subjects gave written informed consent prior to participation. Subjects refrained from additional exercise outside of the study requirements, as well as from alcohol and caffeine intake for at least 48 h prior to any of the testing sessions. None of the subjects had a history of neurological disease or musculoskeletal abnormality, and none were under pharmacological treatment during the course of the study. The study was approved by the Ethics Committee of Liverpool John Moores University.

Study overview. In a randomized cross-over design separated by a minimum of 7 days, subjects performed an acute bout of high-intensity interval (HIT) running either with high (HIGH) or low carbohydrate availability (LOW). In the LOW condition, subjects consumed a diet low in carbohydrate the day before HIT, performed a glycogen-depleting cycling protocol in the evening prior to HIT, and restricted CHO intake before, during, and after undertaking the HIT protocol itself. In the HIGH condition, subjects reported to the laboratory only on the morning of the main experimental trial after consuming a high-CHO diet the day before, a high-CHO breakfast on the morning of testing and were fed CHO immediately before, during, and after exercise. Muscle biopsies and venous blood samples were obtained at regular intervals during the course of testing. Heart rate (Polar S610i; Kempele, Finland) and ratings of perceived exertion (RPE) (7) were monitored continuously throughout the HIT protocol, and CHO and lipid oxidation rates were collected and analyzed according to the calculation of Peronnet and Massicotte (40).

Preliminary testing. On their first visit to the laboratory, subjects performed a maximal incremental cycling test to volitional fatigue on a Lode ergometer (Daum Electronic Premium 8i, Furth, Germany) for determination of peak power output (PPO). The maximal incremental protocol commenced at 50 W for 2 min, and the work rate was increased by 35 W every minute thereafter, until exhaustion (17). On the second visit to the laboratory (~3 days after visit 1), subjects performed a V\textsubscript{O2max} test using an incremental exercise test performed on a motorized treadmill (HP Cosmos, Nussdorf-Traunstein, Germany). Oxygen uptake was measured continuously during exercise using a CPX Ultima series online gas analysis system (Medgraphics, St. Paul, MN). The test began with a 3-min stage at a treadmill speed of 10 km/h followed by 3-min stages at 12 km/h, 14 km/h, and 16 km/h. Upon completion of the 16 km/h stage, the treadmill inclined by 2% every 3 min thereafter until volitional exhaustion. The V\textsubscript{O2max} was taken as the highest V\textsubscript{O2} value obtained in any 10-s period and was stated as being achieved by the following end-point criteria: 1) heart rate within 10 beats per minute (bpm) of age-predicted maximum, 2) respiratory exchange ratio > 1.1, and 3) plateau of oxygen consumption despite increased workload (14).

Experimental protocol. In the LOW condition, subjects reported to the laboratory at 1800 the evening before the acute bout of HIT running and performed an intermittent glycogen-depleting cycling protocol lasting 68 ± 5 min. The exercise protocol that is used to deplete muscle glycogen is an adapted version of the protocol used by Pedersen et al. (36). Following a 5-min warm-up at 50 W, subjects commenced cycling at 90% of PPO followed immediately by 2 min of recovery at 50% of PPO. This work-recovery protocol was maintained until the subjects were unable to complete two min at 90% PPO, determined as an inability to maintain a cadence of 60 rpm for 15 s. As the subjects had limited cycling experience, their ability to perform 2 min at 90% of PPO was reduced compared with experienced cyclists; therefore, when subjects could not maintain 2 min at 90%, the work bouts were reduced to 1 min, while maintaining the 2-min recovery bouts at 50% of PPO. When the subjects were unable to maintain the 1-min work-recovery bouts, the work period was reduced to 30 s. Once the subjects could not maintain 90% of PPO for 30 s, the intensity was lowered to 80% of PPO and the exercise bouts returned to 2 min before lowering to 1 min and 30 s. When the subjects could not maintain 80% of PPO at 30 s the intensity was lowered to 70% of PPO, and finally 60% of PPO. Exercise was terminated when subjects could not complete 30 s of cycling at 60% of PPO at a cadence corresponding to >60 RPM. This protocol was chosen so as to maximally deplete both Type I and Type II muscle fibers of the subjects’ glycogen stores (24). The activity pattern and total time to exhaustion were recorded, and water was consumed ad libitum throughout exercise. In the 30 min within completion of the glycogen depletion protocol, the subjects consumed a low-carbohydrate snack (<50 g CHO), so as to minimize any muscle glycogen resynthesis (32). After this point, subjects did not consume any further nutrient intake, so that on the subsequent morning, subjects performed the HIT protocol with reduced CHO availability before, during, and after exercise.

HIT protocol. On the morning of the main experimental trial, subjects were required to perform an acute bout of 50-min, high-intensity interval running (HIT), as described previously in our laboratory (4, 5). Briefly, subjects performed 6 × 3-min bouts at a velocity corresponding to 90% V\textsubscript{O2max} interspersed with 6 × 3-min recovery bouts at 50% V\textsubscript{O2max}. The intermittent protocol started and finished with a 7-min warmup and cool down at 70% V\textsubscript{O2max}, culminating in a total of 50 min of exercise at an average intensity of 70% V\textsubscript{O2max}. We chose this model of HIT, given that this particular mode, duration, and relative exercise intensities are not only applicable to athletes but also to clinical populations, as it has been well tolerated by both cardiovascular (59) and metabolically diseased patients (51). Performing HIT in this way also induces greater feelings of perceived enjoyment compared with CONT (even when both protocols are
matched for average intensity, duration, and work done), thereby demonstrating the potential for improved exercise adherence with this intervention (5). Furthermore, this model of HIT is also of relevance as a training modality for both team sport and endurance-based athletes.

Dietary controls. In the 24 h preceding the main experimental trial, subjects consumed a low-CHO diet consisting of 3 g/kg (6.8 ± 0.71 MJ: CHO, 242 ± 25 g; fat, 37 ± 4 g; protein, 94 ± 10 g) or a high-CHO diet consisting of 8 g/kg CHO (14.5 ± 1.5 MJ: CHO, 647 ± 68 g; fat, 54 ± 6 g; protein, 133 ± 40 g). On the day of testing, subjects in HIGH were also provided with a high-CHO breakfast containing 2 g/kg (3.5 ± 0.37 MJ: CHO, 166 ± 17 g; fat, 15 ± 1.5 g; protein, 24 ± 2.5 g) 2 h before commencing the HIT protocol. In addition, in the 10 min before exercise, subjects consumed 8 ml/kg (39.5 ± 4.1 g of CHO) of a CHO beverage (6.4%, Lucozade Sport; Glaxo Smith Kline, Brentford, UK), as well as 3 ml/kg (14.5 ± 1.5 g of CHO) during active recovery periods 2 (19 min) and 5 (31 min). In order for drink provision to occur, subjects removed respiratory equipment for 1 min and consumed fluids through a straw. To account for the inability to measure respiratory gases during this time, the 1-min drink time and subsequent 1 min were discarded from data analysis, and respiratory exchange ratio (RER) data are presented as an average of the remaining 46 min. When undertaking the HIGH trial, subjects consumed 1.2 g/kg body wt (in the form of CHO drinks and snacks) immediately postexercise (after muscle biopsy) and every subsequent hour until the 3-h biopsy was obtained. In contrast, when undertaking the LOW trial, subjects commenced the HIT protocol in a glycogen-depleted and overnight fasted state and also refrained from CHO intake before, during, and after completion of the HIT protocol (subjects were allowed water ad libitum only in the 3-h recovery period leading to the final biopsy).

Muscle biopsies. Muscle biopsies were obtained from separate incision sites (2–3 cm apart) from the lateral portion of the vastus lateralis muscle pre-, post-, and 3 h after the HIT protocol using a Bard Monopry Disposable Core Biopsy Instrument 12 gauge × 10 cm length (Bard Biopsy Systems, Tempe, AZ). Samples were obtained (~60 mg) under local anesthesia (0.5% Marcaine, without adrenaline: cat. no. MD92672) and immediately frozen in liquid nitrogen and stored at −80°C for later analysis.

Muscle glycogen concentration was determined according to the method described by van Looon et al. (53). Approximately 3–6 mg of freeze-dried sample was powdered, dissected free of all visible nonmuscle tissue, and subsequently hydrolyzed by incubation in 500 μl of 1 M HCl for 3–4 h at 100°C. After cooling to room temperature, samples were neutralized by the addition of 250 μl of a 0.12 mol/l Tris per 2.1 mol/l KOH solution saturated with KCl. Following centrifugation, 150 μl of the supernatant was analyzed in duplicate for glucose concentration, according to the hekoxinase method using a commercially available kit (GLUC-HK; Randox Laboratories, Antrim, UK). Glycogen concentration is expressed as millimoles per kilogram dry weight (dw) and intra-assay coefficients of variation was <5%.

Western blot analysis. Approximately 20 mg of frozen muscle was ground to powder and homogenized in 120 μl of ice-cold lysis buffer [25 mM Tris-HCl (pH 7.4), 50 mM NaF, 100 mM NaCl, 5 mM EGTA, 1 mM EDTA, 10 mM Na-pyrophosphatase, 1 mM Na3VO4, 0.27 M sucrose, 1% Triton X-100, 0.1% 2-mercaptoethanol] and supplemented with a protease inhibitor tablet (Complete mini, Roche Applied Science, West Sussex, UK). Homogenates were centrifuged at 14,000 g for 10 min at 4°C, and the supernatant was collected. The protein content of the supernatant was determined using a bicinechonic acid assay (Sigma, Dorset, UK). Each sample was diluted with an equal volume of 2× Laemmli buffer (National Diagnostics, Atlanta, GA) and boiled for 5 min at 100°C. For each blot, a negative control was loaded along with 50 μg of protein from each sample and then separated in Tris-glycine running buffer (10× Tris/Glycine, Geneflow, Staffordshire, UK) using self-cast 4% stacking and 10% separating gels (National Diagnostics). Gels were transferred semi-dry onto nitrocellulose membrane (Geneflow) for 2 h at 200 V and 45 mA per gel in transfer buffers (anode 1; 0.3 M Tris, 20% methanol, pH 10.4; anode 2; 0.25 M Tris, 20% methanol, pH 10.4; cathode; 0.4 M 6-amino hexanoic acid, 20% methanol, pH 7.6). After transfer, membranes were blocked for 1 h at room temperature in TBS-Tween (TBST: 0.19 M Tris pH 7.6, 1.3 M NaCl, 0.1% Tween-20) with 5% nonfat milk. The membranes were then washed for 3 × 5 min in TBST before being incubated overnight at 4°C with antibodies for anti-phospho ACCSer79 (cat. no. 3661S), p38MAPK Tyr180/Thr182 (cat. no. 9212S), p53Ser15 (cat. no. 9282S), and GAPDH (cat. no. 2118L), all at concentrations of 1:1,000 in 1× TBST (all from Cell Signaling Technology, Danvers, MA). The next morning, membranes were washed for a further 3 × 5 min in TBST and subsequently incubated with anti-species horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hemspstead, UK) for 1 h at room temperature. After a further 3 × 5 min washes in TBST, membranes were exposed in a chemiluminescence liquid (SuperSignal, Thermo Fisher Scientific, Rockford, IL) for 5 min. Membranes were visualized using a Bio-Rad Chemi-doc system, and band densities were determined using ImageLab analysis software. Comparative samples from each subject for both exercise protocols were run on the same gel, and all gels were run in duplicate to verify responses. It should be noted that all raw densitometry data were used for statistical analysis purposes so as to compare within-subject responses to both the HIGH and LOW trials. However, because it is technically incorrect to compare densitometry data between gels (and hence, between subjects), for graphical purposes each subject’s preexercise value in both trials was normalized to 1, no error bars are shown for this time point), such that values at postexercise and 3 h postexercise are subsequently expressed as fold change relative to preexercise values. This approach has been used previously by us (5, 32) and other researchers (10, 37).

Real-time RT-PCR. Total RNA was isolated from small muscle biopsies (20–30 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. For further purification of the RNA, Qiagen All Prep DNA/RNA Micro Kit (Qiagen, Crawley, UK) was used following manufacturer’s recommended guidelines. The final volume of the purified RNA samples were 14 μl. RNA quality and quantity were determined using Implen Nanophotometer, Munich, Germany, and the RNA was stored at −80°C. cDNA was synthesized using random hexamers (Applied Biosystems, Foster City, CA) and Superscript III enzyme (Invitrogen), using manufacturer’s protocol. Gene-specific expression data were obtained using custom-designed primers, supplied by Eurofins (MWG Eurofins, Ebersberg, Germany). The probes used were selected from Universal Probe Library and supplied by Roche (Roche Applied Science, Indianapolis, IN). The primers sequences and corresponding probes are shown in Table 1. One microliter of each sample was analyzed in triplicate with negative controls using AB 7500 real-time quantitative PCR instrument (Applied Biosystems). Muscle content for GAPDH, PGC-1α, PDK4, and COXIV mRNA were analyzed using Agilent Brilliant II qPCR Master Mix with Low ROX (Agilent Technologies, Columbia, MD). One microliter of cDNA, 500 nM of primer, and 200 nM of probe were used for each 20-μl reaction.

Expression of SCO2, CPT-1, and Tfam were analyzed using SYBR Green chemistry (Applied Biosystems), where one microliter of cDNA together with 200 nM of final concentration of primers were used for each reaction with total volume of 20 μl. The following cycling parameters were used for each reaction; 95°C for 10 min, then 40 cycles of 95°C for 15 s and annealing/elongation at 60°C for 1 min. Standard dissociation curve analysis was performed for QC purposes when SYBR Green chemistry was used. Data were collected and analyzed using AB SDS 1.43 Software (Applied Biosystems). Changes in mRNA content were calculated according to the 2−ΔΔCt method, where GAPDH was used as the housekeeping gene (18). mRNA content levels were normalized to GAPDH mRNA levels, and all samples were expressed relative to
HIGH pre, which acted as the control sample (43). Gene expression data from the preexercise and 3-h biopsy samples are presented only as unpublished observations in our laboratory typically show that many of the exercise-responsive genes do not show any significant changes immediately postexercise.

**Blood analyses.** Upon arrival at the laboratory on the morning of the testing, an antecubital cannula (NHS Supply Chain, Alfreton, UK) was inserted, and a preexercise blood sample was drawn. Blood samples were subsequently drawn during active recovery bouts 3 (25 min) and 6 (43 min) during exercise, followed by immediately postexercise, +1 h recovery, +2 h recovery, and +3 h recovery. Samples were collected into Vacutainers containing EDTA, lithium heparin, or serum and stored on ice (or at room temperature for serum samples) until centrifugation at 1,500 g for 15 min at 4°C. Following centrifugation, aliquots of plasma and serum were stored at −80°C for later analysis. Samples were analyzed for plasma glucose, lactate, nonesterified fatty acid (NEFA), glycerol (Randox Laboratories, Ant- rim, UK), and insulin (Cobas, Roche Diagnostics, Indianapolis, MN) concentration using commercially available kits.

**Statistical analysis.** Statistical analysis was conducted using the Statistical Package for Social Sciences software program (SPSS; version 17, IBM, Armonk, NY). Metabolic responses (i.e., blood metabolites and muscle glycogen data), Western blot and mRNA data, and condition were also evident (see Fig. 2).

### RESULTS

**Exercise variables.** There was no difference in average heart rate ($P = 0.08$), $V_{O_2}$ ($P = 0.38$), and RPE ($P = 0.19$) during exercise between the HIGH and LOW conditions (see Table 2). In contrast, there was a significant difference in RER during exercise in HIGH compared with LOW ($P = 0.008$). As such, total CHO oxidation was significantly greater in HIGH compared with LOW ($P = 0.001$), whereas total lipid oxidation was greater in LOW compared with HIGH ($P = 0.004$) (Table 2).

**Muscle glycogen and plasma/serum metabolites.** The exercise and nutritional intervention was effective in ensuring there was a significant difference between HIGH and LOW ($P < 0.001$) for muscle glycogen concentration across all time points (see Fig. 1). Furthermore, the exercise-induced decreases in muscle glycogen ($P = 0.02$) were greater in HIGH compared with LOW ($P = 0.01$), whereby total glycogen utilization during exercise was $142 \pm 34$ and $30 \pm 12$ mmol/kg dw, respectively. In accordance with the provision of CHO before, during, and after exercise, both plasma glucose and serum insulin displayed significant main effects (all $P < 0.01$) for time and condition (see Fig. 2), such that both variables were higher in HIGH compared with LOW across all time points. In contrast, the restriction of CHO feeding in LOW and reduced serum insulin concentrations ensured elevated plasma NEFA and glycerol across all time points in LOW compared with HIGH, such that such main effects (all $P < 0.01$) of time and condition were also evident (see Fig. 2).

**Muscle signaling.** The changes in phosphorylation (P-) of ACCSer79, a downstream marker indicative of AMPK activity, during exercise were dependent on condition ($P = 0.04$), such that exercise in LOW induced a threefold increase immediately postexercise compared with no change in HIGH (see Fig. 3A). Similarly, P-p53Ser15 displayed a significant difference between conditions ($P = 0.02$) with LOW demonstrating a 2.7-fold increase 3 h postexercise compared with no change in HIGH (see Fig. 3B). There was no difference in basal phos-

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### Table 1. Forward and reverse primer sequences used for quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe #</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AGCCACATGCTGAGACAC</td>
<td>GCCCAATAGCCAAATGCC</td>
<td>60</td>
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<tr>
<td>PDK4</td>
<td>CTGCTATACAGAGGGCTGATCA</td>
<td>TGGCTATTTGAACTTTGAGTG</td>
<td>31</td>
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<tr>
<td>PGC-1a</td>
<td>GACATACCTGCCAGCAGTCA</td>
<td>ATATATCGACGCGCTGTT</td>
<td>–</td>
</tr>
<tr>
<td>SCO2</td>
<td>GACGCAAAAGCAGACAGA</td>
<td>GTGACTCGACAGGTGGAAT</td>
<td>–</td>
</tr>
<tr>
<td>COXIV</td>
<td>CCATTGCAAACAGCTGTC</td>
<td>CAGCAAAGCTTTGTAAGT</td>
<td>20</td>
</tr>
<tr>
<td>Tjα</td>
<td>TGCCCAATGTCGCAAAGACCTG</td>
<td>GTTCCCTGCAAAGCTGGA</td>
<td>–</td>
</tr>
</tbody>
</table>

# denotes Roche UPL probe number.

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### Table 2. Exercise variables during the 50-min HIT running protocol

<table>
<thead>
<tr>
<th></th>
<th>High</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR, bpm</td>
<td>163 ± 11</td>
<td>157 ± 11</td>
</tr>
<tr>
<td>$V_{O_2}$ ml·kg$^{-1}$·min$^{-1}$</td>
<td>37.6 ± 3.9</td>
<td>36.6 ± 3.9</td>
</tr>
<tr>
<td>% $V_{O_2_{max}}$</td>
<td>69 ± 4</td>
<td>67 ± 3</td>
</tr>
<tr>
<td>RER, AU</td>
<td>0.08 ± 0.02</td>
<td>0.90 ± 0.05†</td>
</tr>
<tr>
<td>Total CHO oxidation, g</td>
<td>177 ± 17</td>
<td>122 ± 26†</td>
</tr>
<tr>
<td>Total fat oxidation, g</td>
<td>8 ± 1</td>
<td>23 ± 10†</td>
</tr>
<tr>
<td>RPE, AU</td>
<td>14.4 ± 1.2</td>
<td>14.9 ± 0.9</td>
</tr>
</tbody>
</table>

HIT, high-intensity interval running; bpm, beats per minute; HR, heart rate; RER, respiratory exchange ratio; CHO, carbohydrate; RPE, ratings of perceived exertion. †Significant difference between conditions ($P < 0.05$).
phorylation status of both ACC and p53 between HIGH and LOW (P > 0.05). In contrast to the exercise-induced changes observed in P-ACC and P-p53, there was no change in P-p38MAPK \( \text{Tyr}^{180/182} \) following exercise in either the LOW or HIGH condition (data not shown).

**Gene expression.** There was a significant effect of condition for mRNA content of PGC-1α (P = 0.05), COXIV (P = 0.05), Tfam (P = 0.02), PDK4 (P = 0.016), and a trend for CPT1 (P = 0.09), such that both preexercise and postexercise values were higher in LOW compared with HIGH (see Fig. 4). SCO2 mRNA displayed no significant effect of CHO availability (P = 0.1). Exercise increased PGC-1α expression in both HIGH and LOW (P = 0.01), although the magnitude of effect (P = 0.96) was not affected by CHO availability. In contrast, no effects of exercise was observed for COXIV (P = 0.26), Tfam (P = 0.18), PDK4 (P = 0.92), CPT1 (P = 0.29), and SCO2 (P = 0.18) in either the HIGH or LOW trials (see Fig. 4).

**DISCUSSION**

The aim of the present study was to test the hypothesis that exercising with reduced CHO availability enhances p53 signaling and expression of metabolic genes associated with the regulation of mitochondrial biogenesis and substrate utilization in human skeletal muscle. We were motivated by the possibility that early signaling through p53 may be a potential contributing signaling mechanism underpinning the enhanced oxidative training adaptations associated with reduced CHO availability (15, 21, 32, 54-63). To our knowledge, we provide the first report that in contracting human skeletal muscle, p53 phosphorylation is enhanced during conditions of reduced CHO availability before, during, and after exercise. Given the emerging role of p53 in promoting exercise capacity (30, 47) and in the pathology of metabolic disorders, such as cancer, aging, and insulin resistance (56), we, therefore, consider our data to be of relevance for both athletic and clinical populations.

To address our aim, we adopted an experimental model, whereby our LOW trial consisted of exercise performed in a glycogen-depleted, overnight-fasted, and CHO-restricted state. In contrast, exercise in the HIGH condition was performed after a 24-h CHO-loading strategy, as well as consuming CHO before, during, and after exercise in accordance with typical doses advised to athletic populations (1). This model of exercise and nutritional manipulation was successful in achieving two extremes of endogenous and exogenous CHO availability and induced pronounced changes in regulation of substrate utilization. In agreement with previous reports (43, 44), we observed that both resting and postexercise PDK4 and CPT1 mRNA were increased in LOW compared with HIGH, thus reflecting early transcriptional regulation of two key enzymes, regulating both CHO and lipid metabolism. When examined at the whole body level, exercise in LOW resulted in lower RER values and three times greater total lipid oxidation compared with HIGH, despite identical work done and the relatively intense nature of our exercise protocol.

Activation of AMPK is thought to be due to perturbations in the AMP:ATP ratio (16) but also by low muscle glycogen (60), the latter possibly due to the presence of a glycogen binding domain in the β-subunit (31). As a downstream marker of AMPK activity (22, 27, 33, 35), we examined the phosphorylation status of ACC\(^{\text{Ser}79}\). Despite no evidence of greater AMPK activity preexercise, we observed a three-fold increase in ACC\(^{\text{Ser}79}\) in LOW immediately postexercise compared with no change in HIGH, a finding in agreement with other authors (60, 62). Unfortunately, it is difficult to determine from our
increases in human skeletal muscle at 3 h following both studies (47), we also observed that p53Ser15 phosphorylation in cancer cells (6), an emerging function of p53 is its role in the regulation of mitochondrial biogenesis in skeletal muscle (30, 47). In agreement with data from rodent studies (47), we also observed that p53Ser15 phosphorylation increases in human skeletal muscle at 3 h following both CHO restriction, although further studies should now attempt to initially determine whether there was an actual effect of CHO availability on p38MAPK (50), as we also demonstrate herein that p53Ser15 phosphorylation increases at 3 h postexercise. Furthermore, although p38MAPK can also regulate p53 phosphorylation in cell culture (49), this mechanism may not underpin p53 regulation in contracting skeletal muscle given that we observed no consistent effects of exercise or CHO availability on p38MAPK Tyr180/Thr182 phosphorylation (data not shown). Moreover, it is noteworthy that the effects of CHO availability on p38MAPK remain inconsistent with some authors observing no effect (62), yet others observing enhanced p38MAPK Tyr180/Thr182 phosphorylation when preexercise CHO availability is reduced (12). Although we acknowledge that other currently unknown upstream kinases may be regulating p53 activation during exercise, AMPK appears a feasible candidate based on available data at present.

It is also important to consider the potential role of increased free fatty acid (FFA) availability as signaling molecules regulating training adaptation given that when CHO availability is reduced, FFA availability is concomitantly increased. Indeed, Yeo et al. (61) observed that 5 days of a high-fat diet followed by 1 day of CHO restoration increased resting AMPK activity, as well as the magnitude of exercise-induced increase in pACC Ser221. As such, it is possible that increased circulating FFA availability may be regulating increased AMPK activity (through a currently unknown mechanism) during exercise thereby leading to downstream p53 phosphorylation. It is, therefore, difficult to ascertain from the present data whether it is low CHO or increased FFA availability that is the primary signaling mechanism. However, conflicting data for a beneficial role of lipid availability in regulating oxidative adaptation of skeletal muscle also exist, given that 3 days of high-fat feeding reduces expression of genes involved in oxidative phosphorylation (50) and that pharmacological inhibition of plasma FFA availability does not impair exercise-induced increases of those genes involved in regulation of mitochondrial biogenesis or substrate utilization (52, 58).

In regard to downstream targets of p53 related to mitochondrial biogenesis, we observed no effect of exercise on SCO2 or continuous steady-state and high-intensity interval exercise in a temporal pattern that may be related to upstream signaling through both AMPK and p38MAPK (5). The present data confirm our initial observation (5) (subjects in the previous study were fasted before, during, and after exercise, and initial glycogen levels were ~300 mmol/kg dw), as we also demonstrate herein that p53Ser15 phosphorylation increases at 3 h postexercise. However, we also provide novel data by demonstrating that p53 activation as a consequence of muscle contraction appears dependent on CHO availability, so much so that this effect was abolished in conditions of high CHO availability (i.e., with preexercise muscle glycogen levels ~500 mmol/kg dw and CHO provision before, during, and after exercise). Collectively, these data suggest there may be an initial CHO or muscle glycogen threshold for p53 signaling to occur.

Given that glucose deprivation results in p53 phosphorylation in cell culture in a manner that is dependent upon upstream AMPK activation (23), it is possible that this signaling axis may also be regulating p53 activation in contracting skeletal muscle. Such a hypothesis is particularly attractive considering that we only observed phosphorylation of ACC Ser79 immediately postexercise in the LOW trial, which subsequently may result in a temporal and coordinated phosphorylation of p53 at 3 h postexercise. Furthermore, although p38MAPK can also regulate p53 phosphorylation in cell culture (49), this mechanism may not underpin p53 regulation in contracting skeletal muscle given that we observed no consistent effects of exercise or CHO availability on p38MAPK Tyr180/Thr182 phosphorylation (data not shown). Moreover, it is noteworthy that the effects of CHO availability on p38MAPK remain inconsistent with some authors observing no effect (62), yet others observing enhanced p38MAPK Tyr180/Thr182 phosphorylation when preexercise CHO availability is reduced (12). Although we acknowledge that other currently unknown upstream kinases may be regulating p53 activation during exercise, AMPK appears a feasible candidate based on available data at present.
Tfam expression, although it is possible that measurements at 3 h postexercise is not sufficient to detect exercise-induced changes in gene expression. Despite the apparent lack of an exercise effect, we did observe an effect of condition, such that Tfam mRNA levels were higher both before and after exercise in LOW compared with HIGH. However, on the basis of our chosen experimental design, it is difficult to determine whether the enhanced gene expression is due to low CHO availability per se and/or a residual effect of the glycogen-depleting exercise protocol of the evening before. Indeed, the magnitude of elevation of Tfam in LOW compared with HIGH (i.e., ~1.5-fold differences) is consistent with time-course studies, which show significant increases between 4 and 24 h postexercise (37), which is similar to the time course of our depletion protocol given that our preexercise biopsy was sampled within 10–12 h upon exercise completion. We deliberately chose not to include a glycogen depletion protocol in our HIGH trial, as the particular time scale (i.e., <12 h between the evening depletion protocol and the main trial on the subsequent morning) would not likely permit restoration of muscle glycogen to high levels (42), especially in our cohort of recreationally active males as opposed to endurance-trained subjects (19). Although we acknowledge that we could have included a depletion protocol in an earlier timescale (e.g., 24–36 h prior to the main trial), adopting this design would have required the inclusion of an alternative nutritional strategy, such as high-fat or protein feeding, so as to provide appropriate energy intake and as such, would have introduced confounding variables, which would affect gene expression (38, 39).

The potential carryover effect of the evening depletion protocol may also be contributing to the effects of condition on both PGC-1α and COXIV expression in that both genes were also elevated in LOW compared with HIGH. However, in relation to PGC-1α expression, it is noteworthy that exercise increased mRNA levels by similar magnitudes in both trials (~3-fold increases), thus providing further evidence (11, 12, 45) that acute transcriptional regulation of the PGC-1α gene may not be influenced by CHO availability and moreover, p53 regulation. Nevertheless, future studies would benefit from measuring both nuclear and mitochondrial translocation of PGC-1α (46) in conditions of both high- and low-CHO availability so as to provide a more definitive assessment of regulation of this pathway.

**Perspectives and Significance**

We present novel data by demonstrating that exercise-induced p53 phosphorylation in human skeletal muscle appears dependent on CHO availability, which may be regulated, in part, through upstream signaling by AMPK. Given the emergence of p53 as a regulator of mitochondrial biogenesis in skeletal muscle (34, 47), the nutritional modulation of contraction-induced p53 activation is likely to have implications for both athletic and clinical populations. Indeed, numerous investigators have demonstrated a functional role of p53 in improving physical performance, as demonstrated by improved exercise capacity in wild-type animals compared with p53 knockout animals during fatiguing swimming (30), treadmill running...
(34), and electrical stimulation (47) protocols. Furthermore, considering the role of p53 in promoting a shift from glycolysis toward lipid metabolism (6) and the fact that training with reduced CHO availability promotes lipid utilization during submaximal exercise (21, 63), early signaling through p53 may, therefore, represent an additional potential signaling pathway which regulates oxidative adaptations to endurance training (i.e., increased mitochondrial volume and shifts in substrate utilization), thereby exerting potent effects on subsequent endurance performance. Moreover, because reduced p53 function is associated with tumor development (28), insulin resistance (3), and reduced longevity (29), the combined effects of exercise and low CHO availability on p53 activity, therefore, represents potential exercise interventions to improve various indices of human health. Clearly, further longitudinal studies examining the physiological function of p53 in human cells and tissues are now warranted.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Contributions of authors: J.D.B., M.J.G., G.L.C., B.D., and J.P.M. conception and design of research; J.D.B., J.L., Z.I., A.J.C., W.G., and J.P.M. performed experiments; J.D.B., J.L., and J.P.M. analyzed data; J.D.B., J.L., A.J.C., M.J.G., G.L.C., and J.P.M. interpreted results of experiments; J.D.B. and J.P.M. prepared figures; J.D.B. and J.P.M. drafted manuscript; J.D.B., J.L., M.J.G., G.L.C., and J.P.M. interpreted results of experiments; J.D.B. and J.P.M. approved final version of manuscript.

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