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Physiological and molecular responses to an acute bout of reduced-exertion high-intensity interval training (REHIT)

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ABSTRACT

Purpose: We have previously shown that six weeks of reduced-exertion high-intensity interval training (REHIT) improves $\dot{V}O_2max$ in sedentary men and women, and insulin sensitivity in men. Here we present two studies examining the acute physiological and molecular responses to REHIT.

Methods: In Study 1, five men and six women (age: 26±7 y, BMI: 23±3 $kg \cdot m^{-2}$, $\dot{V}O_2max$: 51±11 $ml \cdot kg^{-1} \cdot min^{-1}$) performed a single 10-min REHIT cycling session (60 W and two 20-s 'all-out' sprints), with *vastus lateralis* biopsies taken before and 0, 30 and 180 min post-exercise for analysis of glycogen content, phosphorylation of AMPK, p38 MAPK and ACC, and gene expression of PGC1α and GLUT4. In Study 2, eight men (21±2 y; 25±4 $kg \cdot m^{-2}$; 39±10 $ml \cdot kg^{-1} \cdot min^{-1}$) performed three trials (REHIT, 30 min cycling at 50% of $\dot{V}O_2max$, and a resting control condition) in a randomised cross-over design. Expired air, venous blood samples, and subjective measures of appetite and fatigue were collected before and 0, 15, 30 and 90 min post-exercise.

Results: Acutely, REHIT was associated with a decrease in muscle glycogen, increased ACC phosphorylation, and activation of PGC1α. When compared to aerobic exercise, changes in $\dot{V}O_2$, RER, plasma volume, and plasma lactate and ghrelin were significantly more pronounced with REHIT, whereas plasma glucose, NEFAs, PYY, and measures of appetite were unaffected.

Conclusions: Collectively these data demonstrate that REHIT is associated with a pronounced disturbance of physiological homeostasis and associated activation of signalling pathways, which together may help explain previously observed adaptations once considered exclusive to aerobic exercise.

Keywords:

HIT, glycogen, signalling pathways, AMPK, exercise metabolism, energy balance

Abbreviations:

ACC: Acetyl-CoA carboxylase, AMPK: AMP-activated protein kinase, ANOVA: analysis of variance, ATP: adenosine triphosphate, AUC: area under the curve, β-HAD: β-hydroxy acyl-CoA dehydrogenase, BMI: body-mass index, COX IV: cytochrome c oxidase, EPOC: excess post-exercise oxygen consumption, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, GLUT4: glucose transporter 4, HIT: high-intensity interval training, HRmax: maximal heart rate, p38 MAPK: p38 mitogen-activated protein kinase, NEFA: non-esterified fatty acid, PCR: polymerase chain reaction, PGC1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PYY: peptide YY, REHIT: reduced-exertion HIT, RER: respiratory exchange ratio, RPE: rating of perceived exertion, $\dot{V}O_2max$: maximal oxygen uptake capacity.

INTRODUCTION

Despite clear recommendations on the minimal amount of physical activity for achieving health benefits and reducing risk of chronic disease (Garber et al. 2011), the majority of people in the developed world remain largely sedentary (Tucker et al. 2011; Colley et al. 2011; Allender et al. 2008). As 'lack of time' has been identified as one of the main barriers to becoming and remaining physically active (Korkiakangas et al. 2009; Reichert et al. 2007), over the past decade research has focused on high-intensity interval training (HIT) as a time-efficient alternative to aerobic exercise (Gillen and Gibala 2014). Although initial studies have convincingly demonstrated equal or better health benefits with various HIT protocols compared to much larger volumes of aerobic exercise (Gibala et al. 2006; Rakobowchuk et al. 2008; Shepherd et al. 2013; Cocks et al. 2013; Burgomaster et al. 2008; Trapp et al. 2008; Gillen et al. 2013; Little et al. 2010), these HIT protocols tend to be very strenuous and as such are unlikely to be adhered to by sedentary populations. Furthermore, most HIT protocols are not as time-efficient as sometimes claimed, with the total time per exercise session generally exceeding 20-30 minutes (Gibala et al. 2006; Rakobowchuk et al. 2008; Shepherd et al. 2013; Cocks et al. 2013; Burgomaster et al. 2008; Trapp et al. 2008; Gillen et al. 2013; Little et al. 2010). However, more recently we (Metcalfe et al. 2012) and others (Gillen et al. 2014; Hazell et al. 2010; Tjønna et al. 2013) have demonstrated that performing HIT protocols consisting of fewer and/or shorter sprints does not appear to attenuate the associated training adaptations, as long as the exercise intensity remains high.

To date, the lowest amount of high-intensity exercise demonstrated to be associated with health benefits has been our reduced-exertion HIT protocol (REHIT), which incorporates two 20-s all-out cycle sprints in a 10-min low-intensity exercise session (Metcalfe et al. 2012). Six weeks of three weekly REHIT sessions improved $\dot{V}O_2max$ in sedentary men and women, as well as insulin sensitivity in men. Support for these results was recently provided by Gillen et al. (2014) who used a similar protocol but with three 20-s sprints instead of two. Similar improvements in $\dot{V}O_2max$ compared to our study were associated with further aerobic adaptations in skeletal muscle (increased protein content of COX IV and increased maximal activities of citrate synthase and β-HAD). A sex difference was again apparent in measures of glycaemic control, potentially partially explained by the significantly lower training-induced increase in muscle GLUT4 levels in women compared with men. As the REHIT protocol is both highly time-efficient (total training time of 30 min per week) and manageable (mean RPE ~13 or 'somewhat hard') it may provide a suitable alternative or adjunct to current physical activity recommendations. However, further larger studies will be needed to confirm these initial positive observations (Metcalfe et al. 2012; Gillen et al. 2014).

At the same time it also needs to be established why such a small volume of high-intensity exercise (total 40-60 s per session) appears to be effective for promoting beneficial skeletal muscle and whole-body adaptations. At the molecular level, adaptation to exercise is thought to result from cumulative transient increases in gene transcription (and protein synthesis) that follow each acute exercise bout, with the metabolic perturbations induced by contraction activating various signalling kinases which, in turn, target downstream transcriptional coactivators and regulators (Perry et al. 2010). Previous studies investigating more time-consuming and more strenuous HIT protocols have demonstrated a molecular

response which includes a 20-30% drop in muscle glycogen concentrations, substantial lactate accumulation, an altered ATP:ADP ratio, and increases in AMPK activity (1.4-fold), p38 MAPK activity (1.5-fold), and a 6-fold increase in mRNA expression of PGC1α (Esbjornsson-Liljedahl et al. 1999; Gibala et al. 2009; Parolin et al. 1999). Furthermore, the acute disturbance in muscle metabolism is associated with a substantial disruption of whole-body/systemic homeostasis, including a prolonged increase in post-exercise oxygen consumption (EPOC), and alterations in the appetite regulating hormones acylated ghrelin and peptide YY in a direction which would be expected to suppress appetite (Hazell et al. 2012; Williams et al. 2013; Deighton et al. 2013). Such changes may explain why studies have observed beneficial alterations in body composition following several weeks of HIT (Whyte et al. 2010; Gillen et al. 2013; Heydari et al. 2012; Trapp et al. 2008). However, to date little is known about whether these acute responses to 'classic' HIT are retained with shorter, more manageable protocols such as REHIT. This represents an important area of research since it may provide information about the initial stimuli responsible for the activation of signalling pathways and chronic phenotypic adaptations.

Here we present two studies investigating the acute responses to REHIT. In the first study we measured glycogen degradation, AMPK and p38 MAPK activation, and changes in PGC1α and GLUT4 gene expression in skeletal muscle following REHIT. Given that glycogen utilisation appears to be most prominent during the first 15-s of the first sprint during a repeated sprint protocol (Parolin et al. 1999), and glycogen concentrations appear to play an important role in the regulation of AMPK and p38 MAPK (Philp et al. 2012), we hypothesised that REHIT would be associated with a 20-30% reduction in muscle glycogen, activation of AMPK and p38 MAPK, and increases in GLUT4 and PGC1α gene expression. In study 2, we examined differences between REHIT and a bout of moderate-intensity aerobic exercise in a range of post-exercise physiological responses including oxygen consumption and appetite regulating hormones. As intensity has been highlighted as the key determinant of exercise-induced changes in these variables (Hazell et al. 2012; Deighton et al. 2013), we hypothesised that REHIT would be associated with a more pronounced disturbance of physiological homeostasis and a greater suppression of appetite compared to aerobic exercise.

METHODS

Study 1

Subjects

Twelve healthy active men (n=6) and women (n=6) agreed to take part in Study 1. We were unable to obtain usable muscle biopsies in one male subject so the final sample size was n=11 (men: mean±SD age 26±7 y, BMI 23±2 kg·m^{-2}, $\dot{V}O_2$max 58±13 ml·kg^{-1}·min^{-1}; women: age 26±7 y, BMI 23±3 kg·m^{-2}, $\dot{V}O_2$max 46±5 ml·kg^{-1}·min^{-1}). The study protocol received ethics approval from the NHS South West Research Ethics Committee (NHS REC Reference: 12/SW/0018) and all subjects provided written informed consent after being advised of the full experimental procedures and potential risks.

Pre-experimental procedures

Prior to the main experimental trial subjects visited the lab on three separate occasions. During the initial visit maximal oxygen uptake capacity ($\dot{V}O_2$max) was determined during a continuous incremental ramp test to exhaustion (30 W·min^{-1} ramp) on an electrically-braked cycle ergometer (Lode, Excalibur Sport, the Netherlands) with $\dot{V}O_2$ measured continuously using an online metabolic cart (ParvoMedics TrueOne 2400, Utah, USA). $\dot{V}O_2$max was determined as the highest value of a 15-breath rolling average. In all tests two or more of the following criteria were met: heart rate within 10 beats of age-predicted maximum, RER ≥ 1.10, RPE ≥ 19, and/or volitional exhaustion.

On separate days, with at least 2 days rest in between, each participant performed two REHIT familiarisation sessions on a mechanically-braked cycle ergometer (Monark 894e, Vansbro, Sweden), consisting of 10 min of cycling at 60 W interspersed with two all-out sprints against a resistance equivalent to 7.5% of bodyweight. Just before each sprint subjects increased their pedal cadence to their maximal speed, the braking torque was applied to the ergometer, and subjects sprinted maximally against the braking torque for 10 (first visit) or 20 s (second visit) as previously described (Metcalfe et al. 2012). The second familiarisation session was identical to that used during the main experimental trial.

Experimental procedures

Between 1 and 3 weeks following the final familiarisation, and having refrained from strenuous physical activity (2 days) and alcohol and caffeine (1 day), subjects attended the lab at 8 am following an overnight fast. After resting quietly for 15 min two small incisions (~5 mm) were made in the *vastus lateralis* of one leg under local anaesthetic (1% lidocaine) for collection of muscle biopsy samples using the Bergstrom needle technique adapted for suction (Tarnopolsky et al. 2011). One of these incisions was used for collection of the baseline muscle sample and immediately closed with sterile strips, whilst the second was initially covered with sterile gauze and used for collection of the sample immediately after performing the 10-min REHIT bout (as described above). Power output during the sprints was recorded continuously using Monark Anaerobic Test Software (Monark, Vansbro, Sweden), and used to calculate peak, average and end power output for each sprint. Further muscle biopsy samples were collected at 30 min and 180 min post-exercise through separate incisions and on the opposite leg to the first two samples. The starting leg for collection of

the muscle samples was counterbalanced and the first biopsy on each leg was always taken at a more distal point to the second (~3 cm between incisions). Following each muscle biopsy procedure, the muscle sample was immediately snap-frozen and stored in liquid nitrogen until subsequent analysis. Throughout the recovery period subjects rested and remained fasted (i.e. water only).

Skeletal muscle glycogen analysis

Approximately 20 mg of frozen wet tissue was freeze-dried, powdered, and dissected free of visible blood and connective tissue. Samples were then suspended in dH_2O (0.5 mg/100 µl) and homogenised on ice; first manually using a dounce homogeniser (30-40 passes) and then by sonication (4 x 10-s bursts at 10 microns with 50 s in between bursts). Homogenates were then heated at 95°C for 5 min and centrifuged at 13000 rpm for 5 min before the glycogen content of the resulting supernatant was determined in duplicate using an enzymatic assay adapted for fluorimetry according to the manufacturer's instructions (Abcam Glycogen Assay Kit (ab65620), Cambridge, UK). Glycogen concentrations were expressed as glucosyl units (mmol·kg^{-1} dry weight) and background glucose concentrations were determined and subtracted from the final glycogen readings. The intra-assay coefficient of variation was ~3%. Due to poor sample quality we were unable to analyse glycogen levels for two subjects at t=0. As the level of glycogen did not differ between the three post-exercise time-points the individual glycogen value for t=30 was used for this time-point for the purpose of statistical analysis.

Western blotting

For each sample approximately 30 mg of frozen wet tissue was freeze-dried, powdered and dissected free of visible blood and connective tissue, and added to ice cold lysis buffer (20 mM Tris (pH 7.8), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1% Triton X-100, 10% (w/v) glycerol, 1 mM EDTA, 1 mM dithiothreitol) supplemented with protease (Thermo Scientific) and phosphatase inhibitor cocktail (Millipore). The samples were homogenised on ice using a dounce homogeniser (40-50 passes) and incubated for 1 hr at 4°C with continuous rotation. Samples were then centrifuged at 13000 rpm for 5 min and the supernatants collected. The protein content of the supernatant was determined using a bicinchoninic acid assay (BCA) (Thermo Scientific). For each blot equal amount of protein (50 µg of each sample) was loaded per lane and separated by SDS-PAGE on 7% (p-ACC^{S79} (acetyl CoA carboxylase), p-$AMPK^{T172}$ and total AMPK) or 10% (p-p38 $MAPK^{T180}$ and total p38 MAPK) Tris-glycine SDS-polyacrylamide gels. Gels were electroblotted by semi-dry transfer onto nitrocellulose membranes. After transfer, membranes were washed in TBS-T (Tris-buffered saline buffer containing Tween 20; 0.09% NaCl, 1M Tris-HCl pH 7.4, 0.1% Tween 20,) and then incubated for 1 hr in a blocking solution consisting of 5% skimmed milk (Marvel, Premier International Foods Ltd, UK) made up with TBS-T. Membranes were then incubated overnight at 4°C with primary antibodies (1:1000 dilution) against p-$AMPK^{T172}$, total AMPK, p-ACC^{S79}, p-p38 $MAPK^{T180}$, and total p38 MAPK (all from Cell Signalling Technologies except p-ACC^{S79} which was from Millipore). After incubation, membranes were washed thoroughly in TBS-T and incubated with a 1:4000 dilution of anti-species IgG horseradish peroxidase-conjugated secondary antibodies made up in blocking solution. After further washes,

membranes were exposed to an enhanced chemiluminescence reagent and visualised using a chemiluminescent imager (EpiChemi II Darkroom, UVP, Upland, Canada). Band densities were quantified using Labworks Image Acquisition and Analysis Software Version 4.0.0.8 for Windows (UVP, Upland, Canada). All samples taken from each subject were run on the same gel. Blots for p-AMPK and p-p38 were normalised to the total amount of each respective protein, and p-ACC was normalised to total AMPK content. Post-exercise time-points were expressed as a fold-change relative to pre-exercise values. Due to poor sample quality we were unable to analyse two samples at t=0 and one at t=180. These missing data points have been replaced with the mean response for the group at that time point for the purpose of statistical analysis.

Real-time PCR

Gene expression analysis was performed on muscle samples collected at baseline and 180 min post-exercise for samples with sufficient tissue left after analysis of glycogen and protein levels (n=5). Total RNA was extracted from ~10 mg of frozen muscle using Trizol reagent according to the manufacturer's instructions (Life Technologies Ltd, Paisley, UK). Briefly, samples were suspended in 1 ml of Trizol, homogenized using a mechanical glass-Teflon (or Potter-Elvenheim) homogeniser, and then mixed with chloroform to produce an aqueous RNA phase which was subsequently mixed with isopropanol to precipitate RNA. The resulting RNA pellet was then washed with 75% ethanol, dried and resuspended in nuclease-free water. RNA quantity and quality was subsequently assessed using a NanoVue spectrophotometer (Thermo Fisher Scientific, Leicester, UK). RNA samples with a ratio of absorbance at 260 and 280 nm of <1.6 were considered of poor quality and were not processed further. RNA was stored at -80°C before being reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, Paisley, UK). Quantitative real-time PCR for GLUT4 (Hs00168966_m1) and PGC1α (HS01016719_m1) relative to GAPDH (Hs.pt.39a.22214836; Integrated DNA technologies) was carried out using TaqMan Fast Universal, No AmpErase UNG chemistry in combination with a StepOne Fast 7500 real-time PCR system (all Applied Biosystems, Warrington, UK). cDNA (45 ng) was amplified under the following conditions: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. All PCR reactions were carried out in duplicate and a non-template control (i.e. nuclease-free water) was included for each housekeeping gene/gene of interest on all plates. The resulting amplification plots were used to generate C_T values for genes of interest/housekeeping gene and the ΔC_T was calculated. The comparative C_T method was used to calculate gene expression at 180 min post-exercise relative to baseline.

Study 2

Subjects

Eight healthy young men (mean±SD age: 21±2 y; BMI: 25±4 kg·m^{-2}; $\dot{V}O_2$max: 39±10 ml·kg^{-1}·min^{-1}) volunteered to take part in Study 2. Subjects were requested to maintain their normal diet and patterns of physical activity throughout the study period. The study protocol was given ethics approval by the Research Ethics Approval Committee for Health (REACH

Reference: EP 14/15 214) at the University of Bath and all subjects provided written informed consent after being advised of the full experimental procedures and potential risks.

Pre-experimental procedures

Prior to the main experimental trials subjects visited the laboratory on four separate occasions. During the initial visit subjects underwent a continuous incremental cycling test to determine $\dot{V}O_2max$ as described above for Study 1. Subjects next performed three familiarisation sessions (two for REHIT and one for aerobic exercise (AER)), each lasting approximately 15 min. The AER familiarisation session was mainly used to check the intensity predicted to elicit 50% of $\dot{V}O_2max$. Subjects cycled for 10 min at the prescribed intensity and an expired air sample was collected during the final min. If necessary, adjustments were made to the intensity used during the main trials. The two REHIT familiarisation sessions were performed as described above for Study 1.

Experimental procedures

Subjects completed three main experimental trials (REHIT, AER and REST) in a randomised cross-over design. Subjects were asked to refrain from strenuous physical activity (2 days) and alcohol or caffeine (1 day) prior to each experimental trial. In addition, prior to the first trial a 24-hr food record was completed and subjects were asked to replicate this diet before each subsequent trial. To ensure adequate hydration subjects were asked to drink ~half a litre of water on the morning of each experimental trial.

Subjects reported to the laboratory between 8:30 and 9:30 am having fasted overnight from 10:00 pm the previous evening. After resting quietly for 15 min, a 5-min baseline expired air sample, a venous blood sample, and subjective ratings of appetite and physical tiredness were collected as described below. These measurements were repeated at 0, 15, 30 and 90 min post-exercise. During the post-exercise period subjects rested quietly and remained fasted with water allowed *ad libitum* throughout each trial.

Both exercise trials were performed on a mechanically-braked cycle ergometer (Monark 894E, Varberg, Sweden). In the AER condition subjects cycled continuously for 30 min at an intensity predicted to elicit 50% of $\dot{V}O_2max$. Heart rate, RPE and an expired air sample were collected during the final min of exercise. The REHIT trial involved 20 min of rest followed by the 10-min sprint interval bout described above for Study 1. Power output during the sprints was recorded continuously using Monark Anaerobic Test Software (Monark, Vansbro, Sweden), and used to calculate peak, average and end power output for each sprint. During the REST trial subjects rested quietly for 30 min.

Collection and analysis of expired air

Subjects respired through a rubber mouthpiece connected to a two-way Hans Rudolph valve and expired air was collected into pre-evacuated Douglas bags. During each gas collection, samples of ambient (i.e. inspired) CO_2 and O_2 concentrations were measured within close proximity to the participant in order to account for small changes that could occur throughout the day in an enclosed laboratory environment (Betts and Thompson 2012). Expired concentrations of O_2 and CO_2 were measured using paramagnetic and infrared

analysers respectively (Servomex miniMP 5200). Douglas bags were then completely evacuated using a dry gas meter (Harvard Apparatus, Kent, UK) and the total volume and temperature (Model C, Edale Intruments, Cambridge, UK) were recorded for calculation of $\dot{V}O_2$, $\dot{V}CO_2$, and RER. All values were corrected to reflect standard temperature and pressures. In order to estimate EPOC, $\dot{V}O_2$ from the REST trial was subtracted from both of the exercise trials, excess $\dot{V}O_2$ from each 5-min measurement period was totalled, and then total area under the oxygen curve was calculated.

Blood collection and analysis

Venous blood samples were collected through a cannula in an antecubital vein (BD Venflon Pro, BD, Helsingborg, Sweden). Haematocrit and haemoglobin concentrations were determined from 1-ml aliquots of EDTA-treated whole blood at each time point. Haematocrit was determined manually using a micro-centrifuge and Hawksley reader (Hawksley & Sons Ltd, Sussex, UK). Haemoglobin concentration was analysed in duplicate on an automatic analyser (Sysmex R-3000, Sysmex Ltd, UK). Changes in plasma volume from baseline during each trial were calculated using published equations (Dill and Costill 1974). Hormone responses were corrected for changes in plasma volume. For plasma glucose both the uncorrected and corrected responses are presented.

For glucose, non-esterified fatty acids (NEFA), lactate and total PYY analysis, whole blood was dispensed into a 4-ml EDTA tube which was immediately spun at 3466 g and 4°C for 10 min to separate the plasma for storage at -80°C until subsequent analysis. Automated analysers were used to analyse plasma lactate (YSI Stat 3000, Yellow Springs Instruments, Yellow Spring, OH) and plasma glucose and NEFA (Randox Daytona, Country Antrim, UK). Plasma total PYY concentrations were analysed using commercially available ELISA (Millipore, Hertfordshire, UK). For analysis of acylated ghrelin 2 ml of whole blood was first dispensed into a 4-ml EDTA tube containing 20 µl of p-hydroxymercuribenzoic acid (PHMB). Samples were then spun at 3466 g for 10 min at 4°C, and 500 µl of the supernatant was transferred into a separate plain tube containing 50 µl of 1 M HCl in order to preserve acylated ghrelin (Hosoda et al. 2004). Samples were then centrifuged again at 3466 g for 10 min at 4°C and the supernatant was transferred into a separate tube and frozen at -80°C until subsequent analysis using a commercially available ELISA (Bertin Pharma, Montigny le Bretonneux, France). All plasma analyses were performed in duplicate.

Subjective ratings of tiredness and appetite

Perceptions of hunger and prospective food consumption were assessed using validated 100-mm visual analogue scales with standard descriptors anchored at each end (Flint et al. 2000). Perceptions of physical tiredness were assessed using 100-mm visual analogue scales ranging from "*not at all physically tired*" to "*as physically tired as I have ever felt*".

Statistical analysis

All data are presented as mean±SEM. All data were analysed using the commercially available Statistics Package for Social Sciences (SPSS) software. Area-under-the-curve (AUC) calculations were performed using the trapezoid rule. For Study 1, changes in skeletal

muscle glycogen and signalling proteins were analysed using a one-way repeated-measures ANOVA. *Post hoc* comparisons were made using paired sample t-tests corrected using the Ryan-Holm Bonferroni stepwise adjustment. Changes in gene expression data from baseline to 180 min post-exercise were compared using a Wilcoxon Rank Sum Test as non-parametric tests are more appropriate when using small sample sizes (Siegel and Castellan 1988). For Study 2, two-way repeated measures ANOVAs (condition [REHIT, AER, REST] × time [Pre, 0, 15, 30, 90]) were used to analyse the response of all measured variables over time. *Post hoc* comparisons were made using paired sample t-tests corrected using the Ryan-Holm Bonferonni stepwise adjustment. Hormone AUCs were compared using one-way repeated measures ANOVA with *post hoc* paired sample t-tests. Significance was accepted at $P<0.05$.

RESULTS

Study 1

Peak, average and end power were 9.4±1.5, 8.1±1.1 and 6.2±1.1 W·kg^{-1} for the first sprint, and 8.4±1.6, 7.2±1.2 and 5.5±0.8 W·kg^{-1} for the second sprint. On average *vastus lateralis* glycogen concentration was reduced by ~20% immediately post-exercise and remained reduced to a similar extent throughout the recovery period ($P<0.05$; Figure 1). Phosphorylation of AMPK (Figure 2A) and p38 MAPK (Figure 2C) did not significantly change at any of the post-exercise time points, but ACC phosphorylation was strongly increased both immediately and 30 min post-exercise (both $P<0.05$), returning to baseline levels at 180 min (Figure 2B). At 180 min following exercise, gene expression was increased for PGC1α (16.5±6.2 fold; $P<0.05$) and tended to increase for GLUT4 (2.2±0.6 fold; $P=0.08$; Figure 3).

Study 2

AER was performed at an average intensity of 86±5 W, which elicited 53±5% of $\dot{V}O_2$max and 70±4% of maximum heart rate during the final min of exercise. Peak, average and end power for the REHIT session were 9.2±1.2, 7.3±1.1 and 5.5±1.1 W·kg^{-1} for the first sprint, and 8.2±1.4, 6.5±1.1 and 4.5±1.4 W·kg^{-1} for the second sprint.

Oxygen uptake and respiratory exchange ratio

Both exercise bouts increased $\dot{V}O_2$ and RER relative to REST ($P<0.01$), but the magnitude of the increase was greater following REHIT for both variables ($P<0.01$; Table 1). $\dot{V}O_2$ returned to baseline levels 15 min after AER but remained elevated for at least 30 min following REHIT (all $P<0.05$), resulting in a higher EPOC following REHIT compared with AER (5.1±0.9 L vs. 1.0±0.8 L; $P<0.01$). Whilst RER returned to resting values at 15 min following AER, following REHIT there was a decline in RER to below 0.70 at 30 min ($P<0.01$) and the decrease was still significant at 90 min post-exercise ($P<0.05$ vs. AER and baseline, $P=0.18$ vs. REST).

Blood parameters

Both exercise bouts reduced plasma volume relative to REST ($P<0.001$) but the decrease was more pronounced with REHIT ($P<0.01$; Figure 4A). Whilst plasma volume had normalised at 15 min post-exercise in AER, it remained reduced in REHIT at this time-point ($P<0.001$ vs. REST and AER). When uncorrected for plasma volume change, plasma glucose was increased directly after and 15 min after REHIT ($P<0.05$; Figure 4B) whilst concentrations remained constant throughout the REST and AER trials. However, when plasma glucose concentrations were corrected for changes in plasma volume there were no changes in glucose levels following REHIT and a small reduction immediately post-exercise in AER compared with REST ($P<0.05$; Figure 4C). Both exercise bouts resulted in an increase in plasma lactate concentration relative to REST which persisted for at least 30 min following AER and at least 90 min following REHIT (all $P<0.01$; Table 1). The increase in plasma lactate concentration was greater following REHIT compared with AER and concentrations remained higher at all measured time-points (all $P<0.05$; Table 1). Plasma NEFA concentrations increased over time (main effect of time $P<0.01$), but this effect was not changed by either exercise bout (Table 1).

Plasma acylated ghrelin concentration decreased immediately after AER and REHIT compared with REST (both $P<0.05$). Whilst concentrations were similar in AER and REHIT immediately post-exercise, they continued to decline in the REHIT trial only and were lower at 15 and 30 min post-exercise compared with both REST and AER ($P<0.01$; Figure 5A). Post-exercise total AUC for acylated ghrelin was lower in REHIT (4.6±3.3 $\mu g \cdot L^{-1} \cdot 90$ min) compared with REST (8.8±4.7 $\mu g \cdot L^{-1} \cdot 90$ min; $P<0.01$) and AER (8.9±5.5 $\mu g \cdot L^{-1} \cdot 90$ min; $P<0.01$). Plasma total PYY concentrations decreased over time (main effect of time $P<0.05$) but this effect was not altered by either exercise bout (Figure 5B).

Subjective measures of appetite and tiredness

Perception of physical tiredness was increased at 0 and 15 min following both exercise bouts ($P<0.01$ and $P<0.05$ vs. REST respectively), but tended to increase more following REHIT at t=0 ($P=0.06$; Table 2). Perceptions of hunger and prospective food consumption increased over time (main effect of time $P<0.05$), but these effects were not significantly altered by either exercise bout (Table 2). However, for prospective food consumption there was a tendency for lower ratings following REHIT ($P=0.08$ for the time × condition interaction; Table 2).

DISCUSSION

We have recently demonstrated that an exercise training intervention requiring a maximum of 40 s of high-intensity exercise within a 10-min cycling session (REHIT) can be effective in improving insulin sensitivity and maximal aerobic capacity over a 6-week period (Metcalfe et al. 2012). The aim of the two studies presented in this article was to characterise some of the acute molecular and physiological responses to a single REHIT session. In Study 1 we demonstrate that a 10-min REHIT session is sufficient to reduce skeletal muscle glycogen levels by a fifth and to increase the skeletal muscle mRNA expression of PGC1α, and GLUT4 (trend only). Although we were unable to detect a significant increase in AMPK or p38 MAPK phosphorylation, the robust activation and phosphorylation of the AMPK substrate ACC-β at its AMPK phosphorylation site tentatively suggests that REHIT is associated with AMPK activation. In Study 2 we show that a 10-min REHIT session induces a markedly greater disturbance of homeostasis compared with 30 min of moderate-intensity aerobic exercise. Particularly noteworthy are the large changes in plasma volume, plasma lactate, and respiratory exchange ratio, all of which can directly or indirectly be explained by the rapid glycogen depletion observed in Study 1. Although immediately after cessation of exercise ratings of physical tiredness were moderately higher for REHIT compared to AER, this difference had disappeared within 15 min after exercise, confirming our previous observations that REHIT is not considered overly strenuous by participants (Metcalfe et al. 2012). The substantial disturbance of physiological homeostasis, the activation of signalling pathways ultimately responsible for chronic training adaptations, the extreme time-efficiency, and the low associated perceived exertion and fatigue provide further support for the notion that low volumes of supramaximal exercise (e.g. REHIT) may provide a feasible alternative or adjunct to current physical activity recommendations for the general population.

A crucial role for glycogen depletion?

Whilst skeletal muscle glycogen has long been appreciated as an important acute regulator of skeletal muscle metabolism, accumulating evidence also supports its role as a signalling molecule potentiating exercise-induced adaptation (Philp et al. 2012; Jensen and Richter 2012). We originally designed our REHIT protocol based on the hypothesis that regular glycogen turnover may be sufficient to induce beneficial adaptations to insulin sensitivity, and we (Metcalfe et al. 2012) and others (Gillen et al. 2014) have indeed demonstrated that REHIT is associated with improved insulin sensitivity (at least in men). In the current study we demonstrate that REHIT is associated with an acute 20% reduction in *vastus lateralis* glycogen levels. These data confirm that glycogen degradation with REHIT is similar to that observed with HIT protocols incorporating a greater number of more prolonged (e.g. 3-4 x 30 s) sprint efforts (Parolin et al. 1999; Gibala et al. 2009). This finding is supported by the data of Parolin *et al* (1999) who demonstrated that during three repeated 30-s all-out cycle sprints glycogen phosphorylase (and hence glycogenolysis) is predominantly activated during the first 15 s of the first sprint, and that further sprints break down little or no extra glycogen.

Recent evidence has suggested an important role of glycogen availability in the activation of AMPK (McBride and Hardie 2009; McBride et al. 2009). Specifically, as AMPK has been shown to contain a glycogen-binding domain, a certain amount of AMPK will be bound within glycogen granules in a glycogen-replete state and this is associated with suppression of skeletal muscle AMPK activity (Philp et al. 2012). Likewise, exercise-induced glycogen depletion will result in a release of AMPK into the cytosol and a concomitant rise in AMPK activity (Philp et al. 2012), and this may be further enhanced in response to the expected rise in cytoplasmic AMP concentrations experienced particularly during high-intensity exercise (Karatzaferi et al. 2001b; Karatzaferi et al. 2001a). However, despite the fact that previous studies have observed significant increases in phosphorylation of AMPK with single and repeated Wingate sprint protocols (Gibala et al. 2009; Fuentes et al. 2012; Fuentes et al. 2013), we did not detect a significant increase in AMPK phosphorylation at any time-point following REHIT (although it should be noted that the numerical increase in AMPK phosphorylation 30 min post-exercise was similar to that observed in previous studies). Nonetheless, we did detect a robust activation and phosphorylation of ACC-β at its AMPK phosphorylation site (Ha et al. 1994; Davies et al. 1990), and this suggests there may have been a level of AMPK activation which we were unable to detect. Indeed, some authors rely solely on ACC-β phosphorylation as a marker of AMPK activity following exercise (Cochran et al. 2014; Little et al. 2011).

Another signalling molecule, p38 MAPK, has previously been shown to be activated following four repeated 30-second Wingate sprints (Gibala et al. 2009; Little et al. 2011). However, in accordance with a number of studies investigating single Wingate sprints (Gibala et al. 2009; Fuentes et al. 2012; Fuentes et al. 2013) we did not observe an increase in p38 MAPK phosphorylation. Whilst in the current study we have measured AMPK and p38 MAPK phosphorylation in a whole-muscle biopsy sample, the cellular localisation of these signalling proteins will likely also be of key importance for any adaptive response: regulation of gene transcription will either require direct translocation of the protein to the nucleus or a signalling cascade which transcends the nuclear membrane. Previous studies have observed translocation of AMPK and p38 MAPK to the cell nucleus during or in the recovery from an acute exercise bout (Little et al. 2011; Steinberg et al. 2006; Chan et al. 2004; McGee et al. 2003). Whether a similar response occurs with HIT/REHIT is a question for future research studies.

The significant increase in PGC1α gene expression and the trend towards an increase in GLUT4 mRNA following REHIT also suggest the effective activation of adaptive signalling pathways such as that involving AMPK. This is supported by the recent findings that several markers of oxidative capacity in skeletal muscle, such as citrate synthase and COX IV, as well as protein levels of GLUT4 are increased following a 6-week REHIT intervention (Gillen et al. 2014). Although such responses have commonly been observed following a variety of submaximal and supramaximal HIT protocols (Gibala et al. 2009; Little et al. 2011; Little et al. 2010; Burgomaster et al. 2005; Hood et al. 2011; Gibala et al. 2006), the current study and that by Gillen et al (2014) together suggest that these responses may be achieved with a much smaller volume of exercise than previously thought.

The absolute level of glycogen utilisation with HIT/REHIT is similar to that observed following a 30-min bout of moderate (50% VO_2max) aerobic exercise (Van Loon et al. 2001). However, the speed at which the glycogenolysis occurs varies greatly between these two exercise modes. Indeed, the extremely rapid mobilisation of muscle glycogen is a unique characteristic of 'all-out' Wingate-type sprints, and likely explains much of the difference in the physiological disturbance observed with REHIT compared with aerobic exercise in the current study. Whilst with moderate aerobic exercise the rate of glycogenolysis will be matched closely with glucose oxidation, the considerable amount of glucose-6-phosphate released from glycogenolysis in the early stages of the initial REHIT sprint is likely to pose a substantial challenge to the intramyocellular milieu. Although the glycolytic catabolism of glucose-6-phosphate results in a rapid accumulation of pyruvate, only a small fraction of this is immediately oxidised; most is instead converted to lactate (Parolin et al. 1999), as evidenced by the large spike in plasma lactate concentration observed in our study. The strong reliance on glycolysis for ATP resynthesis will also result in a reduction in both intramyocellular and blood pH levels (Robergs et al. 2004). Consistent with previous observations following more strenuous and time-consuming HIT protocols (Chan and Burns 2013; Hazell et al. 2012) we observed a sharp increase in RER immediately after REHIT, demonstrating the immediate bicarbonate-mediated buffering of the metabolic acidosis associated with the all-out sprints. The subsequent drop in RER below 0.70 reflects the retention of CO_2 to replenish the depleted blood bicarbonate stores during recovery (Laforgia et al. 1997). Given that the majority of glycogenolysis, and hence metabolic acidosis, occurs during the first sprint of a repeated sprint protocol (Parolin et al. 1999) it is not surprising that a protocol involving a smaller number of shorter sprints poses a similar challenge to buffering mechanisms compared with more strenuous HIT protocols, and hence still has this effect on RER (Hazell et al. 2012; Chan and Burns 2013). It can be speculated that this regular overloading (and recovery) of muscle and blood buffering mechanisms provides an explanation for the adaptations in buffering capacity observed following six weeks of HIT (Gibala et al. 2006).

A final consequence of the rapid glycogenolysis with REHIT is that the accumulation of metabolic derivatives will result in a hypertonic intramyocellular environment leading to an influx of water (Raja et al. 2006), which is consistent with the considerable drop in plasma volume we observed following REHIT. Potentially this subsequently places the myocyte in a transient state of osmotic stress (Haussinger 1996). Intriguingly, in various cell types osmotic swelling has previously been shown to independently activate various signalling cascades known to be involved in cellular remodelling (Haussinger 1996; Liu and Cao 2009). It is interesting to speculate whether this type of osmotic stress could be a mechanism of adaptation with HIT. From a practical research perspective however, a consequence of the substantial decrease in plasma volume following REHIT (which is presumably similar following other supramaximal HIT protocols), is that exercise-induced changes in plasma protein concentration should be corrected for plasma volume changes. Similarly, it seems reasonable to suggest that hemoconcentration due to the rapid drop in plasma volume may be responsible for previously observed increases in plasma glucose concentrations with

sprint exercise (Vincent et al. 2004; Stokes et al. 2013; Esbjornsson et al. 2009), rather than an increase in glucose rate of appearance.

REHIT may affect energy balance

The current study demonstrates that circulating levels of the appetite-stimulating hormone acylated ghrelin are significantly reduced following REHIT, which with a total duration of 10 min makes it the lowest volume of exercise shown to be associated with such an effect to date. Furthermore, we demonstrated that EPOC is significantly greater following 10 min of REHIT compared to 30 min of moderate intensity aerobic exercise. These findings are of importance as several previous studies have observed reductions in fat mass following HIT with some evidence for superior reductions compared with traditional aerobic training (Whyte et al. 2010; Gillen et al. 2013; Heydari et al. 2012; Trapp et al. 2008). Since direct exercise energy expenditure is much lower with HIT compared with aerobic exercise, the (potentially superior) reductions in fat mass following HIT have been hypothesised to be a result of either an increase in energy expenditure through a more prolonged EPOC and/or a reduction in energy intake through suppression of appetite (Boutcher 2011). There are now several studies reporting that HIT results in an increase in total daily energy expenditure (Hazell et al. 2012; Skelly et al. 2014; Beaulieu et al. 2015; Sevits et al. 2013), and this increase is similar to that elicited by 30-50 minutes of moderate-vigorous aerobic exercise (Hazell et al. 2012; Skelly et al. 2014). However, the magnitude of the increase is typically modest and is only evident for 1-2 hours into the recovery period (Hazell et al. 2012; Skelly et al. 2014; Beaulieu et al. 2015; Sevits et al. 2013; Kelly et al. 2013; Williams et al. 2013; Chan and Burns 2013). In the present study we show that REHIT is associated with a 5-fold greater EPOC compared with 30 min of moderate aerobic exercise, and the duration and magnitude of EPOC was similarly modest to protocols utilising a greater number and/or duration of sprints (Hazell et al. 2012; Skelly et al. 2014; Beaulieu et al. 2015; Sevits et al. 2013; Kelly et al. 2013; Williams et al. 2013; Chan and Burns 2013). On balance, the current state of evidence suggests that EPOC would only make a small contribution to any reduction in fat mass with HIT and certainly wouldn't explain any superior effects compared with continuous aerobic exercise (Hazell et al. 2012; Skelly et al. 2014).

It has also been suggested that HIT may result in suppression of appetite after exercise and hence lead to a reduction in energy intake across a training intervention (Boutcher 2011). The gut hormones acylated ghrelin (orexigenic effects) and PYY (anorexigenic effects) have emerged as important episodic regulators of hunger, feeding latency and caloric intake and may therefore play a role in mediating any changes in appetite with exercise (Stensel 2010; Wynne et al. 2005). Following REHIT systemic concentrations of acylated ghrelin were reduced by >50% in the immediate post-exercise period, with little or no change observed following a bout of continuous cycling at a moderate intensity. This is consistent with other HIT studies (Sim et al. 2013; Deighton et al. 2013) and fits with previous research which highlights the key effect of relative exercise intensity on modulating the response of acylated ghrelin to aerobic exercise, with suppression occurring at higher (≥70% $\dot{V}O_2max$) but not lower (≤50% $\dot{V}O_2max$) exercise intensities (King et al. 2010; Ueda et al. 2009; Broom et al. 2007; Broom et al. 2009; Wasse et al. 2013). Conversely, circulating levels of total PYY

appear to be unaffected by REHIT. The effect of other HIT protocols on total PYY is currently inconsistent with two studies demonstrating an increase in the immediate post-exercise period (Deighton et al. 2013; Beaulieu et al. 2015) and another showing no detectable changes (Sim et al. 2013). It is worth pointing out however that PYY values were not corrected for plasma volume changes in these studies (Deighton et al. 2013; Beaulieu et al. 2015); considering the large drop in plasma volume following REHIT in the current study any increase in PYY levels may partly have been caused by hemoconcentration.

Despite the strong attenuation of acylated ghrelin levels following REHIT there was no significant suppression of subjective ratings of appetite (although it is noteworthy that ratings of prospective food consumption tended to be lower in the REHIT trial). To date, there is limited data on the effects of HIT on indices of appetite and findings are equivocal (Deighton et al. 2013; Williams et al. 2013; Sim et al. 2013; Beaulieu et al. 2015). In young healthy males subjects two studies have observed a clear 'anorexic' effect of HIT on appetite-regulating hormones as well as subjective appetite ratings immediately post exercise, but this did not translate into a reduction in caloric intake during laboratory controlled *ad libitum* buffet meals (Deighton et al. 2013; Beaulieu et al. 2015). On the other hand, in a group of overweight men, there was a less robust effect of HIT on gut hormone responses and no effect on subjective appetite ratings, yet *ad libitum* energy intake during a laboratory-controlled breakfast meal and over the following day were both substantially reduced (Sim et al. 2013). Although the physiological relevance of short-term suppression of circulating levels of acylated ghrelin with HIT remains unclear, we can confirm this effect and demonstrate that it can be achieved with a substantially smaller amount of exercise than previously reported. Future research should look to further characterise the effect of REHIT on appetite and examine changes in body composition following a training intervention.

Conclusions

In conclusion, we demonstrate that REHIT is associated with a pronounced disturbance of homeostasis as evidenced by the rapid ~20% reduction in skeletal muscle glycogen levels and the resulting large excursions in plasma volume, lactate levels and acid-base balance. This is accompanied by activation of adaptive signalling pathways as well as hormonal responses which together may explain previously observed training adaptations once thought to be exclusive to classic aerobic exercise. Together these data add further support to the growing awareness that the protocols used in previous HIT studies might be longer and more strenuous than necessary. A shift of focus towards studying lower volumes of high-intensity exercise may advance our quest to enable a larger proportion of the general population to reap the health benefits of regular physical activity.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 1 Changes in $\dot{V}O_2$, RER, plasma lactate and plasma NEFA

			Post-exercise time (min)			
		Pre	**0-5**	**15-20**	**30-35**	**90-95**
$\dot{V}O_2$ ($L \cdot min^{-1}$)	**REST**	0.31±0.02	0.29±0.02	0.29±0.02	0.28±0.02	0.30±0.02
	AER	0.31±0.02	0.46±0.04†	0.31±0.02	0.30±0.02	0.30±0.02
	REHIT	0.30±0.02	0.62±0.04$^{*\#}$	0.38±0.02$^{*\#}$	0.34±0.02$^{*\#}$	0.31±0.02
RER	**REST**	0.84±0.01	0.82±0.01	0.81±0.02	0.82±0.01	0.81±0.01
	AER	0.86±0.02	1.04±0.02†	0.82±0.03	0.81±0.02	0.83±0.02
	REHIT	0.82±0.02	1.29±0.03$^{*\#}$	0.76±0.02$^{*\#}$	0.68±0.01$^{*\#}$	0.75±0.02$^{\#}$
		Pre	**0**	**15**	**30**	**90**
Plasma lactate ($mmol \cdot L^{-1}$)	**REST**	1.45±0.20	1.03±0.11	1.01±0.12	1.00±0.13	1.00±0.12
	AER	1.88±0.39	3.54±0.64†	1.88±0.27†	1.41±0.16†	1.12±0.13
	REHIT	1.51±0.35	15.37±1.25$^{*\#}$	12.51±0.99$^{*\#}$	7.73±0.73$^{*\#}$	1.88±0.18$^{*\#}$
Plasma NEFA ($mmol \cdot L^{-1}$)	**REST**	0.46±0.09	0.45±0.07	0.47±0.07	0.54±0.08	0.66±0.08
	AER	0.43±0.09	0.46±0.11	0.60±0.14	0.46±0.10	0.57±0.12
	REHIT	0.46±0.05	0.34±0.04	0.39±0.06	0.36±0.05	0.58±0.06

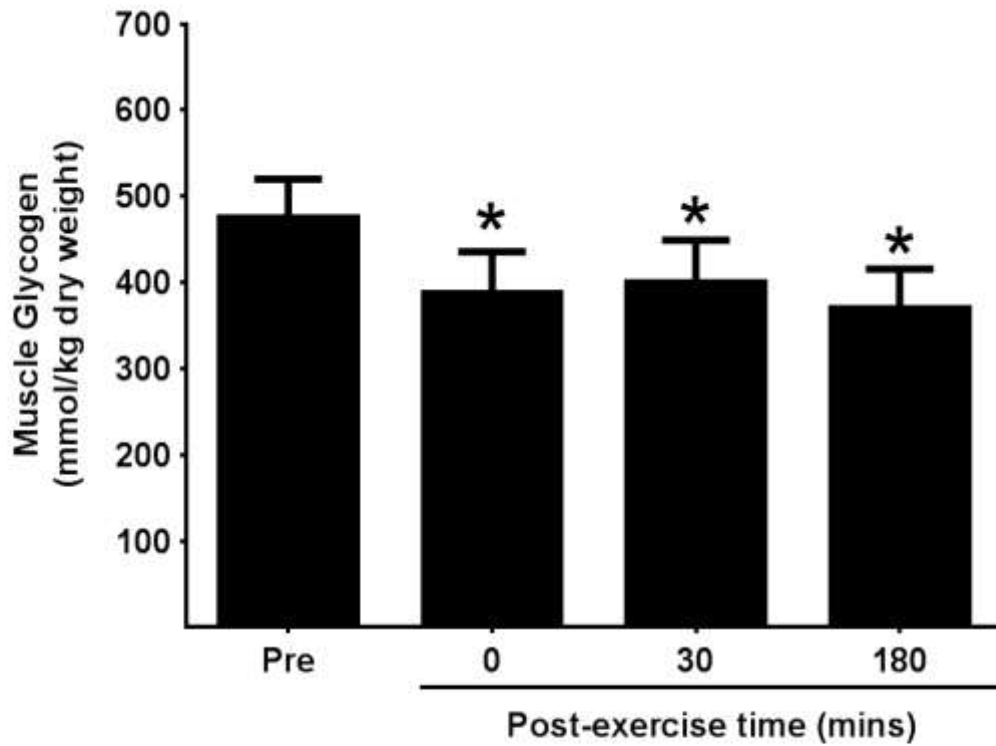
*Data are presented as mean±SEM (n=8). *P<0.05 for REHIT vs. REST; †P<0.05 for AER vs. REST; #P<0.05 for REHIT vs. AER.*

Table 2 Changes in subjective perceptions of physical tiredness, hunger and prospective food consumption

			Post-exercise time (min)			
		Pre	**0**	**15**	**30**	**90**
Physical tiredness (0-100 mm)	REST	15±3	16±7	14±6	14±7	16±8
	AER	16±6	41±7†	28±7†	21±7†	16±7
	REHIT	13±5	58±6*#	27±7*	22±7*	16±7
Hunger$ (0-100 mm)	REST	36±11	42±11	45±12	49±11	55±10
	AER	33±10	45±10	46±10	46±10	53±8
	REHIT	30±8	37±9	38±9	37±9	42±8
Prospective food consumption $ (0-100 mm)	REST	43±10	46±11	50±10	55±10	59±9
	AER	37±10	54±9	53±9	54±8	59±7
	REHIT	36±8	42±8	38±8	39±9	45±8

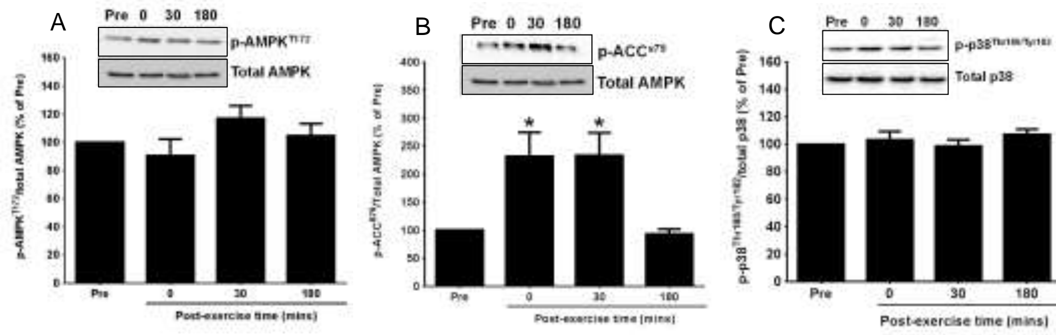
*Data are presented as mean±SEM (n=8). $P<0.05 for time main effect; *P<0.05 for REHIT vs. REST; †P<0.05 for AER vs. REST; #P<0.05 for REHIT vs. AER.*

Figure 1 The effect of REHIT on *vastus lateralis* glycogen concentration



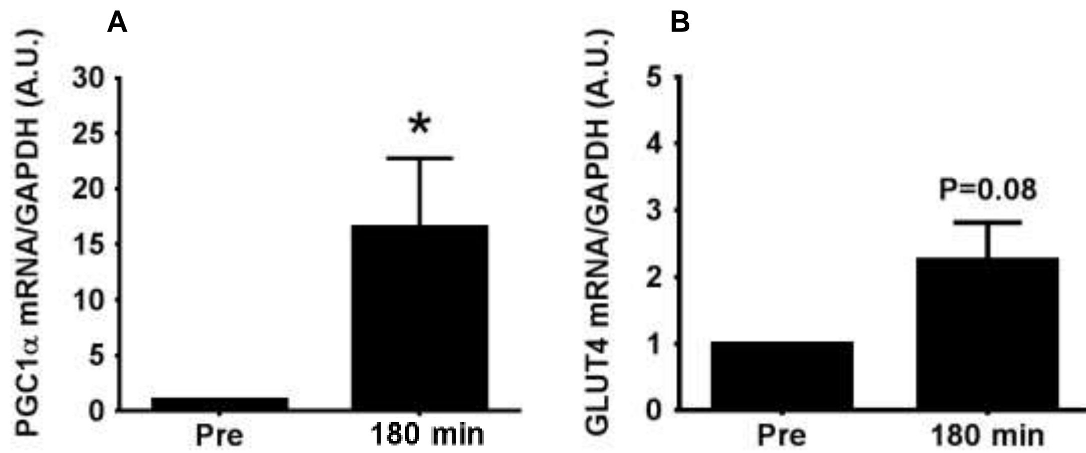
*Data are presented as mean±SEM (n=11). *P<0.05 vs. pre.*

Figure 2 The effect of REHIT on phosphorylation of AMPK (A), ACC (B), and p38 MAPK (C)



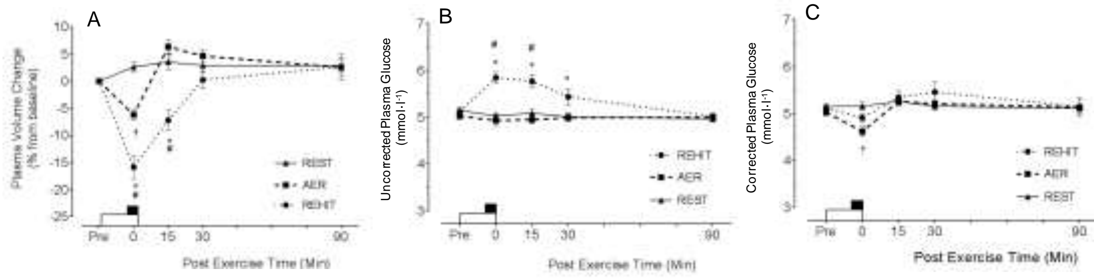
*Data are presented as mean±SEM (n=11). *P<0.05 vs. pre. Representative blots are shown above each respective figure.*

Figure 3 Gene expression changes for PGC1α (A) and GLUT4 (B) at 180 min post-exercise



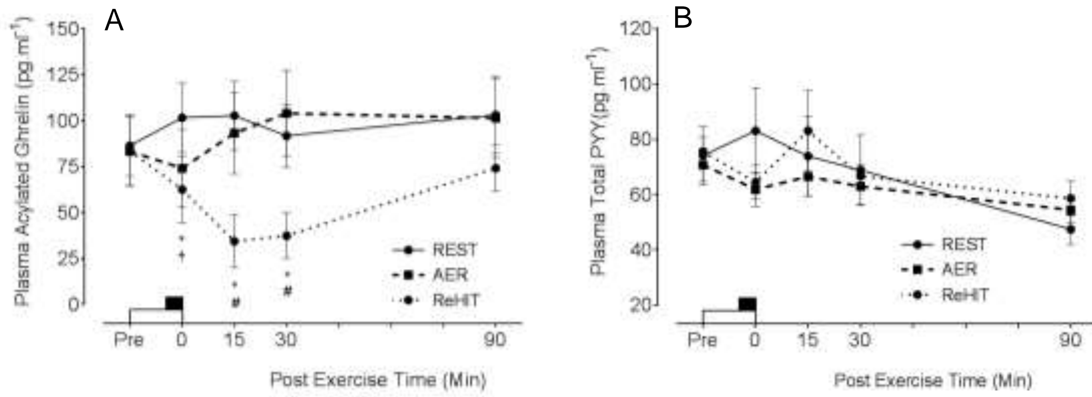
*Data are presented as mean±SEM (n=5). * denotes P<0.05 vs. pre*

Figure 4 Changes in plasma volume (A), and uncorrected (B) and corrected (C) plasma glucose responses



*Data are presented as mean±SEM (n=8). White box: aerobic exercise; black box: REHIT. *P<0.05 for REHIT vs. REST; #P<0.05 for REHIT vs. AER; †P<0.05 for AER vs. REST.*

Figure 5 Changes in plasma acylated ghrelin (A) and PYY (B) concentrations



*Data are presented as mean±SEM (n=8). White box: aerobic exercise; black box: REHIT. * denotes P<0.05 for REHIT vs. REST; # denotes P<0.05 for REHIT vs. AER; † denotes P<0.05 for AER vs. REST. Values have been corrected for changes in plasma volume.*