Post-exercise cold-water immersion modulates skeletal muscle PGC-1α mRNA expression in immersed and non-immersed limbs: evidence of systemic regulation

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Running Head: Cold-water immersion and PGC-1α

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New & Noteworthy

We report for the first time that post-exercise cold-water immersion of one limb also enhances PGC-1α expression in a contra-lateral non-immersed limb. We suggest increased systemic β-adrenergic stimulation, and not localised cooling per se, exerts regulatory effects on local signalling cascades thereby modulating PGC-1α expression. These data therefore have important implications for research designs that adopt contralateral non-immersed limbs as a control condition, whilst also increasing our understanding of potential mechanisms underpinning cold-mediated PGC-1α responses.

Author Contributions

Conception and design of the experiments: RA, WG, JM, APS, BD; Collection, analysis and interpretation of data: RA, APS, JD, JM, WG, SS, GC; Drafting the article and Critical Revision of the article for important intellectual content: RA, APS, GC, SS, BD, JD, JM, WG. All authors approved the final version for publication and agree to be accountable for all aspects of the work.

Abstract

Mechanisms mediating post-exercise cold-induced increases in PGC-1α gene expression in human skeletal muscle are yet to be fully elucidated, but may involve local cooling effects on AMPK and p38 MAPK related signalling and/or increased systemic β-adrenergic stimulation. We aimed to therefore examine whether post-exercise cold-water immersion enhancement of PGC-1α mRNA is mediated through local or systemic mechanisms. Ten subjects completed acute cycling (8x5 min at ~80% peak power output) followed by seated-rest (CON) or single-leg cold-water immersion (CWI; 10 min, 8°C). Muscle biopsies were obtained pre-, post- and 3 h post-exercise from a single limb in the CON condition but from both limbs in CWI (thereby providing tissue from a CWI and non-immersed limb, NOT). Muscle temperature decreased up to 2 h post-exercise following CWI (-5°C) in the immersed limb, with lesser changes observed in CON and NOT (-3°C; $P<0.05$). No differences between limbs were observed in p38MAPK phosphorylation at any time point ($P<0.05$), whilst a significant interaction effect was present for AMPK phosphorylation ($P=0.031$). Exercise (CON) increased gene expression of PGC-1α 3 h post-exercise (~5-fold; $P<0.001$). CWI augmented PGC-1α expression above CON in both the immersed (CWI; ~9-fold; $P=0.003$) and NOT limbs (~12-fold; $P=0.001$). Plasma Normetanephrine concentration was higher in CWI vs. CON immediately post-immersion (860 vs. 665 pmol/L; $P=0.034$). We report for the first time that local cooling of the immersed limb evokes transcriptional control of PGC1-α in the non-immersed limb, suggesting increased systemic β-adrenergic activation of AMPK may mediate, in part, post-exercise cold-induction of PGC-1α mRNA.

Key Words: PGC-1α, CWI, Normetanephrine, AMPK

Introduction

It is well established that the transcriptional co-activator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), the proposed "master regulator" of skeletal muscle mitochondrial biogenesis (37), is sensitively controlled by acute and chronic exercise (3, 4, 10, 36). Upstream control of PGC-1α includes phosphorylation by energy and stress sensing kinases AMP-activated protein kinase (AMPK) (23) and p38 mitogen-activated protein kinase (p38 MAPK) (2). Consistent with its initial discovery as cold-inducible (34, 38), recent studies have examined the potential of acute post-exercise cold exposure to also modulate PGC-1α expression. For example, in human tissue, both cold-ambient temperatures (42, 43) and post exercise cold-water immersion (18, 20) enhances the skeletal muscle PGC-1α gene transcription and protein translational response versus exercise alone. The precise mechanism(s) mediating cold-induced regulation of the PGC-1α transcriptional pathway have yet to be fully determined, though the cooling-induced alterations in muscle blood flow (14, 30) are unlikely to mediate these effects (33, 45). It is noteworthy, however, that chronic cold-induced changes in PGC-1α protein content arise in conjunction with increased activity of signalling kinases AMPK and p38 MAPK (18). These data suggest that cooling of the skeletal muscle tissue may play a role in mediating the post-exercise cold-induction of PGC-1α mRNA through activation of local signalling kinases.

Alternatively, systemic control via increased β-adrenergic activity is suggested to play a potent role in mediating the effects of cold exposure on PGC-1α expression via AMPK (29) and β2-adrenergic receptor (31) mechanisms. Indeed, plasma norepinephrine concentrations remain higher following high-intensity exercise after cold-water immersion versus control conditions (13). Studies to date have utilised the non-immersed limb as the control condition without the use of a true control (no cooling) condition (11, 18, 20). By assuming the response to cold-water immersion is mediated locally, such experimental designs do not

permit examination of the role of systemic versus localised mechanisms in mediating cold-induced changes in PGC-1α. Indeed, Ihsan et al. (20) observed that PGC-1α gene transcription was not induced in a non-immersed control limb, despite the limb having previously been exercised, and exercise being a potent stimulus to induce PGC-1α expression. This would therefore suggest that an induction of PGC-1α expression in an immersed limb occurred by way of cold-induced mechanisms. In this regard, we suggest that the increased systemic β-adrenergic activity associated with post-exercise cooling of the immersed limb could also modulate PGC-1α expression in the non-immersed contralateral limb.

Therefore, the aim of the present study was to examine whether the cold-water induced increase in PGC-1α mRNA observed post exercise is mediated through local or systemic mechanisms. To this end, we employed a novel experimental design (in a repeated measures crossover design) where ten recreationally active males completed an acute cycling protocol (8x5 min at ~80% peak power output) followed by a seated-rest condition (CON) or single-leg cold-water immersion (CWI; 10 min, 8°C). Muscle biopsies were obtained pre-, post- and 3 h post-exercise from a single limb in the CON condition but from both limbs post-exercise in the CWI trial (thereby providing tissue from a CWI and non-immersed limb, NOT). In this way, our design allowed us to obtain tissue from true control conditions but yet, also sample tissue from an immersed and non-immersed limb that was subject to the same hormonal milieu.

Methods

Subjects. Ten recreationally active healthy males (age 26 ± 4 y; body mass 79.29 ± 6.73 kg; height 180 ± 5 cm; $\dot{V}O_{2peak}$ 51.46 ± 9.07 mL.kg^{-1}.min^{-1}; peak power output (PPO) 265.2 ±

38.33 W; mean ± SD) participated in this study. Subjects were instructed to refrain from exercise, alcohol and caffeine 48 hours prior to the test day. All subjects gave written informed consent to participate after details and procedures of the study had been fully explained. Subjects had no history of neurological disease or musculoskeletal abnormality and none were under any pharmacological treatment during the course of the study. Each subject was medically screened by a practising GP prior to participation for their individual risk associated with high-intensity exercise, muscle biopsy procedures and immersion in cold water. All procedures performed in the study were approved by the Ethics Committee of Liverpool John Moores University and in accordance with the 1964 Helsinki declaration and its later amendments.

Experimental Design. In a repeated-measures crossover design, subjects completed two high-intensity intermittent cycling protocols followed either by 10 min seated rest (CON) or single-leg CWI (10 min at 8°C). Muscle biopsies were obtained from the vastus lateralis immediately before, post- and 3 h post-exercise. During the CWI trial muscle biopsies in the post-exercise period were taken from both the immersed and non-immersed leg. This design allowed us to assess the impact of the cold stimulus locally (immersed leg) and systemically (non-immersed leg) against a relevant resting control. Both experimental trials were conducted in a counterbalanced, randomized order with at least 10 days between conditions.

Experimental Protocol. Subjects attended the laboratory on 3 separate occasions. On the first occasion, subjects completed an incremental exercise test to fatigue for the determination of $\dot{V}O_{2max}$ and PPO (15). Results from this test were used to determine the power output necessary for cycling at a proportion of PPO on subsequent test days (detailed below). Prior to the first test day, subjects completed a 24-hour food diary to be replicated before the second trial. Upon arrival at the laboratory (0900h) subjects were fitted with a heart-rate monitor (Polar RS400, Kempele, Finland), skin and rectal temperature probes (MHF-18050-

161 A and MRV-55044-A, Ellab, Rodovre, Denmark). Legs were marked for subsequent
162 insertion of muscle temperature needles; area of insertion was calculated as half the length of
163 the femur, over the 'belly' of the vastus lateralis. The needle thermistor was then placed at a
164 depth of 3cm, plus one-half of the skinfold measurement, for the determination of deep
165 muscle temperature (3cm). Following 10-min resting in a supine position, baseline measures
166 of heart rate (HR), temperature and oxygen uptake ($\dot{V}O_2$; Oxycon Pro, Jaeger, Wuerzberg,
167 Germany) were assessed. Resting venous blood samples were drawn from a superficial vein
168 in the anti-cubital crease of the forearm using venepuncture cannulation (BD Nexiva Closed
169 IV Catheter 22G Blue, Becton Dickinson, Oxford, UK). Resting muscle temperature was
170 assessed using a needle thermistor (13050; Ellab, Rodovre, Denmark) inserted into the vastus
171 lateralis at 3cm depth as previously described (30). Finally, resting muscle biopsy samples
172 from the vastus lateralis (~30–50 mg wet wt) were obtained under local anaesthesia (0.5%
173 marcaine) using a Pro-Mag 2.2 biopsy gun (MD-TECH, Manan Medical Products,
174 Northbrook, IL). At rest only a single leg was biopsied in the cooling trial (CWI) in attempt
175 to reduce the stress experienced by subjects. This leg was randomised between the immersed
176 and non-immersed limbs to exclude potential variation caused by leg dominance; all
177 subsequent biopsies were completed in both legs, at each time point, 2cm proximal to the
178 previous incision.

179 Following the resting biopsy, subjects completed a high-intensity intermittent cycling
180 protocol consisting of 8 × 5min bouts at 82.5% PPO separated by 1 min rest (adapted from
181 44) followed by either single-legged CWI (CWI: 10 min at 8°C; NOT: non-immersed leg i.e.
182 10 min at room temperature) or a control condition (CON; seated rest). CWI was conducted
183 using a specialised seated mechanical hoist to lower the subject so that one leg was placed
184 inside the cold-bath, allowing the other (NOT) to remain outside, and dry. Subjects then
185 recovered in a semi-reclined position under normal laboratory temperatures (~21°C) until 3-

hours post-exercise. Measures of heart rate, skin temperature (thigh and calf) and rectal temperature were recorded throughout the exercise and recovery periods. Oxygen uptake was measured during the final minute of each high-intensity bout of exercise, during immersion, immediately post-immersion and again at 1, 2 and 3 h post-exercise. Ratings of perceived exertion (RPE) were assessed during the final minute of each exercise bout (5), whilst subjective measures of perceived shivering were assessed throughout water immersion and the 3h recovery period using a visual analogue scale from 1 (No shivering) to 5 (Intense Shivering) (24). Laboratory temperatures remained stable throughout (21.18 ± 0.76^{o}C) and at no point were subjects allowed to rub themselves dry or shower (changing into dry shorts after immersion was allowed). Subjects were advised to wear the same clothes between trials. Muscle temperature was assessed post-exercise, immediately post-immersion and at 1, 2 and 3h post-exercise. Venous blood samples were also drawn at these times. Bi-lateral muscle biopsies occurred immediately after exercise and 3h post-exercise.

Venous blood samples were drawn from a superficial vein in the anti-cubital crease of the forearm using standard venepuncture techniques (BD Nexiva Closed IV Catheter 22G Blue, Becton Dickinson, Oxford, UK). Blood samples (~10ml) were collected into vacutainer tubes (Becton Dickinson, Oxford, UK) containing EDTA and stored on ice until centrifugation at 1500 rev.min^{-1} for 15-min at 4^{o}C. Following centrifugation, aliquots of plasma were stored at -80^{o}C for later analysis. Plasma metanephrine and Normetanephrine concentrations were measured using liquid chromatography tandem mass spectrometry as previously described (35). All samples were analysed in duplicate, with the mean value employed.

rt-qRT-PCR. Skeletal muscle samples (~30 mg) were transferred to 2 ml lysing tubes containing 1.4 mm ceramic beads (Lysing Matrix D, MP Biomedicals, UK) containing 1 ml ice-cooled TRI- reagent (Life Technologies Ltd, UK) and homogenised at 6 m/s for 3 x 40 seconds, separated by 5 minutes cooling on ice (MP Fastprep-24, MP Biomedicals, UK).

RNA was extracted according to the TRI-reagent manufacturer's instructions. RNA concentration and purity were assessed by UV spectroscopy at ODs of 260 and 280 nm using a Nanodrop 3000 (Fisher, Rosklide, Denmark). A target of A_{260} / A_{280} ratio was set at 1.8 to 2.2. Seventy ng RNA was used for each PCR reaction. Primer sequences (Table 1) were identified using Gene (NCBI, http://www.ncbi.nlm.nih.gov.gene) and designed using Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast). Sequence homology searches ensured specificity and that all primers had no potential unintended targets. The primers were ideally designed to yield products spanning exon-exon boundaries to prevent any amplification of gDNA. Three or more GC bases in the last five bases at the 3' end of the primer were avoided. Secondary structure interactions (hairpins, self-dimer and cross dimer) within the primer were avoided. All primers were between 16 and 25 bp, and amplified a product of between 67-212 bp. Primers were purchased from Sigma (Suffolk, UK).

rt-qRT-PCR amplifications were performed using QuantiFast™ SYBR® Green RT-PCR one step kit on a Rotor-gene 3000Q (Qiagen, Crawley, UK) supported by rotor-gene software (Hercules, CA, USA). rt-qTR-PCR was performed as follows: hold 50°C for 10 min (reverse transcription/cDNA synthesis), 95°C for 5 min (transcriptase inactivation and initial denaturation step) and PCR steps of 40 cycles; 95°C for 10s (denaturation), 60°C for 30s (annealing and extension). Upon completion, dissociation/melting curve analysis were performed to reveal and exclude non-specific amplification or primer-dimer issues (all melt analysis in this study presented single reproducible peaks for the reference gene and each target gene suggesting amplification of a single product). Following initial screening of suitable reference genes, GAPDH showed the most stable C_t values across all RT-PCR runs, subjects and regardless of experimental condition (23.54 ± 1.69 C_t; 7% Co-efficient of variation) and was selected as the reference gene in all RT-PCR assays. The average PCR efficiency was 90% and variation for all genes was less than 4.3%. The relative gene

236 expression levels were calculated using the comparative C_t ($^{\Delta\Delta}C_t$) equation (41) where the
237 relative expression was calculated as $2^{-\Delta\Delta ct}$ and where C_t represents the threshold cycle.
238 mRNA expression for all target genes was calculated relative to the reference gene (GAPDH;
239 subject's own samples reference) within same subject and condition and to a calibrator of
240 Pre-exercise.

241 *SDS-PAGE and Western Blotting.* Approximately 30 mg of frozen muscle was homogenized
242 using 2.4 mm ceramic beaded tubes (6 m/s for 3 x 40 seconds, separated by 5 minutes
243 cooling on ice; MP Fastprep-24, MP Biomedicals, UK), in 500 µl of ice-cold lysis buffer [25
244 mM Tris·HCl (pH 7.4), 50 mM NaF, 100 mM NaCl, 5 mM EGTA, 1 mM EDTA, 10 mM
245 Na-pyrophosphatase, 1% Triton X-100] and supplemented with a protease phosphatase
246 inhibitor cocktail (Halt Protease and Phosphatase Inhibitor 186 Cocktail, Thermo Scientific, #
247 78442). The resulting homogenates were centrifuged at 14,000 g for 10 min at 4°C, and the
248 supernatant was collected. The protein content of the supernatant was determined using a
249 bicinchoninic acid assay (Sigma, UK). Each sample was diluted with an equal volume of 2X
250 Laemmli buffer (National Diagnostics) and boiled for 10 min at 100°C. Forty µg of total
251 protein from each sample was loaded (65 µg for phosphorylated analyates) and then
252 separated in Tris-glycine running buffer (10x Tris/glycine, Geneflow, Staffordshire, UK)
253 using self-cast 10% separating [33% Protogel; (30% w/v acrylamide: 0.8% (w/v) Bis-
254 Acrylamide stock solution (37.5:1)), 25% Protogel resolving buffer (1.5M Tis-HCL, 0.4%
255 SDS, pH 8.8), 41% ddH_2O, 100µl 10% APS, 20µl TEMED] and 4% stacking [13% Protogel,
256 25% Protogel Stacking buffer (0.5M Tris HCL, 0.4% SDS, pH 6.8), 61% ddH_2O, 100µl 10%
257 APS, 20µl TEMED] gels (National Diagnostics, Geneflow, UK). Gels were transferred
258 semidry onto nitrocellulose membrane (Transblot Turbo, BioRad) for 30 min at 25V and 1.0
259 mA in transfer buffer [10% TRIS/glycine (Sigma), 20% methanol, 70% ddH_2O). After
260 transfer, membranes were briefly washed in TBST (0.19 M Tris pH 7.6, 1.3 M NaCl, 0.1%

Tween-20] before being blocked for 1 h at room temperature in TBST with 1% BSA. The membranes were then washed for 3 x 5 min in TBST before being incubated overnight at 4°C with antibodies for anti-phospho-AMPK Thr172 (cat no: 2532), p38 MAPKThr180/Tyr182 (cat no: 9211) (Cell Signalling) as well as total protein content of AMPK (cat no: 2531), p38 MAPK (cat no: 9212) (Cell Signalling, UK), GAPDH (25778; Santa Cruz), and PGC-1α (Calbiochem, Merck Chemicals, UK) all at concentrations of 1:1000 in 1 x TBS. The following morning, membranes were washed for a further 3 x 5 min in TBST and subsequently incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, UK) for 1h at room temperature. After a further 3 x 5 min washes in TBST, membranes were exposed in a chemiluminescence liquid (SuperSignal, Thermo Fisher Scientific, Rockford, IL) for 5 min (30 sec for GAPDH). Membranes were visualized using a Bio-Rad Chemi-doc system, and band densities were determined using Image Lab image-analysis software (Bio Rad, UK). Samples from each subject for all exercise conditions were run on the same gel and statistical analysis conducted on raw densitometry data. Phosphorylated AMPK ^{Thr172} and p38 MAPK $^{Thr180/Tyr182}$ were normalised to their total protein, as these did not change significantly across blots or samples ($P > 0.05$). PGC-1α was normalised to GAPDH.

Statistical analysis. All data are presented as mean ± SD, unless otherwise stated. Baseline data, distance cycled, exercise HR and RPE were compared between conditions using a Paired Samples T-test. A two-factor (two condition × time) within-participants general linear model was used to evaluate the effect of time (baseline v post exercise) with shared baseline data used for NOT and CWI (Statistical Package for the Social Sciences version 21.0; SPSS Inc., Chicago, IL). A two-factor (three condition × time) within-participants general linear model was subsequently used to evaluate the influence of the cooling intervention following exercise and the 3h post exercise period. The main effects for condition and time was

followed up using planned LSD multiple contrasts. Where a significant condition by time interaction was observed, the post exercise to 3h post exercise change scores were calculated and compared across the 3-conditions using LSD multiple contrasts. The ES magnitude was classified as trivial (<0.2), small (>0.2-0.6), moderate (>0.6-1.2), large (>1.2-2.0) and very large (>2.0-4.0) (17). The α level for evaluation of statistical significance was set at $P < .05$.

Results

Exercise Response.

Distance cycled (CON 32.52 ± 4.21 km, CWI 32.33 ± 4.33 km; $P = 0.629$, ES 0.04 Trivial), heart rate ($P = 0.309$, ES 0.13 Trivial), $\dot{V}O_2$ (ml/kg.min^{-1}; $P = 0.855$, ES 0.02 Trivial) and RPE ($P = 0.637$, ES 0.08 Trivial) were similar between CON and CWI trials (data not shown). Mean HR during the final minute of exercise was 182 ± 8 beats.min^{-1} in CON and 183 ± 8 beats.min^{-1} in CWI, equating to ~94% HR max. The RPE in the final exercise bout was 19 ± 1 AU and 19 ± 1 AU in the CON and CWI trials respectively.

Recovery Response.

Metabolic Responses. Heart rate (ES 0.90 Moderate) and $\dot{V}O_2$, (ES 1.20 Moderate) was higher in CWI vs CON during the post-exercise recovery period (Table 2; $P < 0.001$). The change in HR and $\dot{V}O_2$, over time was also different between conditions ($P <0.001$) with increases in HR and $\dot{V}O_2$, occurring during the initial 2 minutes of immersion. Following immersion, HR and $\dot{V}O_2$ dropped below pre-immersion values and remained lower

throughout the 3h recovery period (HR, ES >0.92 Moderate; $\dot{V}O_2$, ES >1.25 Large) (P < 0.05).

Thermoregulatory Responses. Rectal temperature was similar between conditions throughout the post-exercise period (CON 37.52 ± 0.24°C, CWI 37.48 ± 0.05°C, ES 0.49 Small, P = 0.217. The change in rectal temperature over time was different between conditions, with a small decline in rectal temperature occurring after 3 minutes of immersion until 3h post-exercise (P=0.034, ES >0.22 Small). Thigh skin temperature was generally lower throughout the post-exercise recovery period in CWI versus CON (ES 6.26 Very Large) and NOT (ES 6.46 Very Large) (Figure 1a, P < 0.001). The change over time was also different between conditions, with thigh skin temperature continually decreasing in CWI and remaining lower than pre-immersion values until 1h post exercise (ES 3.0 Very Large, P < 0.001). Values in CON and NOT limbs remained similar to pre-immersion throughout the 3h recovery period (P = 0.10, ES 0.57 Small).

Post-exercise muscle temperature (3 cm depth) was similar between CON (38.75°C), CWI (38.86°C) and NOT (38.54°C) (Figure 1b; P > 0.05). During the 3 h recovery period muscle temperature (3cm) was lower in CWI versus CON (ES 1.60 Large) and NOT (ES 1.77 Large) (P < 0.001). The change in muscle temperature over time was also different between conditions (P < 0.001). Muscle temperature declined to a large extent immediately after immersion in the CWI limb, followed by a further gradual reduction during the remaining 3h post-exercise period (P < 0.001). In CON and NOT conditions, muscle temperature was reduced to a lesser extent immediately following immersion followed by a further gradual reduction during the 3h post-exercise period (P = 0.246, ES 0.34 Small) (See Figure 1b).

Subjective shivering ratings were greater in CWI vs CON during the post-exercise recovery period (ES 1.20 Large, P = 0.067). The change in shivering over time also tended to be

different between conditions (P = 0.062), with 'slight' shivering observed in the CWI condition during the first 2 minutes following immersion (ES >0.60 Moderate). Slight shivering was also observed 2h post exercise in the CWI condition (ES 0.95 Moderate).

AMPK and P38 MAPK activity and total abundance

Phosphorylation of AMPK ^{Thr172} was not increased post-exercise (P = 0.242, ES 0.20 Small). At post-exercise and 3h post-exercise phosphorylation of AMPK^{Thr172} was similar between conditions (P = 0.846, ES 0.03 Trivial). However, the change in AMPK^{Thr172} between these time points was different between conditions (P = 0.031; Figure 2). AMPK^{Thr172} phosphorylation increased in CWI vs. CON (P = 0.027, ES 1.22 Large) with a moderate increase in AMPK^{Thr172} phosphorylation also observed in NOT vs. CON (P = 0.145, ES 0.70 Moderate). Representative Western blots are shown in Figure 5.

Exercise induced a small increase in phosphorylation of p38MAPK$^{Thr180/Tyr182}$ (P = 0.056, ES 0.44 Small, Figure 3). At post exercise and 3h post-exercise phosphorylation of p38MAPK$^{Thr180/Tyr182}$ was similar between conditions (P = 0.672; ES 0.03, Trivial). No differences in the change in phosphorylation between these time points was observed between conditions (P = 0.268, Figure 3). Representative Western blots are shown in Figure 5.

PGC-1alpha mRNA and protein abundance.

PGC-1α mRNA expression was moderately increased with exercise (P = 0.066, ES 0.92 Moderate, Figure 4a). At 3h post-exercise, expression was greater in CWI (ES 1.2 Moderate, P = 0.003) and NOT (ES 1.6 Large, P = 0.001) versus CON, but was similar between CWI and NOT (ES 0.6 Small, P = 0.141) (Figure 4a). This reflected the greater change in expression in CWI and NOT conditions between post exercise and 3h post exercise time

355 points (P = 0.001, Figure 4a). PGC-1α protein content was not influenced by exercise (P =
356 0.092) or CWI (P = 0.471, Figure 4b). Representative Western blots are shown in Figure 5.

357 *Additional gene expression*

358 Exercise induced increases in SIRT1 (P = 0.057, ES 0.8 Moderate) and NRF2 (P = 0.028, ES
359 0.6 Moderate) mRNA (data not shown). No changes were seen between conditions, or
360 between conditions over time (P > 0.05). Gene expression analysis for p53, COXIV, CS,
361 TFAM, SIRT1, NRF2 and ERRα mRNA was not influenced by exercise or CWI (P > 0.05;
362 data not shown).

363

364 *Plasma Metanephrine and NorMetanephrine.*

365 Metanephrine concentrations were similar between conditions (P = 0.159, ES 0.15 Trivial).
366 The change in metanephrine over time was also similar between conditions (P = 0.299).
367 Metanephrine concentration was increased post-exercise (ES 2.46 Very Large) and post-
368 immersion (ES 0.77 Moderate) vs. baseline ($P \leq 0.02$). Normetanephrine values were greater
369 in CWI vs. CON (P = 0.034, ES 0.43 Small) with the largest difference seen post-immersion
370 (860 vs. 665 pmol/L, CWI vs. CON, respectively). The change in Normetanephrine over time
371 was similar between conditions (P = 0.821). Normetanephrine concentrations increased with
372 exercise (ES >4.70; P < 0.001) and remained above baseline post-immersion (ES 1.52 Large)
373 and 1hr post-exercise (ES 1.06 Moderate) (P <0.001). Concentrations returned to baseline at
374 2hr post-exercise (See Table 3).

375

376 **Discussion**

The aim of the present study was to examine whether the post-exercise cold-water induced increase of PGC-1α mRNA is mediated through local or systemic mechanisms. Using a novel experimental design, we report for the first time the appearance of systemic "cross-talk" between immersed and non-immersed limbs, as evidenced by the similar increase in PGC-1α mRNA in these limbs after single-legged CWI. Additionally, we suggest that this effect could be mediated by β-adrenergic induced stimulation of AMPK. In addition to providing novel data on the potential mechanisms mediating post-exercise cold-induced enhancement of PGC-1α expression, our data also have potential implications for research designs that utilise as non-immersed limbs as control conditions.

Since its initial discovery (38) the importance of the post-exercise PGC-1α response to the oxidative adaptive process has been examined extensively, with mRNA increases ranging 5 to 10-fold commonly observed at 3-4 hours following exercise (3, 4, 21, 36). More recently, cold-ambient temperatures (42, 43) and post exercise cold-water immersion (18-20, 24), have also been shown to enhance (~2- to 4- fold greater) the skeletal muscle PGC-1α gene transcription and protein translational response versus exercise alone. In line with such observations, the high-intensity intermittent cycling protocol used in the present study elicited a ~5-fold increase in PGC-1alpha mRNA at 3 hours post-exercise. Furthermore, CWI enhanced this response to a greater extent than exercise alone (~9-fold increase vs. Pre in the CWI immersed limb).

There have been suggestions that reduced tissue temperature may be responsible for the differences observed between cold-treated and control limbs. This stems from the initial discovery that PGC-1alpha was cold inducible in animals (6, 9, 38). In humans, recent data from Ihsan and colleagues (18, 20) implicates a reduction in tissue temperature in the cold-induced increases of PGC-1α, as increases in mRNA (3h post-exercise) and protein content (after 4 weeks of training) were seen only in a cooled limb, and not in the contralateral non-

402 immersed limb. Potential mechanisms underpinning such responses include activation of
403 non-noxious thermoreceptors via reduced skin temperature (Hensel & Boman, 1960, 22).
404 Within the present study, both the NOT and CWI limbs displayed similar acute PGC-1α
405 mRNA expression in the 3 h recovery period, whilst skin and muscle temperature were
406 significantly reduced in the CWI limb only. Indeed, the non-immersed limb (NOT) showed a
407 similar temperature profile (skin and muscle temperature) to that of CON, where the
408 magnitude of PGC-1α mRNA response was almost half when compared with CWI and NOT.
409 When taken together, these data suggest that alterations to local muscle temperature do not
410 play a significant role in cold-induced regulation of PGC-1α expression.

411 As a result of increased local PGC-1α gene expression in the non-immersed exercised limb to
412 a similar magnitude as the immersed limb, we sought to consolidate the role of the upstream
413 kinases AMPK and p38-MAPK (7, 23, 47) in their ability to regulate PGC-1α transcription.
414 p38MAPK is a stress activated kinase that has been shown extensively to be phosphorylated
415 after acute exercise, independent of intensity (10). Moreover, p38MAPK can exert its effect
416 upon PGC-1α transcription via upregulated ATF2 activity at the PGC-1α promoter (2). In the
417 current study, exercise induced a small (ES 0.44, 1.5-fold) increase in phosphorylation with
418 no further response to cooling. Moreover, our data supports previous data showing acute
419 post-exercise phosphorylation of p38MAPK locally in skeletal muscle tissue occurs
420 systemically (46). Exercise-induced intensity dependant AMPK phosphorylation is a well
421 reported phenomenon (8, 12, 28) in rodent and human studies (34, 10). Moreover, AMPK is
422 implicated in PGC-1α activity via direct phosphorylation, initiating many of the important
423 gene regulatory functions of PGC-1α in skeletal muscle (23). The post-exercise increase in
424 phosphorylation of AMPK in the present study was similar in magnitude to previous work (3)
425 from our laboratory, albeit failing to achieve statistical significance. Notwithstanding this,
426 large and moderate effect sizes were observed at 3 h post-exercise in the CWI and NOT limbs

vs. CON, respectively. These greater increases from post-exercise to 3 h post-exercise in the immersion trial (CWI and NOT limbs), compared to a slight decline in CON suggest the increases in phosphorylation of AMPK during the post-cooling period are controlled by a systemic mechanism, possibly adrenergic control via cold-augmented plasma Normetanephrine.

Epinerphrine and norepinephrine are both dual α- and β- adrenergic agonists. Previous in vivo and in vitro incubation techniques utilising α- and β- adrenergic agonists have reported increased AMPK activation in rodent skeletal muscle (32), adipose tissue (26) and cell cultures (48) implicating catecholamines as a potential AMPK activator. Despite this, support for the above hypothesis is currently conflicting, and is limited by distinct differences in methodological design and species studied. In rodent muscle, acute infusion of adrenergic agonists/antagonists have previously shown to be ineffective at altering PGC-1α transcriptional activity (39, 40) and its upstream effector p38-MAPK (25). In contrast, treatment with β-adrenergic agonists/antagonists has been shown to induce and inhibit PGC-1α respectively (31), whilst incubation of rodent skeletal muscle with the adrenergic agonist phenylephrine increased the activity of the upstream regulator of PGC-1α, AMPK (32). When results from these studies are taken together, it could be suggested that adrenergic activation of AMPK is a potential mechanism to explain the systemic increases in PGC-1α gene expression described herein.

In humans, one study has investigated the impact of higher catecholamine levels on AMPK phosphorylation in human skeletal muscle (27). These authors assessed muscle biopsies from an exercised and non-exercised limb in conditions of heightened catecholamine release. Results showed AMPK activity was restricted to contracting muscle only, with no systemic effects notable in the non-exercised limb despite the increased catecholamine levels. Importantly, in our study all limbs were exercised before undergoing cold exposure. It

therefore may be that the cold induction of β-adrenergic pathways (via increased catecholamines) presented in this manuscript allows an additive response to exercise stimulated AMPK phosphorylation. Further studies are now required to verify this signalling response in related experimental conditions in human skeletal muscle.

Another pathway by which increased catecholamine's may enhance PGC-1α transcription is via increased activation of β-adrenergic receptors. Activation of these receptors increases intracellular cAMP, which could ultimately activate CREB function on the PGC-1α promoter (1). However, evidence exists to show β-adrenergic stimulation does not activate a p38MAPK - ATF2 - CREB - PGC-1α signalling axis in skeletal muscle (25). These findings resonate with results from the present study as no changes in phosphorylation of p38MAPK were observed alongside increased plasma Normetanephrine concentrations. Further work is required to investigate the influence of the dual stress of exercise and cold temperature upon this signalling axis.

Downstream of PGC-1α, Slivka and colleagues (42) noted that recovery in cold ambient temperatures (4h in 7°C ambient air) reduced the expression of the transcription factors Nuclear-respiratory factor 2 (NRF2) and estrogen-related receptor α (ERRα), whilst having no effect on mitochondrial transcription factor a (TFAM). Their importance to the adaptive response is highlighted by their roles in oxidative metabolism. Our data suggest the immersion protocol used herein was not sufficient to induce such changes. Indeed, 10 minutes of single-legged immersion offers a much smaller cooling stimulus than the 4 hours in cool ambient temperatures, as used by Slivka et al. (42). In addition, an acute increase in PGC-1α gene expression was not followed by changes in PGC-1α total protein content, perhaps due to the acute time-frame of sampling applied in the present study. With this lack of change in total protein content it is however, unlikely that changes in gene expression of downstream genes such as NRF2, TFAM, COXIV, CS, ERRα presented here, would be

477 affected by its upstream protein function as a transcription factor (PGC-1α). More research is
478 required to understand the effect and dose response of a cold stimulus on downstream targets
479 of PGC-1α.

480 It is difficult to explain the differences in PGC-1alpha mRNA results between the present
481 study and Ihsan et al. (20), particularly the difference in non-immersed limbs. Moreover,
482 reasons as to why Ihsan and colleagues failed to see the expected exercise-induced response
483 in their non-immersed control limb, with minor changes from baseline at 3h post-exercise
484 (1.5-fold increase vs. 3-5-fold increase usually seen), remain unclear. One possible
485 explanation might include the differing muscle recruitment patterns occurring between the
486 exercise protocols utilised (cycling vs. running). Ultimately, results from the present study
487 have future implications on scientific study designs. Those wishing to investigate cold
488 induced post-exercise responses in skeletal muscle must be aware of the evidence that
489 implicates a systemic response of Normetanephrine, and a local response of p-AMPK in both
490 immersed and non-immersed limbs. Further evidence is required to support the impact of
491 systemic transcriptional responses in unilateral research designs, as such designs may be
492 liable to error; potentially underestimating the actual response occurring in the immersed
493 limb if relativized to a non-immersed limb instead of a resting control. Ultimately, the choice
494 of scientific design lies with the question posed, as contralateral designs remain useful for
495 understanding both local and systemic responses.

496 In summary, the present study characterises for the first time the mechanistic control of cold
497 induced PGC-1α mRNA expression. Data herein indicate a reduction in tissue temperature
498 (2-3°C) plays a limited role as similar levels of PGC-1α mRNA expression are observed in an
499 immersed and non-immersed limb despite a reduction in tissue temperature in the CWI limb
500 only. Moreover, a cold-induced systemic increase in plasma Normetanephrine may impact
501 localised phosphorylation events of the signalling kinase $AMPK^{Thr172}$, with potential

downstream effects upon rates of PGC-1α mRNA expression. Future studies should investigate the role of β-adrenergic receptors in Normetanephrine induced AMPK phosphorylation and the signalling role of MEF2 and CRE/ATF2 sites to confirm a link between catecholamines and PGC-1α. Moreover, due to the acute nature of the present study more work is required to investigate whether the response seen herein is maintained over a more chronic term.

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Disclosures

The authors report no conflicts of interest.

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Table 1: Primer sequences used for real-time PCR.

Gene	Forward Primer	Reverse Primer	Product Length (base pairs)
GAPDH NM_002046.5	AAGACCTTGGGCTGGGACTG	TGGCTCGGCTGGCGAC	168
PGC-1alpha NM_013261.3	TGCTAAACGACTCCGAGAA	TGCAAAGTTCCCTCTCTGCT	67
p53 NM_000546.5	ACCTATGGAAACTACTTCCTGAAA	CTGGCATTCTGGGAGCTTCA	141
SIRT1 NM_012238.4	CGGAAACAATACCTCCACCT	CACATGAAACAGACACCCCA	186
COXIV NM_001861.4	CGAGCAATTTCCACCTCTGT	GGTCACGCCGATCCATATAA	94
CS NM_004077.2	CCTGCCTAATGACCCCATGTT	CATAATACTGGAGCAGCACCCC	137
TFAM NM_003201.2	TGGCAAGTTGTCCAAAGAAACCTGT	GTTCCCTCCAACGCTGGGCA	135
NRF2 NM_002040.3	AAATTGAGATTGATGGAACAGAGAA	TATGGCCTGGCTTACACATTCA	95
ERRα NM_004451.4	TGCCAATTCAGACTCTGTGC	CCAGCTTCACCCCATAGAAA	212

Table 2: Heart rate and oxygen uptake during immersion and the post-immersion period (n=10, mean ± SD).

		Immersion						Post-Immersion							
		PreIm	2min	4min	6min	8min	10min	2min	4min	6min	8min	10min	1hr	2hr	3hr
HR (beats. min^{-1})	CON	88 ±10	86$^{\#}$ ±9	86 ±10	86 ±9	87 ±8	86 ±9	85$^{\#}$ ±9	85$^{\#}$ ±9	84$^{\#}$ ±9	83$^{\#}$ ±9	85$^{\#}$ ±10	85$^{\#}$ ±10	76$^{\#}$ ±11	76$^{\#}$ ±10
	CWI	103 ±14	117$^{\#}$ ±18	109 ±16	104 ±13	102 ±14	101 ±13	90$^{\#}$ ±13	90$^{\#}$ ±12	89$^{\#}$ ±12	88$^{\#}$ ±11	88$^{\#}$ ±11	89$^{\#}$ ±11	75$^{\#}$ ±13	73$^{\#}$ ±14
$\dot{V}O_2$ (ml/kg ,min^{-1})	CON	4.6 ±0.9	4.2 ±0.8	4.3 ±0.7	4.4 ±0.8	4.3 ±0.6	4.3 ±0.9	4.4$^{\#}$ ±0.8	4.5$^{\#}$ ±0.8	4.3$^{\#}$ ±0.8	4.2$^{\#}$ ±0.7	4.3$^{\#}$ ±0.9	4.5$^{\#}$ ±0.8	4.3$^{\#}$ ±0.6	4.0$^{\#}$ ±0.6
	CWI	7.05 ±1.4	8.1 ±1.4	6.5 ±1.2	6.3 ±0.7	6.0 ±1.0	5.9 ±0.6	5.2$^{\#}$ ±1.4	4.8$^{\#}$ ±1.0	4.7$^{\#}$ ±0.8	4.4$^{\#}$ ±0.9	4.1$^{\#}$ ±1.2	4.3$^{\#}$ ±1.3	3.8$^{\#}$ ±0.5	3.8$^{\#}$ ±0.5

Values are mean ± SD. A main effect for condition and time along with a significant interaction between condition and time were found for HR and $\dot{V}O_2$ ($P < 0.05$).

$^{\#}$ Significant difference from pre-immersion ($P < 0.05$).

Table 3: Catecholamine concentrations (pmol/L) pre and post exercise, with and without CWI (*n*=10, mean ± SD).

					Recovery		
		Baseline	Post-Exercise	Post-Immersion	1hr	2hr	3hr
Normetanephrine	CON	326.7	1711.9#	665#	615.7#	489.3	426.9
		±165.6	±411.10	±291.8	±368.3	±407.0	±260.0
	CWI	478.2*	1774.1*#	859.8*#	716.8*#	621.2*	517.2*
		±189.2	±358.4	±264.5	±257.3	±247.5	±191.0
Metanephrine	CON	199.7	489.2#	231.4#	193.4	167.9#	145.8#
		±62.0	±234.4	±90.5	±75.2	±62.1	±68.4
	CWI	201.6	509.6#	281.8#	245.6	176.5#	157.5#
		±36.7	±153.9	±104.8	±78.3	±43.9	±46.4

Values are mean ± SD. A main effect for condition was found for NorMetanephrine ($P = 0.034$). A main effect for time was found for both NorMetanephrine and Metanephrine ($P < 0.001$). # Significant difference from baseline ($P < 0$ .05). *Significant difference versus the CON condition ($P < 0$ .05).

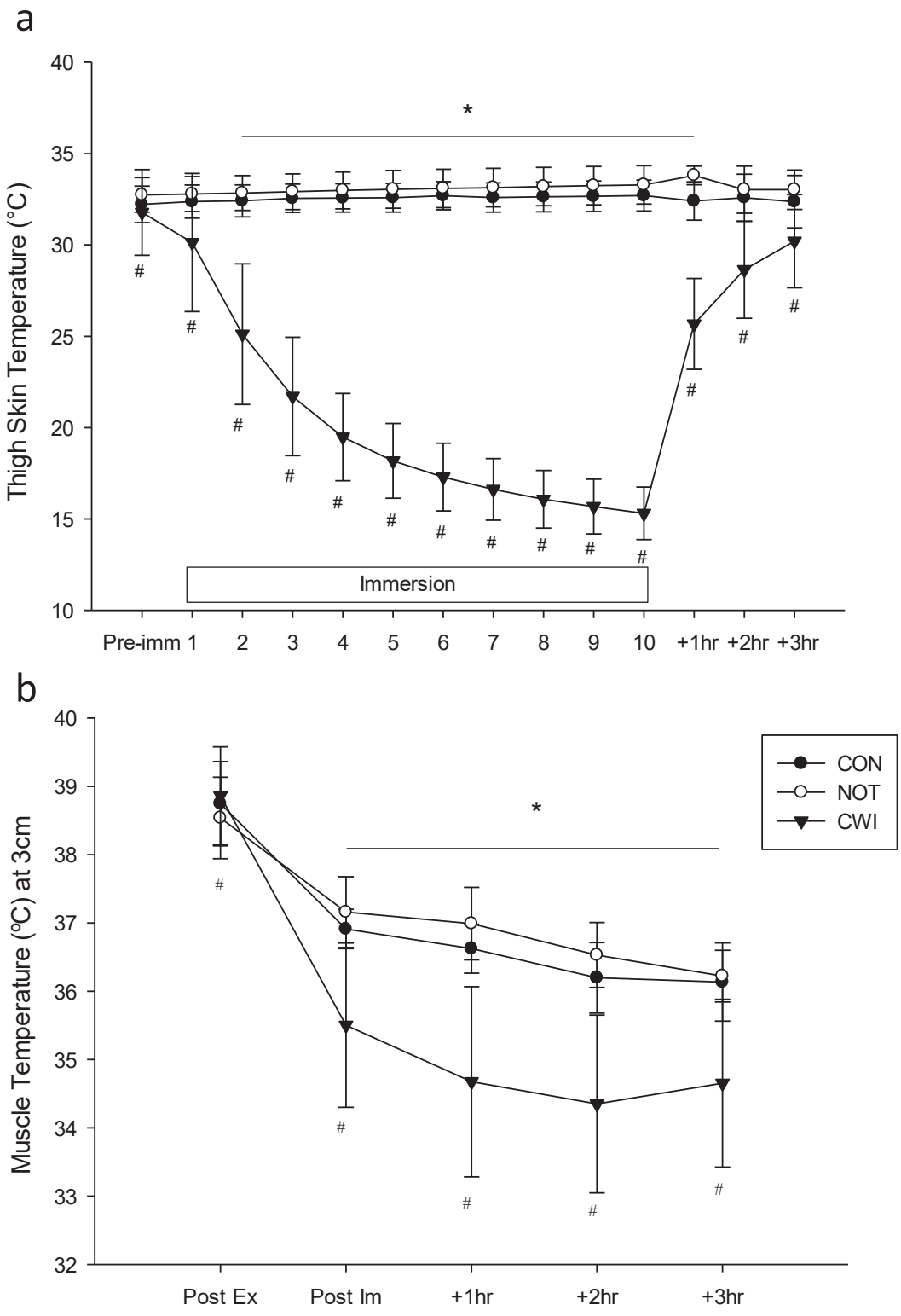

Figure 1: Temperature response post-exercise for thigh skin (a) and deep muscle (3cm depth) (b) with/without CWI. Values are mean ± SD. A main effect for time, condition and time*condition interaction was found in both thigh and muscle temperature measures (P<0.05). *Significantly different from pre-immersion (Skin) or post-exercise (muscle) (P < 0.05). # Significantly different from CON and NOT (P < 0.05).

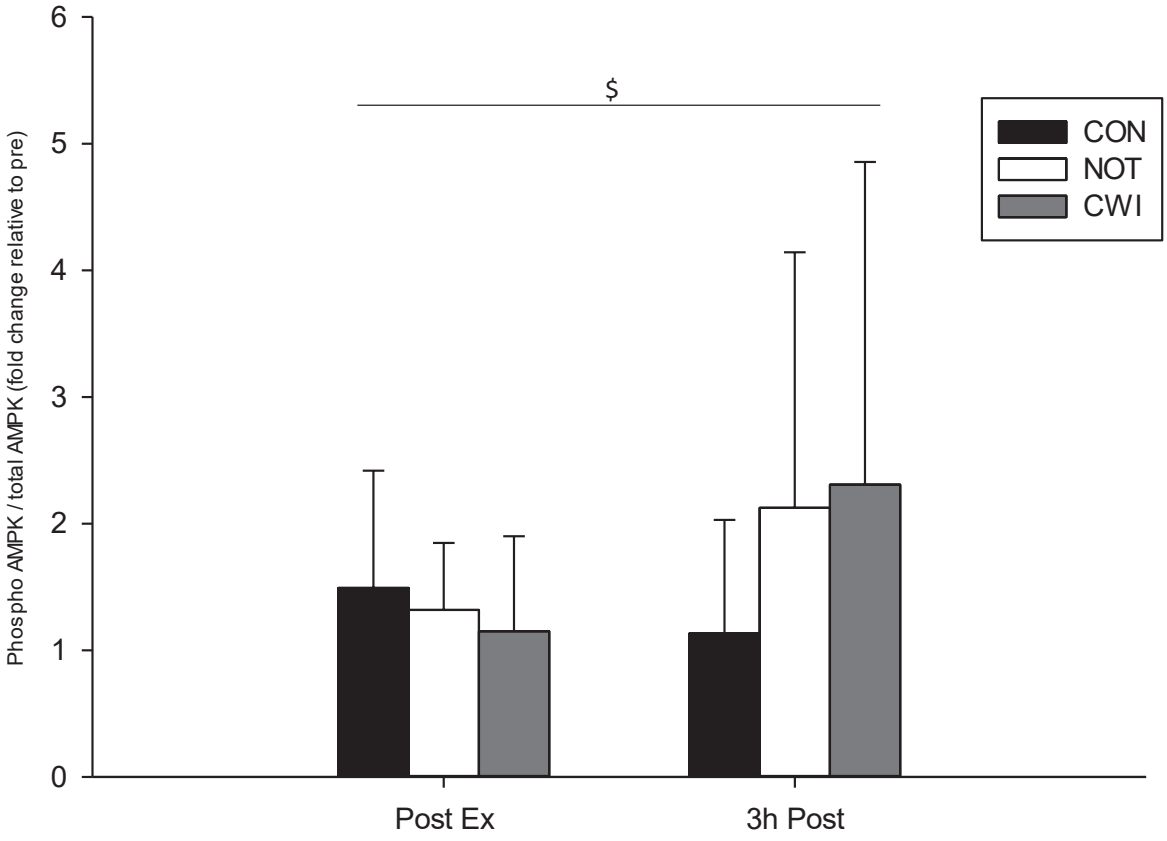

Figure 2: Phosphorylation of AMPK^{Thr172} fold changes from Pre, expressed relative to total AMPK as total AMPK did not change significantly across blots or samples ($P > 0.05$). Values are mean ± SD. $ Significant time*condition interaction ($P = 0.031$).

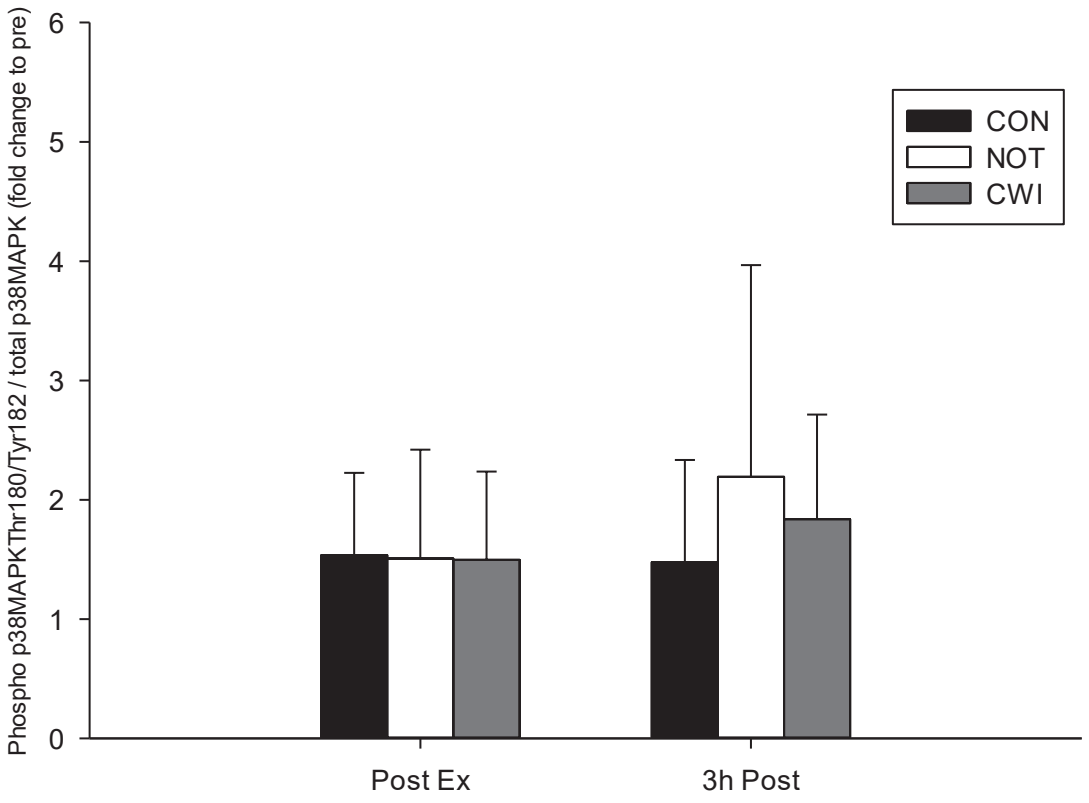

Figure 3: Phosphorylation of p38MAPK$^{Thr180/Tyr182}$ fold change from Pre, expressed relative to total p38MAPK as total p38 MAPK did not change significantly across blots or samples ($P > 0.05$). Values are mean ± SD.

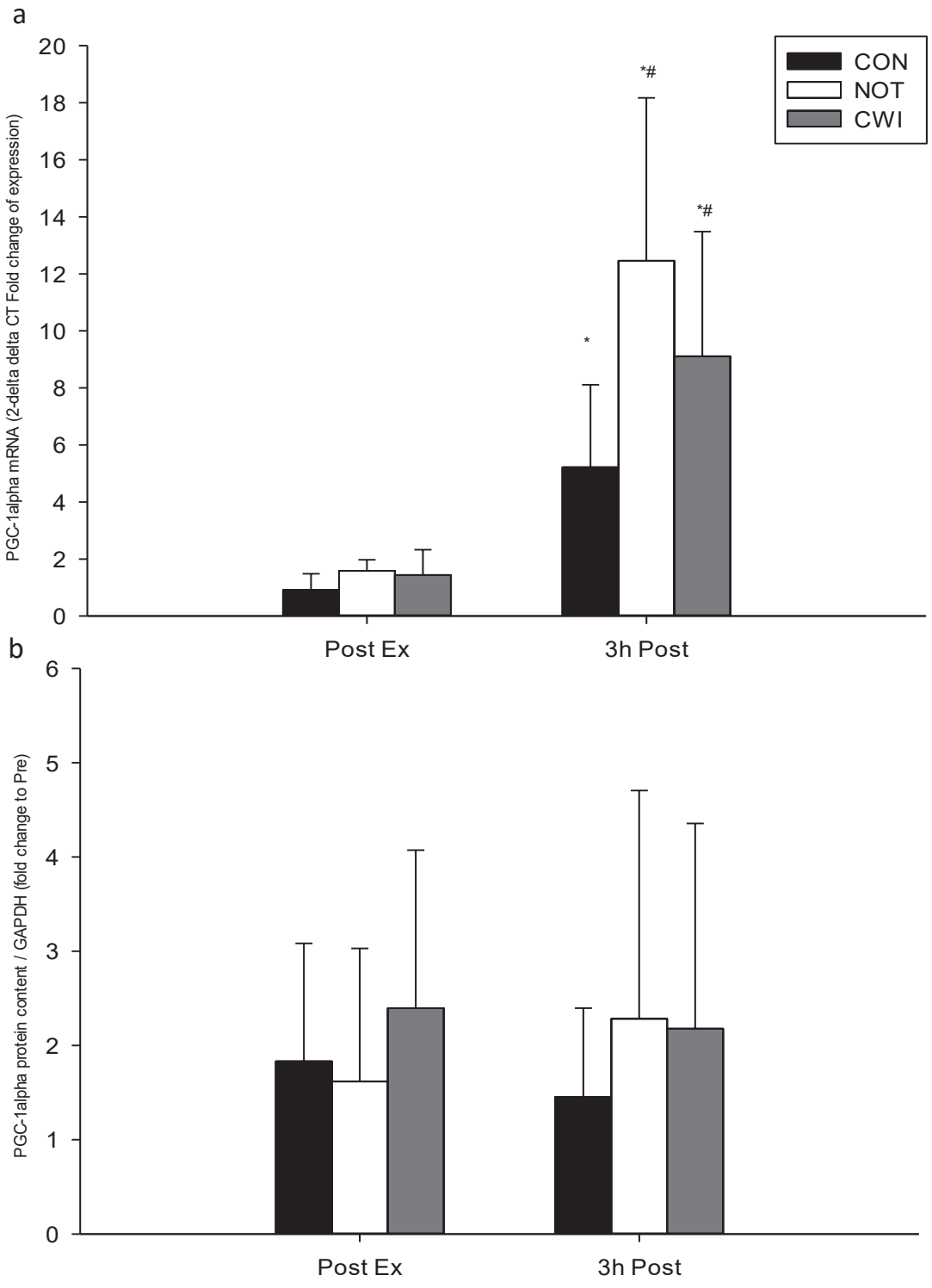

Figure 4: PGC-1α mRNA (A) and protein content (B) $2^{-\Delta\Delta CT}$ fold change in expression with the calibrator as pre-exercise and the reference gene as GAPDH (see methods for details). Values are mean ± SD. A main effect for condition and time was found ($P < 0.001$). There was also a significant interaction between condition and time (P = 0.001) *Significantly different from baseline ($P < 0.05$). # Significantly different from CON ($P < 0.05$).

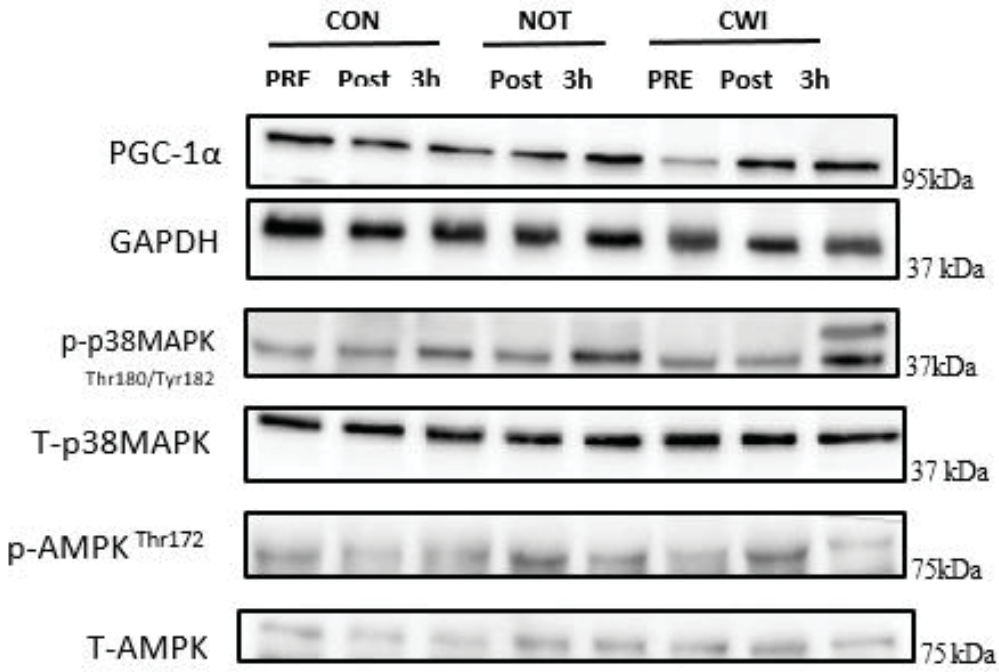

Figure 5: Representative Western Blots from the control (CON) non-immersed (NOT) and immersed (CWI) legs before (Pre), after (Post), and 3h after (3h) exercise. p-, phosphorylated; t-, total. Phosphorylated AMPK Thr172 and p38 MAPK Thr180/Tyr182 were relativized to their total counterpart, as these did not change significantly across blots or samples ($P > 0.05$). PGC-1α was relativized to GAPDH.