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Regular postexercise cooling enhances mitochondrial biogenesis through AMPK and p38 MAPK in human skeletal muscle

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Ihsan M, Markworth JF, Watson G, Choo HC, Govus A, Pham T, Hickey A, Cameron-Smith D, Abbiss CR. Regular postexercise cooling enhances mitochondrial biogenesis through AMPK and p38 MAPK in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 309: R286–R294, 2015. First published June 3, 2015; doi:10.1152/ajpregu.00031.2015.—This study investigated the effect of regular postexercise cold water immersion (CWI) on muscle aerobic adaptations to endurance training. Eight males performed 3 sessions/wk of endurance training for 4 wk. Following each session, subjects immersed one leg in a cold water bath (10°C; COLD) for 15 min, while the contralateral leg served as a control (CON). Muscle biopsies were obtained from vastus lateralis of both CON and COLD legs prior to training and 48 h following the last training session. Samples were analyzed for signaling kinases: p38 MAPK and AMPK, peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), enzyme activities indicative of mitochondrial biogenesis, and protein subunits representative of respiratory chain complexes I–V. Following training, subjects' peak oxygen uptake and running velocity were improved by 5.9% and 6.2%, respectively ($P < 0.05$). Repeated CWI resulted in higher total AMPK, phosphorylated AMPK, phosphorylated acetyl-CoA carboxylase, β-3-hydroxyacyl-CoA-dehydrogenase and the protein subunits representative of complex I and III ($P < 0.05$). Moreover, large effect sizes (Cohen's $d > 0.8$) were noted with changes in protein content of p38 ($d = 1.02$, $P = 0.064$), PGC-1α ($d = 0.99$, $P = 0.079$), and peroxisome proliferator-activated receptor α ($d = 0.93$, $P = 0.10$) in COLD compared with CON. No differences between conditions were observed in the representative protein subunits of respiratory complexes II, IV, and V and in the activities of several mitochondrial enzymes ($P > 0.05$). These findings indicate that regular CWI enhances p38, AMPK, and possibly mitochondrial biogenesis.

cold water immersion; exercise recovery; muscle oxidative adaptations; PGC-1α; nonshivering thermogenesis

ONE OF THE MOST PRONOUNCED consequences of endurance training is an increase in skeletal muscle mitochondrial content (17), which inevitably includes the increase in respiratory chain complexes (15), enzymes of the citric acid cycle (14), and fatty acid oxidation (16). These adaptations, consequently, lead to an improved muscle aerobic function (16), which is associated with enhanced endurance performance (11) and a reduction in risk factors for a variety of chronic diseases (42).

It is currently accepted that mitochondrial phenotypic adaptations following endurance training stems from accumulated changes in gene expression following each exercise session (26). Changes in gene expression, in turn, are triggered by contraction-induced (i.e., changes in phosphagen ratio, reactive oxygen species, and Ca^{2+} flux) alterations in the posttranslational activities of key signaling kinases, including the AMPK and the p38 MAPK (1, 13). Moreover, the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) has been identified as a downstream target of both the AMPK and p38 MAPK cascades (1, 22) and has shown to be essential in the regulation and coordination of the mitochondrial biogenesis program (27, 44). For instance, PGC-1α has been shown to regulate the expression and activity of the nuclear respiratory factors and the mitochondrial transcription factor (27, 44), which are key transcription factors involved in encoding the nuclear and mitochondrial genomes, respectively (28, 38). Moreover, in rodent (8) and cell culture (44) models, overexpression of PGC-1α paralleled adaptations similar to that seen following endurance training (11, 14–17), including an increase in the expression of respiratory chain components (8, 44), mitochondrial enzyme activity (8), mitochondrial content (44), improved endurance capacity, and performance (8). In contrast, mitochondrial biogenesis has been shown to be impaired in response to exercise or chronic electrical stimulation in PGC-1α knockout rodents and cultured myotubes (12, 35). Taken together, PGC-1α along with upstream regulators, such as AMPK and p38 MAPK, are key molecular targets for interventional strategies, as its increase is invaluable to both enhanced athletic performance and clinical outcomes.

Cryotherapy is a well-recognized treatment for acute musculoskeletal injuries, which recently in the form of cold water immersion (CWI), has been utilized as a post-exercise recovery strategy to improve sport/training performances (21, 25, 36). Apart from the physiological benefits conferred by CWI in promoting postexercise recovery (39), CWI may be a potential strategy to enhance exercise-induced mitochondrial adaptations, as cold exposure per se has been shown to upregulate PGC-1α similarly to that observed following exercise (7, 19, 27, 44). For instance, cold exposure, through adrenergic mechanisms, has been shown to induce PGC-1α in adipose tissue and myotube cultures, where it has been implicated in the regulation of uncoupling proteins (UCP) and adaptive (i.e., nonshivering) thermogenesis (27, 44). More recently, an increase in PGC-1α protein expression, citrate synthase (CS), and β-3-hydroxyacyl-CoA-dehydrogenase (β-HAD) activities, in line with increased resting intracellular Ca^{2+} content, was demonstrated in rodents following 8 wk of cold acclimatization (7).

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While it is evident that both exercise and cold exposure independently induce PGC-1α and mitochondrial biogenesis, the interaction between exercise and postexercise CWI on mitochondrial adaptations is unclear and has been the subject of much debate (20, 21, 45). For instance, we recently showed that a single CWI (15 min at 10°C) treatment performed following endurance exercise enhanced the muscle mRNA expression of PGC-1α in physically active males (20). Although such responses in PGC-1α would likely result in favorable mitochondrial adaptations and, consequently, contribute to enhanced endurance performance in the longer term, Yamane et al. (45), in contrast, reported attenuated improvements in maximal oxygen uptake ($\dot{V}O_{2\,max}$) and cycling time to exhaustion following regular CWI (2 × 20 min at 5°C) during 4 wk of endurance training. Alterations in AMPK and/or p38 signaling may possibly explain the observed differences between acute responses and chronic adaptations to postexercise CWI. For instance, cooling of the legs following intense exercise has shown to significantly reduce postexercise muscle oxygen demand (21), an important stimulus for activating AMPK (3, 40). Moreover, accelerating the decline in muscle temperature following exercise might attenuate both p38 and AMPK signaling, as postexercise heat exposure or heat exposure alone has shown to induce mitochondrial biogenesis in rodents and myotube cultures, respectively, through increased p38 and AMPK signaling (24, 33).

To the best of our knowledge, the influence of regular postexercise CWI application on exercise-induced mitochondrial biogenesis has yet to be determined. Moreover, mechanisms upstream of PGC-1α following postexercise CWI are at best unclear. As such, the purpose of our study was to investigate the influence of regular CWI on training-induced changes in AMPK, p38 MAPK, PGC-1α, and indices of mitochondrial biogenesis.

METHODS

Subjects

Eight physically active healthy males [means ± SD: age: 21.4 ± 2.8 yr, height: 177 ± 7 cm, mass: 76.6 ± 8.2 kg, peak oxygen uptake ($\dot{V}O_{2\,peak}$): 46.7 ± 5.7 ml·kg^{-1}·min^{-1}] were recruited for this study. Subjects were participating in a recreational team sport, such as soccer, floorball, or hockey for ~2 to 3 h/wk, for at least a year at the time this study was conducted. Subjects were not using medication and had no history of lower limb musculoskeletal injuries at the time of testing. Participants were asked to refrain from all exercise, apart from that conducted within the study, as well as alcohol and caffeine for at least 48 h prior to the pretraining and posttraining testing sessions. Subjects were also fully informed of the requirements and risks associated with the study, and written informed consent was obtained prior to participation. This study was approved by the Edith Cowan University human research ethics committee.

Experimental Design and Procedures

Incremental exercise testing. All subjects performed two identical incremental treadmill (Trackmaster, JAS fitness systems) running tests prior to the commencement of training and 48–72 h following their last training session, respectively. The tests were conducted at the same time of the day, following an 8-h fast. The subjects' diet in the 24 h preceding the pretraining test was recorded and replicated in the 24 h preceding the posttraining test. The test commenced at either 9 or 10 km/h depending on the subjects' comfort and increased by 1 km/h every 2 min until volitional exhaustion. The gradient of the treadmill was maintained at 1%, while the heart rate (S610, Polar, Finland) and gas exchange (TrueOne, ParvoMedics, Sandy, UT) were continuously recorded throughout the test. Prior to all tests, the gas analyzer and the ventilometer were calibrated using gases of known concentrations and a 3-l syringe (5530 series; Hans Rudolph, Shawnee, KS), respectively. Subjects' $\dot{V}O_{2\,peak}$ was determined as the highest value attained in any 30-s average, while their maximal aerobic velocity ($\dot{V}_{max}$) was calculated using the equation by Kuipers et al. (23): $\dot{V}_{max} = V_f + (t/120 \times 1)$ where V_f is the velocity achieved during the last completed stage in km/h, and t is the time of the incomplete stage in seconds.

Training. All subjects performed three supervised treadmill running training sessions·per week for 4 wk. The three training sessions included long- (6–8 min), moderate- (2 min), and short- (30 s) interval bouts performed at 80–110% $\dot{V}_{max}$, respectively. The progressive overload in intensity, interval duration, and repetitions are detailed in Table 1, while absolute running speeds were increased weekly, on the basis of an estimated 2% increase in $\dot{V}O_{2\,peak}$/wk and assuming that the relationship between running velocity and $\dot{V}O_2$ was linear (10). Heart rate was monitored during all training sessions, and subjects' adherence to refrain from training external to the study was verbally confirmed on a weekly basis.

Within 3 min following the cessation of all training sessions, subjects immersed one leg (COLD) to the level of their gluteal fold into a plastic water bath (47 × 41 × 87 cm) maintained at 10.1 ± 0.3°C for 15 min, while their contralateral leg rested outside the water tank. The contralateral leg received no cooling treatment and, thus, served as the control (CON). The limb cooled was randomized between subjects' dominant and nondominant leg. Although recovery CWI usually involves whole body or waist-deep immersions, we utilized this one-legged protocol to control for shivering thermogenesis, which possibly influences AMPK activation and mitochondrial biogenesis (30, 31). This protocol has also been shown to effectively reduce postexercise muscle temperatures (<30°C), while having minimal influence on the natural decline in postexercise core temperature (20, 21), hence, isolating the effects of a lowered muscle temperature per se on training-induced mitochondrial biogenesis. Finally, this one-legged cooling protocol controls for systemic changes induced by CWI and, hence, allows for the controlled examination of localized adaptations (21).

Muscle biopsy. Muscle biopsies were performed on both legs prior to undertaking the pretraining and posttraining incremental performance tests. Muscle samples were extracted using a disposable, spring-loaded microbiopsy system (MAX-CORE, Bard Biopsy Sys-

Table 1. *Training program for weeks 1–4*

	Intensity, % V_{max}			Work Interval Duration			Interval Repetitions			Work-Rest Ratio			Rest Interval Mode		
Interval Type	Long	Medium	Short	Long	Medium	Short	Long	Medium	Short	Long	Medium	Short	Long	Medium	Short
Week 1	80%	90%	100%	6 min	2 min	30 s	3	4	6	1:0.33	1:1	1:0.5	AR	AR	PR
Week 2	80%	90%	100%	6 min	2 min	30 s	3	6	8	1:0.33	1:1	1:0.5	AR	AR	PR
Week 3	85%	95%	105%	8 min	2 min	30 s	3	8	10	1:0.2	1:1	1:0.5	AR	AR	PR
Week 4	85%	95%	110%	8 min	2 min	30 s	3	10	12	1:0.2	1:1	1:0.5	AR	AR	PR

AR, active recovery undertaken at 50% of V_{max}; PR, passive recovery.

tems, Tempe, AZ) from the vastus lateralis muscle. Biopsy site was standardized (i.e., pretraining and posttraining) at the midpoint between the greater trochanter and patella border. Following the application of topical anesthesia (5% lidocaine) around the sampling region, a 13-gauge cannula was inserted 3 cm into the muscle belly. Then a 14-gauge biopsy needle was inserted into the cannula and two to three muscle samples (30–40 mg total) were subsequently extracted per biopsy. The tissue samples were immediately frozen in liquid nitrogen upon extraction and stored in a −80°C freezer for later analysis.

Tissue processing and immunoblotting. Muscle biopsy tissue (~20 mg) were homogenized in ice cold 1× RIPA lysis buffer (15 μl/mg tissue) (Millipore no. 20-188: 50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA) supplemented with a commercially available protease and phosphatase inhibitor cocktail (Halt protease and phosphatase inhibitor cocktail; no. 78442; Thermo Fisher Scientific, Waltham, MA) using 2.8-mm ceramic beads in a bead mill homogenizer (OMNI Ruptor, 5.65 m/s, 2 × 30 s). Resulting homogenates were collected and agitated for 1 h at 4°C prior to centrifugation at 13,000 g at 4°C for 10 min. The supernatant was collected and stored at −80°C until further analysis. Total protein content of the muscle homogenate was determined with a BCA protein kit, as per the manufacturer's instructions (no. 23225; Pierce, Rockford, IL). Total protein aliquots were diluted to 2 μg/μl, suspended in Laemmli buffer, boiled, and subjected to SDS-PAGE (20 μg protein per well). The four muscle biopsy samples collected from each participant were always run in contiguous lanes on the same gel. Proteins were transferred to a PVDF membrane, using the semi-dry Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA) prior to membrane blocking in 5% BSA/Tris-buffered saline/0.1% Tween 20 (TBST) for 2 h at room temperature. Membranes were then incubated overnight at 4°C with gentle agitation in blocking solution containing primary antibodies: total AMPKα (no. 2603, 1:1,000; Cell Signaling, Beverly, MA), phosphorylated-AMPKα (p-AMPKα) (Thr-172) (Cell Signaling, no. 2535, 1:1,000), phosphorylated-acetyl-CoA carboxylase (p-ACC) (Ser-79) (no. 3661, 1:1,000; Cell Signaling), total p38 MAPK (no. 9212, 1:1,000; Cell Signaling), PGC-1α (ab3242; Millipore), mitochondrial complexes I–V (ab110411; MitoProfile Total OXPHOS human WB antibody cocktail), succinate dehydrogenase (SDH; no. 11998, 1:2,000; Cell Signaling), β-HAD (ab81492; Abcam), citrate synthase (ab96600; Abcam), and peroxisome proliferator-activated receptor α (PPARα; ab24509; Abcam). The following morning membranes were washed for 30 min with TBST and probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (H + L) or goat anti-mouse (H + L) HRP-conjugated secondary antibodies (Jackson ImmunoResearch; 1:20,000) for 1 h at room temperature. Following 30 min of further washing in TBST, antibody binding was visualized using enhanced chemiluminescence (ECL Select Western blotting detection reagent; Amersham, Little Chalfont, Buckinghamshire, UK), and signals were captured using a ChemiDoc MP imaging system (Bio-Rad). Densitometry analysis of protein bands of interest was performed using Image Lab 4.1 software (Bio-Rad). The abundance of proteins of interest was normalized to total protein loading per lane, as determined by staining membranes with Coomassie brilliant blue. Representative blots for all analyzed proteins are presented in Fig. 1.

Enzymatic analysis. Frozen tissues (−80°C) were thawed, minced, weighed, and homogenized using a Molecular Devices tissue lyser in 1:10 (μl/mg) ice-cold buffer A consisting of (in mM unless otherwise stated): 25 Tris·HCl at pH 7.8, 1 EDTA, 2 $MgCl_2$, 50 KCl, and 0.50% vol/vol Triton X-100. The tissue homogenates were centrifuged at 14,000 *g* for 10 min at 4°C (Eppendorf centrifuge 5417R), and the supernatant was frozen at −80°C until use. Prior to use, samples were rapidly thawed, placed on ice, and diluted according to requirements. All assays were followed using a Molecular Devices (Sunnyvale, CA) Spectramax-340 96-well microplate reading spectrophotometer. Enzyme activities were measured per milligram wet weight of tissue. For CS activity, homogenate (5 μl of 5 × diluted supernatants in buffer A) was incubated (in mM) in 50 Tris·HCl (pH 8), 0.1 acetyl coenzyme A, and 0.2 DTNB. Twenty microliters of oxaloacetate (5) was added to initiate reactions and followed at 412 nm at 25°C. Rates of reaction were standardized to purified porcine heart citrate synthase (Sigma C3260). For CPT1 activity, a 5-μl sample was incubated (in mM unless otherwise noted in 50 Tris·HCl pH 7.4, 0.1 palmitoyl-CoA, 0.2 DTNB, 150 KCl, 0.2 g/l BSA) and was added to the plate and following supernatants in the first column of the plate. Twenty microliters of L-carnitine (5) was added to initiate activity. CPT activity per wet weight of tissue was determined on the basis of slope (Δabsorbance/Δmin). For succinate dehydrogenase (SDH) activity, tissue homogenates were centrifuged at 14,000 *g* for 10 min at 4°C, and the pellet was retained from the supernatant collection and frozen with mixture of 1:10 (wt/vol) buffer A. Five microliters of resuspended pellet was assayed with (in mM) 50 Tris·HCl (pH 7.4), 50 KCl, 1 azide, 1 antimycin A, 0.001 rotenone, 3.3 phenazine methosulfate, 0.2 dichlorophenolinphenol, and 0.02 EDTA. The reaction was initiated by the addition of 10 mM succinate and followed at 600 nm. SDH activity per wet weight of tissue was determined on the basis of slope (Δ absorbance/Δ min). Total protein was estimated using Biuret reagent with BSA (10 mg/ml) as a standard.

Statistical Analysis

Data distribution was assessed using the Shapiro-Wilk test, which demonstrated no deviations from normality in all variables. Changes in $\dot{V}O_{2\,peak}$ and $\dot{V}_{max}$ during incremental test were analyzed by a paired-sample *t*-test (Pre vs. Post). Fold changes in enzyme activity and protein expression were analyzed using a two-way mixed-model ANOVA (condition × time), where the within-subject factor was time (pre vs. post) and the between-subject factor was condition (CON leg vs. COLD leg). Where significant main effects (i.e., $P < 0.05$) were evident, secondary analysis using Fisher's LSD were undertaken to locate the differences. Where values for main effects were between $P > 0.05$ and $P \leq 0.10$, Cohen's effect size (*d*) was calculated to determine the magnitude of changes over time or between conditions and assessed as 0.2 = small effect, 0.5 = moderate effect, and 0.8 = large effect. Only changes with large effect sizes (where main effects were $P > 0.05 <$ and $P \leq 0.10$) are included in the discussion. All statistical analysis was performed using SPSS version 19 (IBM SPSS, Chicago, IL), and all data are presented as means ± SD.

RESULTS

Training Data and Performance during Incremental Treadmill Test

Mean heart rate (expressed as a % of maximal heart rate determined during pretraining incremental test) during training for *weeks 1*, *2*, *3*, and *4* were 83 ± 4%, 82 ± 4%, 81 ± 3%, and 82 ± 3%, respectively. Training load [heart rate × duration (min)] was increased by 17–20% (Fig. 2) per week throughout *weeks 1–4*. As a measure of overall training stimulus, subjects $\dot{V}O_{2\,peak}$ and $\dot{V}_{max}$ improved by 5.9% (pre: 46.7 ± 5.7 $ml \cdot kg^{-1} \cdot min^{-1}$ vs. post: 49.5 ± 5.9 $ml \cdot kg^{-1} \cdot min^{-1}$, $P = 0.01$) and 6.2% (pre: 13.5 ± 0.9 km/h vs. post: 14.3 ± 1.1 km/h, $P = 0.002$), respectively.

Muscle Protein Content and Enzyme Activity

Main effects for condition ($P = 0.048$) and interaction ($P = 0.048$) were determined for changes in total AMPK, where protein content was higher in COLD compared with CON following training (Fig. 3*A*). Main effects for changes in p38 MAPK expression (Fig. 3*A*) for time, condition, and interaction were $P = 0.095$, $P = 0.064$, and $P = 0.064$, respectively.

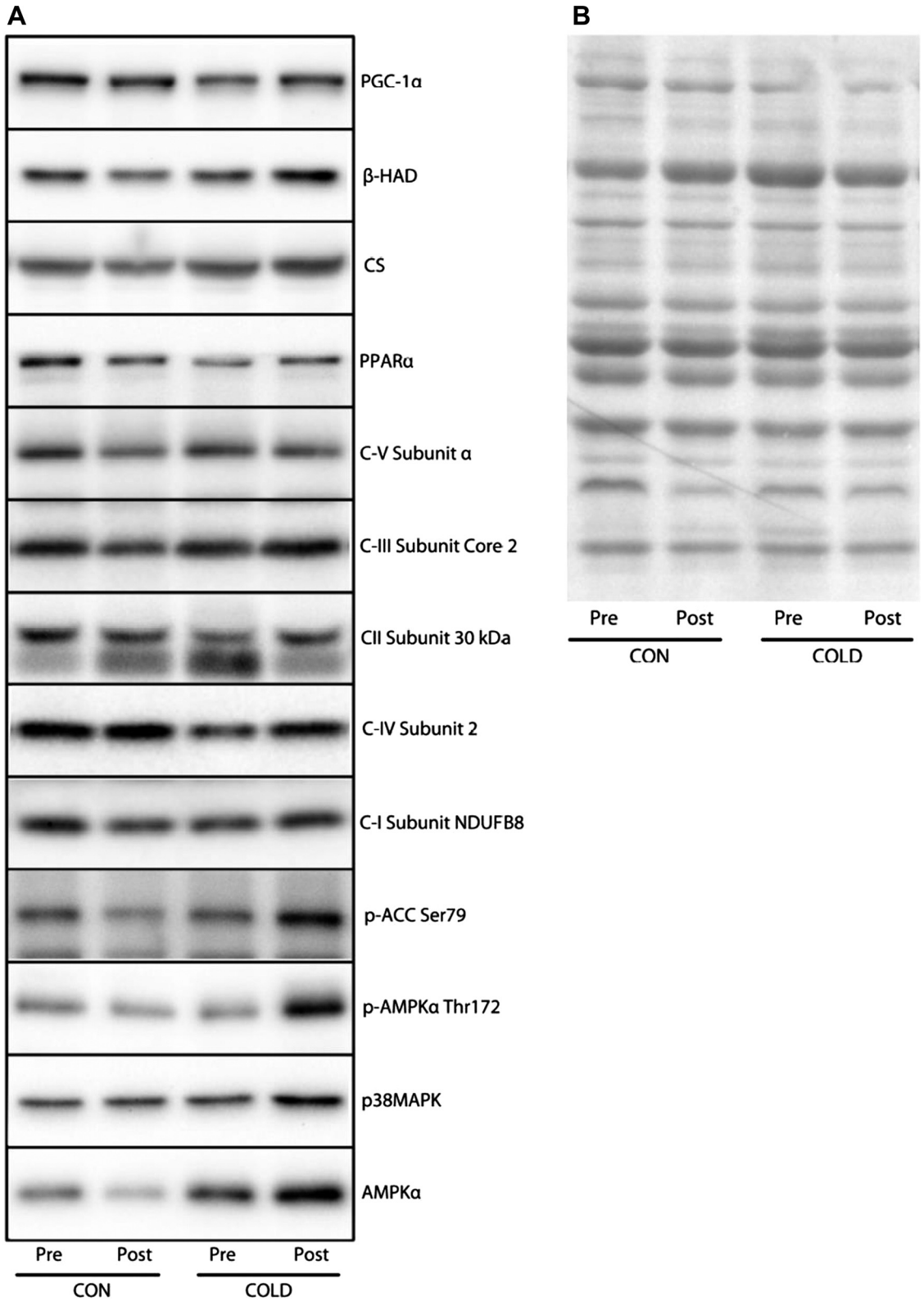

Fig. 1. Representative Western blots for proteins of interest (*A*) and Coomassie stain loading controls (*B*). See text for abbreviations and antibody descriptions.

Further analysis revealed large effect sizes for changes over time in COLD ($d = 1.02$) and between conditions posttraining ($d = 1.07$) but small effect sizes for changes over time in CON ($d = 0.44$). Changes in phosphorylated AMPK (p-AMPK) at Thr-172 demonstrated time ($P < 0.001$), condition ($P = 0.004$), and interaction ($P = 0.004$) effects (Fig. 3*B*). Compared with baseline values, an increase in p-AMPK content was observed in COLD ($P < 0.001$) but not CON ($P = 0.154$),

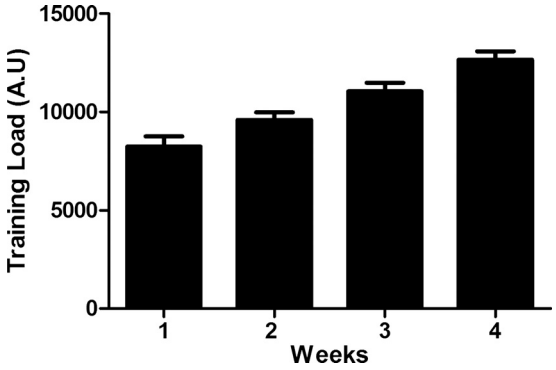

Fig. 2. Training load [heart rate × training time (min)] for *weeks 1–4*.

with changes in COLD being higher compared with CON ($P = 0.004$) posttraining. Main effects for time, condition, and interaction for changes in p-ACC content were $P = 0.059$, $P = 0.035$, and $P = 0.035$, respectively, where p-ACC protein abundance was higher in COLD compared with CON following training. Moreover, compared with pretraining values, changes in p-ACC over time were higher in COLD ($P = 0.007$) but not in CON ($P = 0.850$) (Fig. 3*B*). Condition ($P = 0.079$) and interaction ($P = 0.079$) effects for changes in PGC-1α were not significant (Fig. 4). However, large effect size changes ($d = 0.99$) were noted for PGC-1α in COLD compared with CON following training. Changes in β-HAD

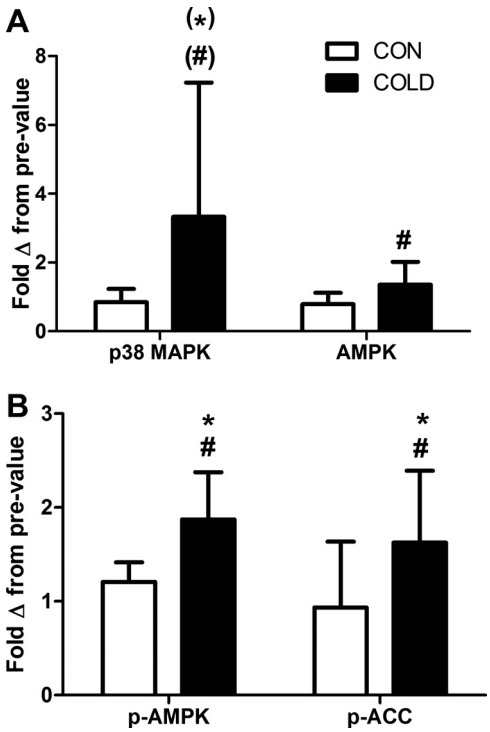

Fig. 3. Changes in total protein content of p38 MAPK and AMPK (*A*) and phosphorylation status of AMPK at Thr-179 and ACC at Ser-79 (*B*) following 4 wk of endurance training with (COLD) or without (CON) regular postexercise cooling. Data are presented as fold change from pretraining values. *Significantly higher compared with pretraining value in the COLD condition. #Significantly different between CON and COLD posttraining. (*)Large effect size vs. pretraining ($d > 0.8$ where main effects were between $P > 0.05$ and $P \leq 0.10$) in the COLD condition. (#)Large effect size ($d > 0.8$ where main effects were between $P > 0.05$ and $P \leq 0.10$) between conditions posttraining.

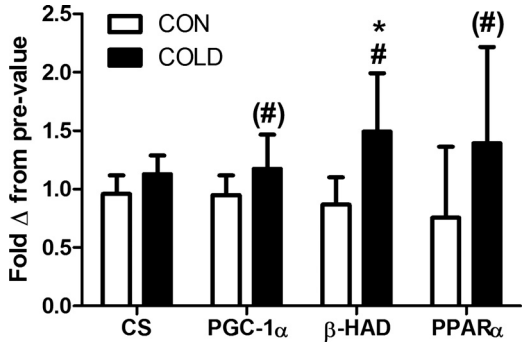

Fig. 4. Changes in total protein content of CS, PGC-1α, β-HAD, and PPARα following 4 wk of endurance training with (COLD) or without (CON) regular postexercise cooling. Data are presented as fold change from pretraining values. *Significantly higher compared with pretraining value in the COLD condition. #Significantly different between CON and COLD. (#)Large effect size ($d > 0.8$ where main effects were between $P > 0.05$ and $P \leq 0.10$) between conditions posttraining.

(Fig. 4) protein content demonstrated time ($P = 0.019$), condition ($P = 0.001$), and interaction effects ($P = 0.001$). Specifically, compared with pretraining values, changes in β-HAD following training was higher in COLD ($P < 0.001$) but not CON ($P = 0.341$). Moreover, β-HAD content was higher in COLD compared with CON following training ($P < 0.001$). Nonsignificant effects for condition ($P = 0.100$) and interaction ($P = 0.100$) were observed for changes in PPARα (Fig. 4). However, large effect sizes were noted between conditions, where changes in PPARα were higher in COLD compared with CON ($d = 0.93$).

Changes in protein subunits representative of respiratory complex I ($P = 0.044$) and III ($P = 0.039$) demonstrated condition and interaction effects, in which complex I and III subunits were higher in COLD compared with CON following training (Fig. 5). Compared with pretraining values, increases in protein abundance were observed in COLD for complex I ($P = 0.009$) and III ($P = 0.011$), but not in CON (complex I:

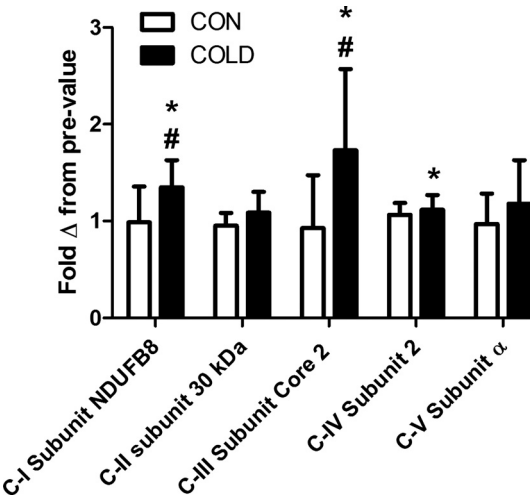

Fig. 5. Changes in total protein content of mitochondrial subunits representative of respiratory complexes I-V following 4 wk of endurance training with (COLD) or without (CON) regular postexercise cooling. Data presented as fold change from pretraining values. *Significantly higher compared with pretraining value in the COLD condition. #Significantly different between CON and COLD posttraining.

$P = 0.903$, complex III: $P = 0.769$). Changes in complex IV (Fig. 5) demonstrated time effects ($P = 0.021$), and compared with pretraining values, complex IV following training was higher in COLD ($P = 0.032$) but not CON ($P = 0.221$). No training effects were noted for changes in protein abundance for complexes II, IV, and V ($P > 0.05$, Fig. 5), as well as CPT1, SDH, or CS activities ($P > 0.05$, Fig. 6).

DISCUSSION

The purpose of this study was to investigate the effect of regular CWI on training-induced changes in AMPK, p38 MAPK, PGC-1α, and indices of mitochondrial biogenesis. Despite CWI being a popular postexercise recovery strategy, it is currently unknown how this intervention might influence mitochondrial adaptations to exercise. Indeed, Yamane et al. (45) reported attenuated improvements in $\dot{V}O_{2\,max}$ and cycling time to exhaustion following regular CWI performed during 4 wk of endurance training. In contrast, we recently demonstrated increased muscle PGC-1α mRNA following a single postexercise CWI intervention (20), suggesting that regular use of this intervention might potentially enhance mitochondrial biogenesis. In the present study, total AMPK and p38 protein content, as well as AMPK signaling (p-AMPK and p-ACC), were upregulated in basal muscle tissue posttraining only in the leg undergoing regular repeated CWI treatments. Downstream, the transcriptional coactivator PGC-1α ($P = 0.079$, $d = 0.99$) along with PPARα ($P = 0.1$, $d = 0.93$) tended to increase, while several mitochondrial proteins such as β-HAD ($P = 0.001$) and subunits representing respiratory chain complexes I ($P = 0.044$), III ($P = 0.039$), and IV ($P = 0.021$, time effects evident in COLD only) were upregulated following repeated CWI treatments. However, no differences between conditions were evident in the protein expression of CS, other respiratory chain complexes (C-II and C-V), or mitochondrial enzyme activities [CPT, SDH (C-II), and CS]. As such, regular postexercise CWI appeared to enhance some, but not all, indices of mitochondrial biogenesis, possibly through upregulation of components of the AMPK and/or p38 MAPK-mediated signaling pathways.

Postexercise CWI utilizing a similar cooling protocol has been previously shown by us to acutely decrease muscle tissue temperature and metabolic activity (20, 21), both important precursors for activating p38 MAPK and AMPK, respectively (3, 19, 33, 40). As such, regular use of this recovery modality might be hypothesized to attenuate increases in signaling during recovery from exercise, which, in turn, might attenuate mitochondrial biogenesis, lending support to the findings by Yamane et al. (45). In contrast, the present study shows that postexercise CWI had no detrimental effect on muscle AMPK and p38 protein content, or AMPK signaling following endurance training. This indicates that decreasing postexercise muscle tissue temperature and/or metabolic activity via CWI has little influence on p38 or AMPK signaling cascades, respectively, as the activation of these cascades are likely determined during exercise (5). In turn, it is intriguing that regular cooling appeared to have upregulated basal muscle total levels of both p38 and AMPK, and phosphorylation status of AMPK (Fig. 3). To the best of the authors' knowledge, the present study is the first to demonstrate a cold-induced increase in p38 and AMPK in human skeletal muscle. Increased protein abundance of these kinases is generally associated with enhanced phosphorylation potential and, consequently, interactions with downstream targets (3). In this regard, the use of postexercise cooling seems a viable strategy to enhance exercise-induced mitochondrial biogenesis. However, these findings also highlight the need for appropriate periodization of training and cooling regimens to maintain an overall potent training stimulus, as a higher absolute exercise stimulus may be required to achieve the same activation/phosphorylation following increased protein abundance of p38 and AMPK (6, 46). While the mechanisms involved remain to be fully elucidated, increased adrenergic activation and/or mitochondrial uncoupling may potentially underpin how postexercise cooling activates both p38 and AMPK. For instance, chronic adrenergic stimulation to the skeletal muscles has been shown to increase Ca^{2+} leakage from the sarcoplasmic reticulum through modifications/destabilization to the ryodine receptor 1 channel complex (4, 7). Accordingly, an increase in cytosolic Ca^{2+} has been shown to phosphorylate both p38 MAPK and AMPK through the Ca^{2+}/calmodulin protein kinase (43) and Ca^{2+}/calmodulin protein kinase kinase, respectively (18).

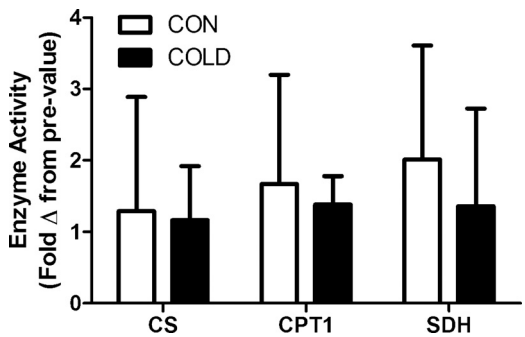

Fig. 6. Changes in the enzyme activities of CS, CPT1, and SDH following 4 wk of endurance training with (COLD) or without (CON) regular postexercise cooling.

Downstream of p38 MAPK and AMPK, PGC-1α content, along with several mitochondrial proteins (i.e., complex I, complex III, possibly complex IV, and β-HAD) were upregulated by CWI, extending some support that regular CWI might have enhanced training-induced mitochondrial biogenesis (Figs. 4 and 5). The increase in PGC-1α protein content is in agreement with our previous study (20) and others (30, 31), where PGC-1α mRNA expression was shown to be acutely upregulated following a single postexercise CWI treatment (15 min at 10°C) or when postexercise recovery was undertaken in cold ambient environments (2–3 h at 7°C), respectively. However, evidence of enhanced mitochondrial biogenesis per se has yet to be established, as studies so far have focused on transient mRNA responses in response to a single acute session of postexercise cold exposure (20, 30, 31). Moreover, postexercise cold exposure has been shown to attenuate the mRNA expression of nuclear respiratory factor 2 and estrogen-related receptor α (despite an increase in PGC-1α) (30), which are key transcription factors downstream of PGC-1α, and are responsible for the transcriptional activation of many nuclear genes. Taken together, the effects of postexercise cold exposure on exercise-induced mitochondrial biogenesis is, indeed, inconclusive. Results from the present study elucidate some aspects of these uncertainties, in that we demonstrated enhanced expression of several mitochondrial proteins (i.e., complex I,

complex III, possibly complex IV, and β-HAD). Moreover, no analyzed variable seemed to be attenuated by regular CWI, although it is acknowledged that some of the markers indicative of mitochondrial biogenesis did not reach statistical significance in this study. Perhaps, our sample size and/or training duration might have limited the attainment of statistical significance for some of the variables analyzed in this study.

While the molecular adaptations to exercise seem to be augmented by CWI, Yamane et al. (45) reported attenuated improvements in $\dot{V}O_{2\,max}$ and cycling time to exhaustion during single-leg cycling following 4 wk of endurance training in the leg subjected to regular postexercise cooling (2 × 20 min at 5°C) (45). It is, indeed, difficult to reconcile the findings between the present study and the study by Yamane et al. (45), considering that increases in mitochondrial proteins (indicative of increased mitochondrial content) should ideally translate into improved aerobic capacity and/or exercise performance. However, it must be considered that the relationship between mitochondrial content and exercise performance might be dissociated in the situation where mitochondrial biogenesis was induced by cold exposure, as in the case with CWI treatments. For instance, in cold-induced mitochondrial biogenesis, the increase in mitochondrial content could be counteracted by increases in uncoupled mitochondrial respiration through Ca^{2+}-mediated pathways (2) and/or through the increase in the expression of UCP (44). Specifically, cold exposures result in the expression of UCPs (27, 44), which facilitates the leak of protons across the inner mitochondrial membrane, bypassing ATP synthase (coupled respiration). This effectively decreases the proton gradient necessary for ATP synthesis and, hence, uncouples O_2 consumption from ATP production, dissipating energy as heat. Moreover, repeated cold exposures have been shown to increase the expression and activity of Ca^{2+} ATPase I, an enzyme that hydrolyzes ATP to pump Ca^{2+} back into the sarcoplasmic reticulum, as well as increase heat production (i.e., uncoupled from Ca^{2+} transport) (2). These parallel adaptations might consequently lead to a decrease in mitochondrial efficiency and ATP turnover, where O_2 consumption and ATP hydrolysis is directed toward dissipating energy as heat rather than muscle force production (2, 44). In support, it was recently shown in rodents that 28 days of 2,4-dinitrophenol (mitochondrial uncoupler) administration resulted in diminished running economy, maximal running velocity, and mitochondrial respiratory efficiency, despite increases in the mRNA of key regulators of mitochondrial biogenesis and overall increase in mitochondrial content (29). This consequently indicates that mitochondrial uncoupling may trigger quantitative mitochondrial adaptations (i.e., increased mitochondrial content) to compensate for qualitative impairments at the oxidative phosphorylation level. Somewhat similarly, in the current study, we did not observe enhanced activities of key mitochondrial enzymes (i.e., SDH, CPT1, and CS, Fig. 6) despite protein abundance of several respiratory chain components and other mitochondrial proteins (Figs. 4 and 5). As such, divergent qualitative and quantitative mitochondrial adaptations following cold-induced uncoupling may, in part, explain the mismatch between mitochondrial content and exercise performance, as evident in the current study and the study by Yamane et al. (45).

The present study also demonstrates that regular CWI significantly increased p-ACC content (Fig. 3*B*), a protein that not only reflects overall AMPK activity (i.e., activation by both allosteric and covalent mechanisms) (40) but also is indicative of increased fatty acid oxidation (40, 41). Increased p-ACC has shown to decrease the synthesis of malonyl-CoA, a major inhibitor of CPT1 (40, 41). Relieving the inhibitory effect on CPT1 consequently enhances the transport of fatty acid into the mitochondria, increasing fatty acid oxidation (3, 40). Also evident were increases in β-HAD (Fig. 4), the rate-limiting enzyme in the β-oxidation cycle and PPARα (Fig. 4), a nuclear receptor with which PGC-1α interacts to regulate the expression of several enzymes involved in fatty acid oxidation, including CPT1 (37). However, despite an increase in p-ACC content and other markers indicative of increased capacity for fatty acid oxidation, we did not see a consequential increase in CPT1 activity (Fig. 6). It must be mentioned that malonyl-CoA synthesis is also regulated by malonyl-CoA decarboxylase (3, 40) and may account for the lack of CPT1 activity evident in this study. Further research is certainly needed with regard to adaptation to CWI and fatty acid oxidation.

The authors also acknowledge the lack of molecular responses observed in the control condition. Such results are somewhat surprising given that exercise has shown to be a potent upregulator of mitochondrial biogenesis (5, 6, 11, 13, 26, 34). Nevertheless, several studies (9, 32) have not observed an upregulation in mitochondrial content following short-term endurance training of similar intensity to the present study. Such inconsistencies could be the result of variations in the type of endurance training undertaken (i.e., continuous vs. intervals vs. sprint intervals), initial fitness of participants, analysis methods, and the antibody used. Conversely, it may be speculated that unilateral CWI might have influenced some of the molecular responses in the control leg. However, the authors believe this is highly unlikely, as pilot work from our laboratory demonstrated no differences in postexercise muscle oxygenation, blood volume, and skin and muscle temperatures between the control leg during unilateral CWI compared with when no cooling was administered.

Perspectives and Significance

This study investigated the effect of regular CWI on p38 MAPK, AMPK, PGC-1α, and mitochondrial biogenesis during 4 wk of endurance training. Despite CWI being a popular postexercise recovery modality, there was little evidence for how this intervention might influence muscle adaptations to training. The novel finding of this study was that regular postexercise cooling of the muscles enhanced exercise training-induced increases in p38 MAPK, AMPK content, as well as AMPK signaling in skeletal muscle at rest. Downstream, an increase in PGC-1α abundance was noted along with several mitochondrial proteins, indicating that mitochondrial biogenesis may be enhanced. Regardless, we advocate caution with regard to regular use of this intervention, as some preliminary evidence suggests that cold-induced mitochondrial biogenesis may concomitantly decrease mitochondrial efficiency. We did not include any unilateral exercise tests within the present study, which would have potentially clarified relationships between the observed molecular responses and subsequent performance. As such, studies with detailed measurements of performance variables in deconditioned individuals, as well as highly trained athletes, are very much warranted to further

improve our understanding in how CWI may be utilized to enhance muscle oxidative adaptations to exercise.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: M.I., G.W., H.C.C., A.G., A.J.H., D.C.-S., and C.R.A. conception and design of research; M.I., H.C.C., A.G., and C.R.A. performed experiments; M.I., J.F.M., T.P., A.J.H., and C.R.A. analyzed data; M.I., J.F.M., H.C.C., A.G., T.P., A.J.H., D.C.-S., and C.R.A. interpreted results of experiments; M.I. and J.F.M. prepared figures; M.I., J.F.M., G.W., D.C.-S., and C.R.A. drafted manuscript; M.I., J.F.M., G.W., A.J.H., D.C.-S., and C.R.A. edited and revised manuscript; M.I., J.F.M., G.W., H.C.C., A.G., T.P., A.J.H., D.C.-S., and C.R.A. approved final version of manuscript.

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