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

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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 mitochondrial 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, β3-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 Ca2+ 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 β3-hydroxyacyl-CoA dehydrogenase (β-HAD) activities, in line with increased resting intracellular Ca2+ content, was demonstrated in rodents following 8 wk of cold acclimatization (7).
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 \(\times\) 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 \(\pm\) SD: age: 21.4 \(\pm\) 2.8 yr, height: 177 \(\pm\) 7 cm, mass: 76.6 \(\pm\) 8.2 kg, peak oxygen uptake (\(\dot{V}O_2\) peak): 46.7 \(\pm\) 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.

Table 1. Training program for weeks 1–4

<table>
<thead>
<tr>
<th>Interval Type</th>
<th>Intensity, % (V_{\text{max}})</th>
<th>Work Interval Duration</th>
<th>Interval Repetitions</th>
<th>Work-Rest Ratio</th>
<th>Rest Interval Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Long</td>
<td>Medium</td>
<td>Short</td>
<td>Long</td>
<td>Medium</td>
</tr>
<tr>
<td>Week 1</td>
<td>80%</td>
<td>90%</td>
<td>100%</td>
<td>6 min</td>
<td>2 min</td>
</tr>
<tr>
<td>Week 2</td>
<td>80%</td>
<td>90%</td>
<td>100%</td>
<td>6 min</td>
<td>2 min</td>
</tr>
<tr>
<td>Week 3</td>
<td>85%</td>
<td>95%</td>
<td>105%</td>
<td>8 min</td>
<td>2 min</td>
</tr>
<tr>
<td>Week 4</td>
<td>85%</td>
<td>95%</td>
<td>110%</td>
<td>8 min</td>
<td>2 min</td>
</tr>
</tbody>
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AR, active recovery undertaken at 50% of \(V_{\text{max}}\); PR, passive recovery.
tens, 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, no. 78442; Thermo Fisher Scientific, Waltham, MA) for 20 min at 4°C prior to centrifugation at 13,000 g at 4°C for 10 min. The supernatant was collected and stored at 4°C prior to centrifugation at 13,000 g at 4°C for 20 min.

Ceramic beads (OMNI Ruptor, 5.65 m/s, no. 78442; Thermo Fisher Scientific, Waltham, MA) were used to homogenize the 2.8-mm ceramic beads in a bead mill homogenizer at 5.65 m/s. 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.

**CPT activity** per wet weight of tissue was determined on the basis of slope (Δ absorbance/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 antimony A, 0.001 rotenone, 3.7 phenazine methosulfate, 0.2 dichlorphenolindophenol, 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 Bicinchoninic acid 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 VO₂ peak and Vmax 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 ≤ 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 ≤ 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 VO₂ peak and Vmax improved by 5.9% (pre: 46.7 ± 5.7 ml·kg⁻¹·min⁻¹ vs. post: 49.5 ± 5.9 ml·kg⁻¹·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 (F = 0.048) and interaction (F = 0.048) were determined for changes in total AMPK, where protein content was higher in COLD compared with CON following training (Fig. 3A). Main effects for changes in p38 MAPK expression (Fig. 3A) for time, condition, and interaction were P = 0.095, F = 0.064, and P = 0.064, respectively.
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. 3B). Compared with baseline values, an increase in p-AMPK content was observed in COLD ($P < 0.001$) but not CON ($P = 0.154$).
with changes in COLD being higher compared with CON (P = 0.004) postraining. 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. 3B). 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. 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:...
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 \( (P > 0.05, \text{Fig. 5}) \), as well as CPT1, SDH, or CS activities \( (P > 0.05, \text{Fig. 6}) \).

**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 \( \dot{V}O_2 \) \( \text{max} \) 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 (\( \alpha \)-AMPK and \( \alpha \)-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 \( \beta \)-HAD \( (P = 0.001) \) and subunits representing respiratory chain complexes I \( (P = 0.044) \), III \( (P = 0.039) \), and IV \( (P = 0.021, \text{time effects evident in COLD only}) \) 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 \( \text{Ca}^{2+} \) leakage from the sarcoplasmic reticulum through modifications/distabilization to the ryodine receptor 1 channel complex (4, 7). Accordingly, an increase in cytosolic \( \text{Ca}^{2+} \) has been shown to phosphorylate both p38 MAPK and AMPK through the \( \text{Ca}^{2+}/\text{calmodulin protein kinase (43)} \) and \( \text{Ca}^{2+}/\text{calmodulin protein kinase kinase, respectively (18).} \)

Downstream of p38 MAPK and AMPK, PGC-1α content, along with several mitochondrial proteins (i.e., complex I, complex III, possibly complex IV, and \( \beta \)-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 $V_{\text{O2max}}$ 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. 3B), 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
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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


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