Ingesting a Combined Carbohydrate and Essential Amino Acid Supplement Compared to a Non-Nutritive Placebo Blunts Mitochondrial Biogenesis-Related Gene Expression after Aerobic Exercise

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Abstract

Background: Whether load carriage (LC), an endurance exercise mode composed of the aerobic component of traditional endurance exercise [e.g., cycle ergometry (CE)] and contractile forces characteristic of resistive-type exercise, modulates acute mitochondrial adaptive responses to endurance exercise and supplemental nutrition [carbohydrate + essential amino acids (CHO + EAA)] is not known.

Objective: The aim of this study was to examine the effects of LC and CE, with or without CHO+EAA supplementation, on acute markers of mitochondrial biogenesis.

Methods: Twenty-five adults performed 90 min of metabolically matched LC (treadmill walking, wearing a vest equal to 30% of body mass) or CE exercise during which CHO+EAA (46 g carbohydrate and 10 g essential amino acids) or non-nutritive control (CON) drinks were consumed. Muscle biopsy samples were collected at rest (pre-exercise), post-exercise, and after 3 h of recovery to assess citrate synthase activity and the expression of mRNA (reverse transcriptase–quantitative polymerase chain reaction) and protein (Western blot).

Results: Citrate synthase and phosphorylated p38 mitogen-activated protein kinase (p38 MAPK)Thr180/Tyr182 were elevated postexercise compared with pre-exercise (time main effect, P < 0.05). Peroxisome proliferator-activated γ-receptor coactivator 1α (PGC-1α) expression was highest after recovery for CE compared with LC (exercise-by-time effect, P < 0.05). Sirtuin 1 (SIRT1) expression postexercise was higher for CON than for CHO+EAA treatments (drink-by-time, P < 0.05). Tumor suppressor p53 (p53), mitochondrial transcription factor A (TFAM), and cytochrome c oxidase subunit IV (COXIV) expression was greater for CON than for CHO+EAA treatments (drink-by-time, P < 0.05). PGC-1α and p53 expressions were positively associated (P < 0.05) with TFAM (r = 0.629 and 0.736, respectively) and COXIV (r = 0.465 and 0.461, respectively).

Conclusions: Acute mitochondrial adaptive responses to endurance exercise appear to be largely driven by exogenous nutrition availability. Although CE upregulated PGC-1α expression to a greater extent than LC, downstream signaling was the same between modes, suggesting that LC, in large part, elicits the same acute mitochondrial response as traditional, non-weight-bearing endurance exercise. This trial was registered at clinicaltrials.gov as NCT01714479. CDN 2017;1:e000893.

Introduction

Skeletal muscle is a malleable tissue that responds to acute exercise and diet-induced alterations in molecular signaling, resulting in long-term aerobic adaptations with training (1, 2). The peroxisome proliferator-activated γ-receptor coactivator 1α (PGC-1α) is the key regulator of skeletal muscle adaptations to endurance-type exercise, responsive to exogenous and endogenous
carbohydrate availability (3–5). Increased expression of PGC-1α has been linked to enhanced oxidative capacity by activating mitochondrial transcription factor A (TFAM) and upregulating mitochondrial biogenesis (6, 7). Exercise- and nutrition-induced mitochondrial biogenesis may also be stimulated by the tumor suppressor p53 (p53), which targets TFAM to stimulate mitochondrial DNA expression (8, 9). Multiple upstream targets, which include AMP-activated protein kinase (AMPK), p38 mitogen-activated protein kinase (p38 MAPK), and sirtuin 1 (SIRT1), govern the activation of PGC-1α and p53 and are responsive to muscle contractile activity and diminished intracellular energy availability (Figure 1) (9–14). Muscle aerobic capacity is enhanced with repeated stimulation of this pathway and subsequent increases in mitochondrial biogenesis with training, resulting in improved endurance exercise performance (15, 16).

It is well accepted that mitochondrial biogenesis is stimulated by traditional endurance exercise (1), and acute responses appear to be sensitive to exogenous and endogenous carbohydrate availability and exercise mode. Maintaining endogenous carbohydrate availability (i.e., glycogen) by manipulating dietary carbohydrate intake, or by ingesting carbohydrate with exercise, is generally recommended to sustain or improve endurance exercise performance (17). However, exercising in a carbohydrate-restricted state (i.e., fasted with low muscle glycogen status) (18, 19) may produce greater acute mitochondrial adaptive stimulus because PGC-1α expression is upregulated to a greater extent than when exercise is performed in a carbohydrate-replete state (7, 9, 20). Interestingly, PGC-1α mRNA expression is upregulated to a greater extent after endurance exercise when resistance exercise is performed concurrently (21), suggesting the contractile forces of traditional resistance exercise may modulate mitochondrial biogenesis signaling (22). Studies have also reported that performing resistance exercise in a carbohydrate-restricted state potentiates resistance exercise–induced changes in PGC-1α transcription (23). These studies suggest that combining the mechanical strain of traditional resistance exercise and the metabolic stress of traditional endurance exercise, particularly when carbohydrate availability is limited, may enhance activation of mitochondrial biogenesis-related signaling.

Load carriage (LC), an endurance exercise mode commonly performed by military personnel (24), elicits mechanical forces that exceed traditional, non-weight-bearing endurance exercise. We recently reported greater myofibrillar muscle protein synthetic responses to LC than to cycle ergometry (CE) (25). This suggests that acute mitochondrial biogenesis-related signaling may be more pronounced for LC than for CE, particularly when carbohydrate availability is limited, although to our knowledge this has never been studied. This study compared molecular markers of mitochondrial biogenesis in response to LC and CE, with or without consuming a combined carbohydrate and essential amino acids (CHO+EAA) drink during exercise (i.e., reflecting restricted and adequate carbohydrate availability). We hypothesized that LC would stimulate a greater upregulation in the expression of various markers of mitochondrial biogenesis than CE, and that this response would be greatest when exogenous energy and carbohydrate were not consumed during the exercise bout.

FIGURE 1 PGC-1α pathway schematic. AMPK, AMP-activated protein kinase; PGC-1α, peroxisome proliferator-activated γ-receptor coactivator 1α; p38 MAPK, p38 mitogen-activated protein kinase; p53, tumor suppressor p53; RER, respiratory exchange ratio; SIRT1, sirtuin 1; TFAM, mitochondrial transcription factor A.

Methods

Experimental design
After providing informed consent, 25 adults (23 men and 2 women; mean ± SD age, 22 ± 1 y; height, 177 ± 2 cm; weight, 82 ± 2 kg; VO2peak, 50 ± 1 mL kg−1 min−1) participated in this randomized, double-blind, placebo-controlled study (25). The data presented in this study were derived from a larger investigation; however, the sample size is smaller due to limited tissue availability. Volunteers were randomly assigned to 1 of 2 exercise experimental groups. One group performed CE and the other performed LC (weighted vest equal to 30% of body mass) on a treadmill. Within each exercise group, participants consumed either combined CHO+EAA [223 kcal, 46 g carbohydrate, and 10 g essential amino acids (EAA); 0.7 g histidine, 0.7 g isoleucine, 3.6 g leucine, 1.2 g lysine, 0.3 g methionine, 1.4 g phenylalanine, 1.0 g threonine, and 1 g valine] or flavor-matched, non-nutritive control (CON; 22 kcal, 5 g carbohydrate) drinks at 0,
30, 60, and 90 min during the exercise session (LC-CHO+EAA: \( n = 6 \); CE-CHO+EAA: \( n = 7 \); LC-CON: \( n = 5 \); CE-CON: \( n = 7 \)).

Exercise intensity (2.2 ± 0.6 L/min) and energy expenditure (1000 ± 26 kcal/90 min) were matched between modes as described elsewhere (25). Muscle biopsies of the vastus lateralis were performed at rest (pre-exercise), postexercise, and after 3 h of recovery and analyzed for markers of mitochondrial biogenesis-related signaling and activity. Dietary intake and physical activity were controlled as previously described (25). This study was approved by the Institutional Review Board at the US Army Research Institute of Environmental Medicine and registered at www.clinicaltrials.gov as NCT01714479.

Substrate oxidation

Respiratory exchange ratio (RER; \( \dot{V}CO_2/\dot{V}O_2 \)) was determined by using indirect calorimetry (ParvoMedics) every 30 min during exercise. Nonprotein substrate oxidation was calculated from \( \dot{V}CO_2 \) and \( \dot{V}O_2 \) measurements during exercise (ParvoMedics) (26, 27), as follows:

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\text{Carbohydrate Oxidation (g/90 min)} = [4.585 \times \dot{V}CO_2] - [3.226 \times \dot{V}O_2 (L/min)] \times 90
\]

\[
\text{Fat Oxidation (g/90 min)} = [1.695 \times \dot{V}O_2 (L/min)] - [1.701 \times \dot{V}CO_2 (L/min)] \times 90
\]

Citrate synthase activity assay

Citrate synthase activity was assessed to determine mitochondrial activity by using pre-exercise and postexercise time points due to limited sample availability. The analysis was performed by using whole-cell lysate from ~30 mg muscle homogenized in ice-cold RIPA (radio-immunoprecipitation assay) (ThermoFisher) homogenization buffer (1:10 wt:vol) containing 1 mM DTT, phosphatase (PhosSTOP; Roche), and protease (complete, ULTRA Tablet; Roche) inhibitors. Enzyme activity was determined by using a colorimetric assay by combining 10 ¼L diluted (1:10; 0.1 M Tris HCl, pH 8.1) sample to 150 ¼L reaction master mix (1 mL DNTB [5,5-dithio-bis-(2-nitrobenzoic acid)], 3 mg acetyl-CoA, and 8 mL 0.1 M Tris HCl, pH 8.1) and 10 ¼L 10 mM oxaloacetate. Samples were analyzed at 412 nm on a SpectraMax 3 microplate reader (Molecular Devices). Data were normalized to protein content.

Western blotting

Western blot was only performed for pre- and postexercise time periods due to limited sample availability. Homogenates used for the citrate synthase activity assay were centrifuged for 15 min at 10,000 \( \times g \) at 4 °C, the supernatant (lysatge) was collected, and protein content was analyzed (ThermoFisher). Equal amounts of total protein (15 ¼g) were used in precast 4–20% Mini-PROTEAN TGX gels (Bio-Rad Laboratories). Proteins were transferred to polyvinylidene fluoride membranes and exposed to phosphorylated p38 MAPK\(^{\text{Thr180/Tyr182}} \) and phosphorylated AMPK\(^{\text{Thr172}} \) (Cell Signaling Technology) primary antibodies at 4 °C overnight. Labeling was performed by using secondary antibody (anti-rabbit IgG conjugate with HRP; Cell Signaling Technology) and chemiluminescent reagents (Super Signal, West Pico Kit; Pierce Biotechnology). Blots were quantified by using a phosphoimager (ChemiDoc XRS; Bio-Rad) and Image Lab software (Bio-Rad). GAPDH was used to confirm equal protein loading per well. All data are presented as fold changes relative to the pre-exercise time period.

mRNA expression

Total RNA was isolated from ~20 mg muscle (pre-exercise, postexercise, and recovery period) by using a miRNeasy Mini kit (Qiagen) and assessed for quantity and quality with the use of a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). Equal amounts of RNA were reverse transcribed by using a TaqMan cDNA with RNase Inhibitor Reverse Transcription kit (Applied Biosystems). Real-time PCR with the use of individual TaqMan Gene Expression Assays (PGC-1\( \alpha \), SIRT1, \( p53 \), and TFAM; Applied Biosystems) were performed in triplicate. Data were normalized to the geometric mean of \( 18S \) and \( RNU48 \). Fold changes for mRNA were calculated by using the \( \Delta \Delta \text{Ct} \) method (28) and expressed relative to the pre-exercise time period.

Statistical analyses

A univariate ANOVA was used to determine the main effect of exercise mode (LC compared with CE) and drink (CHO+EAA compared with CON) on RER. Mixed-model repeated-measures ANOVA was used to determine main effects and interactions of exercise mode, drink, and time (pre-exercise, postexercise, and recovery period) for mRNA expression. Mixed-model repeated-measures ANOVA was also used to determine main effects and interactions for Western blotting and citrate synthase activity; however, due to the limited sample availability, analyses were only included at pre- and postexercise time periods. Bonferroni adjustments were used for post hoc comparisons if interactions were observed. Correlations between mRNA data were assessed by using Pearson correlation coefficients. Data were log transformed (log2) for correlations to maintain equal scale between positive and negative values. Significance was set at \( P < 0.05 \). Data were analyzed by using SPSS (version 21.0, 2010; SPSS, Inc) and expressed as means ± SEMs.

Results

Independent of drink, RER was higher (\( P < 0.05 \)) during CE (0.06 ± 0.02 \( \dot{V}CO_2/\dot{V}O_2 \)) than during LC (Table 1). Carbohydrate oxidation was higher (\( P < 0.05 \)) during CE (64 ± 16 g/90 min) than during LC, regardless of drink. Fat oxidation was lower (\( P < 0.05 \)) during CE (22 ± 6 g/90 min) than during LC, independent of drink.

Citrate synthase activity was higher postexercise than pre-exercise (14.6 ± 5.4 \( \mu \)mol · g\(^{-1} \) · min\(^{-1} \); \( P < 0.05 \)), regardless of exercise mode (Figure 2A). Postexercise phosphorylated p38 MAPK\(^{\text{Thr180/Tyr182}} \) was 2.41 ± 0.55-fold higher than at pre-exercise (\( P < 0.05 \)), independent of exercise mode and drink (Figure 2B). No changes were observed in phosphorylated AMPK\(^{\text{Thr172}} \).

PGC-1\( \alpha \) expression was highest with CE compared with LC during the recovery period (exercise-by-time interaction; \( P < 0.05 \); Figure 3A). SIRT1 expression was elevated in CON to a greater extent than CHO+EAA at postexercise (drink-by-time interaction; \( P < 0.05 \)). The expression of \( p53 \) and TFAM was higher during the
recovery period than at pre-exercise (time main effect; \( P < 0.05 \), p53, TFAM, and cytochrome c oxidase subunit IV (COXIV) expression was higher with CON than with CHO+EAA (drink main effect; \( P < 0.05 \); Figure 3B). No other main effects of interactions were observed. PGC-1α and p53 expressions were positively associated \(( P < 0.05 \) with TFAM \(( r = 0.629 \) and 0.736, respectively) and COXIV \(( r = 0.465 \) and 0.461, respectively) expressions during the recovery period (Figure 4).

### TABLE 1  Substrate oxidation

|                      | CHO+EAA | CON          |
|----------------------|---------|--------------|
|                      | Load carriage | Cycle ergometry | Load carriage | Cycle ergometry |
| RER, VCO₂/VO₂        | 0.85 ± 0.02 | 0.91 ± 0.02*  | 0.87 ± 0.02  | 0.93 ± 0.02*   |
| Carbohydrate oxidation, g/90 min | 123 ± 16  | 196 ± 16*    | 140 ± 15     | 195 ± 15*      |
| Fat oxidation, g/90 min | 52 ± 7   | 30 ± 7*      | 45 ± 6       | 24 ± 6*        |

\(^1\)Values are means ± SEMs. *Different from load carriage, \( P < 0.05 \). CHO+EAA, carbohydrate (46 g) plus essential amino acids (10 g); CON, control (non-nutritive); RER, respiratory exchange ratio; VCO₂, volume of carbon dioxide produced; VO₂, volume of oxygen consumed.

FIGURE 2  Citrate synthase activity (A) and p-p38 MAPK\(^{\text{Thr180/Tyr182}}\) and p-AMPK\(^{\text{Thr172}}\) (B). Representative bands correspond to x axis labels below in panel B. Phosphorylation status was normalized to GAPDH, with data presented as fold changes relative to the pre-exercise time period for each group. Values are means ± SEMs. *Different from pre-exercise, \( P < 0.05 \). CE, cycle ergometry; CHO+EAA, carbohydrate plus essential amino acids; CON, control; LC, load carriage; p-AMPK, phosphorylated AMP-activated protein kinase; p-p38 MAPK, phosphorylated p38 mitogen-activated protein kinase; POST, postexercise; PRE, pre-exercise.
**Discussion**

The primary outcome of this study was that mRNA expression of PGC-1α was markedly greater after a bout of CE than after LC. However, despite the marked differences in PGC-1α expression between LC and CE, SIRT1, p53, TFAM, and COXIV transcription was not affected by exercise mode. SIRT1, p53, TFAM, and COXIV expressions were, however, upregulated to a greater extent when participants ingested the non-nutritive CON drink than after the CHO+EAA drink. These findings suggest that the expression of metabolic genes associated with regulating mitochondrial biogenesis is largely driven by substrate availability and only modestly affected by mode.

Contrary to our hypothesis, PGC-1α mRNA expression during recovery was upregulated to a greater extent for CE than for LC. One explanation is that the localized contractile forces and metabolic stress imposed on the quadriceps biopsied may have differed between LC and CE. We previously reported that the muscle protein synthetic response to LC exceeded the response to CE, which suggests that, in the current analysis, the metabolic stress with CE exceeded LC. This theory is supported, in part, by RER, which was greater for CE, despite matching the exercise modes for oxygen consumption (2.2 L/min) and energy expenditure (1000 kcal/90 min). Elevated RER resulted in higher rates of carbohydrate oxidation and lower rates of fat oxidation for CE than for LC. Similarly, others have reported lower fat oxidation with CE than with a more whole-body endurance exercise mode (e.g., running) when exercise was matched at 60% VO2max (29). In the study that showed that PGC-1α expression was higher after performing concurrent CE (65% VO2max) and resistance exercise compared with CE alone, the participants performed 6 sets of leg presses (70–80% 1-repetition maximum) isolating the quadriceps, which likely generated contractile forces different from LC (21). Taken together, the difference between the metabolic and contractile stressors of LC (i.e., whole body) and the more localized stress of CE likely explains the observed differences in PGC-1α.

Performing LC and CE in a fasted state upregulated SIRT1, p53, TFAM, and COXIV expressions to a greater extent than when the CHO+EAA drinks were consumed. These findings are consistent

**FIGURE 3** mRNA expressions of SIRT1, PGC-1α, and p53 (A) and TFAM and COXIV (B). Data are presented as fold changes relative to mean pre-exercise values for each group. Values are means ± SEMs. *Different from pre-exercise, *P < 0.05. †Different from postexercise, †P < 0.05. ‡Different from LC, *P < 0.05. §Different from CHO+EAA, *P < 0.05. CE, cycle ergometry; CHO+EAA, carbohydrate plus essential amino acids; CON, control; COXIV, cytochrome c oxidase subunit IV; LC, load carriage; PGC-1α, peroxisome proliferator-activated γ-receptor coactivator 1α; POST, postexercise; PRE, pre-exercise; p53, tumor suppressor p53; REC, recovery; SIRT1, sirtuin 1; TFAM, mitochondrial transcription factor A.
with past studies that showed that the provision of exogenous energy and carbohydrate during endurance exercise dampens transcriptional activation regulating mitochondrial biogenesis (5, 9, 20). Although we were unable to detect diet differences in PGC-1α expression between the CON and CHO+EAA treatments, there was a trend toward significance (P = 0.09) and PGC-1α and p53 were strongly associated with downstream targets, TFAM and COXIV, during the recovery period. These correlation data, together with previous reports (30, 31), provide further evidence that after endurance exercise, PGC-1α and p53 likely stimulate the transcription of TFAM to upregulate mitochondrial DNA expression (6, 7, 32), as well as COXIV, an indicator of mitochondrial count (30, 33). The lack of an effect on transcription of TFAM and COXIV suggests that LC likely does not inhibit mitochondrial biogenesis compared with CE.

It is unlikely that the addition of EAA to the carbohydrate altered the effects of carbohydrate availability on mitochondrial biogenesis-related gene expression. In vitro analysis has suggested that the EAA leucine may increase mitochondrial size in skeletal muscle through the activation of SIRT1 and subsequent stimulation of TFAM to upregulate mitochondrial DNA expression (6, 7, 32), as well as COXIV, an indicator of mitochondrial count (30, 33). The lack of an effect on transcription of TFAM and COXIV suggests that LC likely does not inhibit mitochondrial biogenesis compared with CE.

Despite endurance exercise mode and nutrient supplementation effects on the transcription of PGC-1α, SIRT1, p53, TFAM, and COXIV during the recovery period, no treatment effects were observed for phosphorylation status of AMPK and p38 MAPK, or citrate synthase activity. Previous work has shown that the activation of AMPK and p38 MAPK (9, 10, 23) and citrate synthase (40, 41) is sensitive to exercise and carbohydrate availability. The lack of an effect between treatment groups in the present study may potentially be due to our limited sample size per group. In addition, the lack of a recovery muscle sample to assess signaling and citrate synthase activity may have also contributed to no treatment effects being observed. Because transcriptional modifications of the mitochondrial markers were most pronounced at recovery, treatment effects on signaling and citrate synthase may have been missed due to a lack of tissue to sample at this time point. Furthermore, reliance on citrate synthase to assess mitochondrial activity may have also contributed to no treatment effects being observed. Because transcriptional modifications of the mitochondrial markers were most pronounced at recovery, treatment effects on signaling and citrate synthase may have been missed due to a lack of tissue to sample at this time point. Furthermore, reliance on citrate synthase to assess mitochondrial activity may have limited our ability to distinguish differences between exercise mode and dietary supplementation. Additional assessments of isocitrate dehydrogenase and α-ketoglutarate dehydrogenase, rate-limiting enzymes of the TCA cycle, may have provided further insight into the impact of our intervention on mitochondrial activity. However, the current study was limited by sample availability.

In conclusion, results from this investigation further suggest that performing endurance exercise when energy and carbohydrate availability is low stimulates the expression of metabolic genes associated with mitochondrial biogenesis. Although LC resulted in a lower expression of PGC-1α than CE, the change in

**FIGURE 4** Correlation of PGC-1α to TFAM (A) and COXIV (B) expression and of p53 to TFAM (C) and COXIV (D) expression. Data are reported as log2 values for correlations to maintain equal scale between positive and negative values. COXIV, cytochrome c oxidase subunit IV; PGC-1α, peroxisome proliferator-activated γ-receptor coactivator 1α; p53, tumor suppressor p53; TFAM, mitochondrial transcription factor A.
PGC-1α expression was proportional to change in expression of its downstream targets TFAM and COXIV, which were not negatively affected by exercise mode. This finding indicates that LC may not inhibit mitochondrial biogenesis. Future investigation should focus on the long-term training impact of LC compared with CE on mitochondrial biogenesis and adaptations to training.

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