Optimizing Intramuscular Adaptations to Aerobic Exercise: Effects of Carbohydrate Restriction and Protein Supplementation on Mitochondrial Biogenesis\textsuperscript{1,2}

Lee M. Margolis and Stefan M. Pasiakos\textsuperscript{*}
Military Nutrition Division, United States Army Research Institute of Environmental Medicine, Natick, MA

ABSTRACT
Mitochondrial biogenesis is a critical metabolic adaptation to aerobic exercise training that results in enhanced mitochondrial size, content, number, and activity. Recent evidence has shown that dietary manipulation can further enhance mitochondrial adaptations to aerobic exercise training, which may delay skeletal muscle fatigue and enhance exercise performance. Specifically, studies have demonstrated that combining carbohydrate restriction (endogenous and exogenous) with a single bout of aerobic exercise potentiates the beneficial effects of exercise on markers of mitochondrial biogenesis. Additionally, studies have demonstrated that high-quality protein supplementation enhances anabolic skeletal muscle intracellular signaling and mitochondrial protein synthesis following a single bout of aerobic exercise. Mitochondrial biogenesis is stimulated by complex intracellular signaling pathways that appear to be primarily regulated by S\textsuperscript{5'-AMP-activated protein kinase} and p38 mitogen-activated protein kinase mediated through proliferator-activated \( \gamma \) receptor co-activator 1 \( \alpha \) activation, resulting in increased mitochondrial DNA expression and enhanced skeletal muscle oxidative capacity. However, the mechanisms by which concomitant carbohydrate restriction and dietary protein supplementation modulate mitochondrial adaptations to aerobic exercise training remains unclear. This review summarizes intracellular regulation of mitochondrial biogenesis and the effects of carbohydrate restriction and protein supplementation on mitochondrial adaptations to aerobic exercise.

Introduction
Skeletal muscle is highly adaptive and sensitive to a variety of external stimuli, particularly exercise (1). Skeletal muscle adaptations to exercise training (i.e., multiple bouts of exercise over time) are dependent on the frequency, intensity, duration, and mode (i.e., resistance vs. aerobic) of exercise performed (2). One pronounced i.m. adaptive response to aerobic exercise training is mitochondrial biogenesis, which increases mitochondria mRNA expression, protein content, number, and oxidative activity (2–4). The peroxisome proliferator-activated \( \gamma \) receptor co-activator 1 \( \alpha \) (PGC-1\( \alpha \))\textsuperscript{3}, which is activated in response to a single bout of aerobic exercise through S\textsuperscript{5'-AMP-activated protein kinase} and p38 mitogen-activated protein kinase (p38 MAPK) intracellular signaling, is thought to be the primary regulator of mitochondria biogenesis (5,6). Upregulated PGC-1\( \alpha \) activity and subsequent increases in mitochondrial biogenesis may enhance aerobic capacity by increasing fatty acid

\textsuperscript{1} Supported by United States Army Military Research and Material Command. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense.

\textsuperscript{2} Author disclosures: L. M. Margolis and S. M. Pasiakos, no conflicts of interest.

\textsuperscript{*} To whom correspondence should be addressed. E-mail: stefan.pasiakos@us.army.mil.

\textsuperscript{3} Abbreviations used: Akt, protein kinase B; AMPK, AMP-activated protein kinase; ATF-2, activating transcription factor 2; CaMK, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase; COX, cytochrome c; COX IV, cytochrome c oxidase subunit IV; CREB, cAMP response element-binding protein; 4E-BP1, eukaryotic initiation factor 4E-binding protein; eEF2, eukaryotic elongation factor 2; eEF2K, eukaryotic elongation factor 2 kinase; eIF4E/eIF4G, eukaryotic initiation factor 4E/4G; ERR\( \alpha \); estrogen-related receptor \( \alpha \); MEF2, myocyte enhancer factor 2; mTORC1, mammalian target of rapamycin complex 1; MNK, mitogen and stress activated kinase; mTORC1, mammalian target of rapamycin complex 1; NRF-1/2, nuclear respiratory factor-1/2; p38 MAPK, p38 mitogen-activated protein kinase; p53, tumor suppressor protein; p70 S6K, p70 S6 kinase; p160 Mbp, p160 myb binding protein; PGC-1\( \alpha \), proliferator-activated \( \gamma \) receptor co-activator; rpS6, ribosomal protein S6; SIRT1, silent mating type information regulation 2 homolog 1; Tlam, mitochondrial transcription factor A; YY1, yin yang 1.

β-oxidation and attenuating muscle glycogenolysis, thus delaying the onset of muscle fatigue and improving aerobic exercise performance (7–9).

Dietary intake modulates skeletal muscle adaptations to aerobic training (10). It is generally accepted that dietary carbohydrate intake and endogenous glycogen availability must be adequate to sustain aerobic performance and delay the onset of muscle fatigue (11,12). However, evidence now suggests that periodic restriction of carbohydrate intake prior to aerobic exercise can influence skeletal muscle oxidative capacity by enhancing mitochondrial biogenesis (13,14). Although deliberate carbohydrate restriction may potentiate metabolic adaptations to aerobic exercise, likely to improve physical performance, combining glycogen-depleting aerobic exercise with dietary carbohydrate restriction can also increase skeletal muscle proteolysis, resulting in negative muscle protein balance (15). Additionally, although consuming a low-carbohydrate (<2.5 g · kg⁻¹ · d⁻¹), high-fat (65–70% kcal · d⁻¹) diet may improve lipid oxidation, manipulating dietary carbohydrate and fat intake to that extent may not necessarily translate to improved aerobic exercise performance (16). However, increasing dietary protein intake at the expense of carbohydrate, while maintaining dietary fat at recommended levels (≈35% kcal · d⁻¹), is perhaps the more appropriate dietary manipulation. Recently, several investigations have demonstrated that combining high-quality protein supplementation with aerobic exercise increases mixed muscle protein synthesis, mitigating protein loss associated with carbohydrate restriction and resulting in positive protein balance (17,18). However, whether increased mixed muscle protein synthesis in response to aerobic exercise and protein consumption results from enhanced mitochondrial protein synthesis is not well described.

This manuscript provides a contemporary review of mitochondrial biogenesis and the mitochondrial adaptive responses to aerobic exercise training. This manuscript will also highlight dietary strategies to optimize aerobic exercise–induced mitochondrial biogenesis. Specifically, the mechanistic advantages by which carbohydrate restriction modulates skeletal muscle oxidative capacity and the effects of protein supplementation on i.m. regulators of mitochondrial biogenesis will be explored.

Intracellular Signaling and the Regulation of Mitochondrial Biogenesis
Mitochondria are often described as the “powerhouse” of the cell given their ability to generate chemical energy in the form of ATP through fatty acid β-oxidation, the tricarboxylic acid cycle, and oxidative phosphorylation. Continuous ATP generation is essential to maintain function, particularly in response to cellular stress, such as exercise (10). Mitochondrial adaptations to aerobic exercise training are salient to the metabolic plasticity of skeletal muscle. The biosynthesis of mitochondria enhances skeletal muscle oxidative capacity, allowing for greater generation of ATP, thereby delaying muscle time to fatigue and improving aerobic exercise performance. This dramatic phenotypic alteration is known as mitochondrial biogenesis, which results in increased mitochondrial size, content, number, and function in response to changes in energy status, contractile activity, and metabolic stress.

Regulation of mitochondrial biogenesis appears to be mediated at the level of transcription initiation by a complex intracellular signaling cascade. Central to the activation of this signaling cascade is PGC-1α, often referred to as the master regulator of mitochondrial biogenesis (19,20). The expression of PGC-1α regulates interaction and coactivation of nuclear respiratory factor-1 (NRF-1) and NRF-2, which control the expression of genes involved in oxidative phosphorylation through the electron transport chain by encoding cytochrome c (COX) and COX oxidase subunit IV (COX IV), mitochondrial DNA transcription and replication, protein import machinery, and protein assembly (21–23). The activity of PGC-1α also modulates the activity of various nuclear transcription factors, including the PPARs and estrogen-related receptors (ERRs) involved in the regulation of mitochondrial fatty acid β-oxidation, the tricarboxylic acid cycle, and the electron transport chain (24).

Activation of PGC-1α occurs at both the transcriptional and post-translational levels (Fig. 1) (23). Transcriptional PGC-1α expression is regulated through PGC-1α promoter binding activity of transcription factors myocyte enhancer factor 2 (MEF2), cAMP response element-binding protein (CREB), and activating transcription factor 2 (ATF-2) (25,26). Interestingly, although MEF2 enhances PGC-1α transcription, it is also a target of PGC-1α, which is indicative of an autoregulatory loop by which PGC-1α regulates PGC-1α expression (27). Post-translational activation of PGC-1α is regulated through direct phosphorylation by AMPK and p38MAPK as well as deacetylation through silent mating type information regulation 2 homolog 1 (SIRT1) (26,28).

Effects of Aerobic Exercise on the Regulation of Mitochondrial Biogenesis
The cumulative effects of aerobic training generally increase skeletal muscle mitochondria amount and activity with concomitant increases in PGC-1α mRNA expression and protein content (26,29–31). The mechanism by which aerobic exercise training modulates mitochondrial biogenesis is dependent on disruption of cellular homeostasis. Contractile-induced increases in cytosolic Ca²⁺ and increased ratios of AMP:ATP and NAD+:NADH regulates PGC-1α activity by triggering intracellular signaling. Contractile-induced Ca²⁺ release from the sarcoplasmic reticulum results in increased cytosolic Ca²⁺ concentrations, which upregulate PGC-1α expression and mitochondrial biogenesis through activation of Ca²⁺/calmodulin-dependent protein kinase (CaMK) (32,33). CaMK may indirectly activate PGC-1α by phosphorylating the transcription factors CREB and MEF2, thereby enabling binding of these factors to the PGC-1α promoter site, which enhances PGC-1α transcription (26,27). Increased intracellular Ca²⁺ concentrations may also mediate upregulation of p38 MAPK
through CaMK activation (34). Similar to CaMK, p38 MAPK may also indirectly stimulate PGC-1α activity by phosphorylating the transcription factors ATF-2 and MEF2 and inhibiting the repressor p160 MBP, which blocks PGC-1α and MEF2 autoregulation (26,35–38). Additionally, p38 MAPK directly phosphorylates PGC-1α (36) and although p38 MAPK signaling occurs downstream of CaMK, p38 MAPK appears to activate PGC-1α through a CaMK-independent mechanism (6). CaMK-independent, upregulated p38 MAPK phosphorylation may be attributed to aerobic exercise–induced expression of the upstream regulatory signaling proteins mitogen-activated protein kinase kinase 3 (MKK3) and MKK6. Investigations have shown that aerobic exercise upregulates MKK3 and MKK6 phosphorylation (39), which in turn directly phosphorylates p38 MAPK (40).

In addition to muscle contraction, cellular energy status is also a critical regulator of mitochondrial biogenesis. Prolonged aerobic exercise accelerates ATP utilization, increasing l.m. AMP:ATP ratios (41). Elevated cellular AMP initiates AMPK activation, which maintains cellular energy balance by inhibiting energy-utilizing anabolic pathways and upregulating ATP-yielding catabolic pathways (28,42). The metabolic demand associated with sustained aerobic exercise increases AMPK phosphorylation, which appears to be an upstream intracellular regulator of PGC-1α activity (43,44), because AMPK directly phosphorylates PGC-1α (45). Increased energy utilization during aerobic exercise also activates SIRT1 due to elevations in the cellular ratio of NAD⁺:NADH (46). The activation of SIRT1 results in PGC-1α deacetylation, which in turn activates PGC-1α and subsequent mitochondrial biogenesis (46). The phosphorylation status of AMPK indirectly regulates SIRT1, because AMPK controls the activation of signaling proteins involved in the catabolic energy yielding process, such as acetyl-CoA carboxylase and 6-phosphofructo-2-kinase, which result in increased NAD⁺:NADH levels (47). Together, these findings clearly illustrate the complexity associated with aerobic exercise-induced modulation of mitochondrial biogenesis, with multiple convergent signaling pathways sensitive to contractile force and cellular energy status regulating PGC-1α activity and mitochondrial biogenesis. Ultimately, aerobic training-induced alterations in intracellular signaling enhances mitochondrial content, number, size, and activity.

**Effects of Carbohydrate Restriction on Aerobic Training-Induced Mitochondrial Biogenesis**

Maintaining carbohydrate availability can sustain and perhaps enhance aerobic exercise performance by delaying time to exhaustion (48). However, recent evidence now suggests that periodic reductions in glycogen stores by dietary carbohydrate restriction combined with short-term aerobic exercise training periods (3–10 wk) enhances mitochondrial biogenesis to a greater extent than when aerobic exercise is performed in a glycogen-replete state (13). Specifically, dietary carbohydrate restriction increases markers of mitochondrial activity, including citrate synthase and β-hydroxyacyl-CoA dehydrogenase activity, enhances COX IV total protein

---

**FIGURE 1** PGC-1α regulation of mitochondrial biogenesis. Aerobic exercise and energy utilization initiate mitochondrial biogenesis. This process is centrally regulated by PGC-1α, which can be activated at the transcriptional level through promoter binding activity and at the post-translational level via direct phosphorylation and deacetylation. PGC-1α controls mitochondrial biogenesis through interaction and coactivation of NRF-1, NRF-2, PPARα, and ERRα, which are regulators of mitochondrial DNA expression, fatty acid β-oxidation, the tricarboxylic acid cycle, and the electron transport chain. Stimulators of mitochondrial biogenesis are shown in green. Inhibitors of mitochondrial biogenesis are depicted in red.

AMPK, 5’AMP-activated protein kinase; ATF-2, activating transcription factor 2; CaMK, Ca2+/calmodulin-dependent protein kinase; CRE, cAMP response element; CREB, cAMP response element-binding protein; ERRα, estrogen-related receptor α; MBP, myelin basic protein; MEF2, myocyte enhancer factor 2; MKK, mitogen-activated protein kinase kinase; mtDNA, mitochondrial DNA; NRF-1/2, nuclear respiratory factor-1/2; p38 MAPK, p38 mitogen-activated protein kinase; PGC-1α, proliferator-activated γ receptor co-activator; SIRT1, silent mating type information regulation 2 homolog 1; TCA, tricarboxylic acid cycle.
content, upregulates whole-body fat oxidation, and improves exercise time to exhaustion (14,49). Furthermore, periods of reduced glycogen stores alter the activity of signaling proteins integral to intracellular lipid and glucose metabolism, including carnitine palmitoyltransferase-1, pyruvate dehydrogenase kinase-4, and glucose transporter protein 4 (50–53).

The mechanism by which skeletal muscle oxidative capacity is upregulated in response to aerobic exercise when dietary carbohydrate intake is restricted appears to occur upstream of PGC-1α and is dependent on AMPK and p38 MAPK activation. Phosphorylation of AMPK and p38 MAPK is higher when exogenous carbohydrate availability is restricted following a bout of glycogen-depleting aerobic exercise compared with phosphorylation levels when carbohydrate intake is adequate during recovery (53,54). Recent reports demonstrate that increased AMPK and p38 MAPK phosphorylation in response to carbohydrate restriction upregulates PGC-1α activity following aerobic exercise (30).

However, not all studies support the link between carbohydrate availability and PGC-1α activity. In 2 recent studies, restricting carbohydrate availability with aerobic exercise increases markers of mitochondrial activity compared with aerobic exercise alone, but carbohydrate restriction had no effect on PGC-1α mRNA expression (48,52). These data suggest that although PGC-1α is the central regulator of mitochondrial biogenesis in response to aerobic exercise, the mechanism by which carbohydrate restriction influences mitochondrial adaptations to aerobic exercise is not clear.

The tumor suppressor protein, p53, which is sensitive to carbohydrate availability, has recently been identified as a potential regulator of mitochondrial biogenesis (55). Studies have demonstrated that p53 is phosphorylated by AMPK (56) and p38 MAPK (57) and stimulates the expression of genes that promote and maintain mitochondrial function (58,59). Bartlett et al. (60) demonstrated upregulation in p53, AMPK, and p38 MAPK phosphorylation in glycogen-depleted human skeletal muscle following 50 min of continuous aerobic exercise or high-intensity, interval-type exercise. The same researchers demonstrated that p53 phosphorylation, mitochondrial transcription factor A (Tfam), and COX IV mRNA expression were greater during recovery from 50 min of high-intensity interval cycling when volunteers were restricted from consuming carbohydrate compared with volunteers who consumed carbohydrate before, during, and after exercise (61). This investigation also observed greater PGC-1α mRNA expression during carbohydrate restriction. It is important to note that a glycogen depletion protocol was utilized the evening prior to the experimental session to elicit the low-carbohydrate state. As such, the higher PGC-1α mRNA expression observed during baseline and recovery from the 50-min aerobic exercise bout may have been a carryover effect from the glycogen depletion protocol (61). However, because this investigation utilized a glycogen depletion protocol combined with dietary carbohydrate restriction, it is difficult to interpret the influence on PGC-1α mRNA expression. Thus, although periodic carbohydrate restriction potentiates aerobic exercise–induced mitochondrial biogenesis, whether the increase in mitochondrial biogenesis was due to activation of p53 or PGC-1α remains unclear.

### Effects of Protein Supplementation on Aerobic Training–Induced Mitochondrial Biogenesis

Although carbohydrate restriction may augment mitochondrial adaptations to exercise, it may also impair skeletal muscle repair and recovery from aerobic exercise. Howarth et al. (15) reported that performing aerobic exercise under conditions of limited muscle glycogen availability increases skeletal muscle proteolysis and reduces muscle protein synthesis during recovery compared with responses when aerobic exercise was performed in a glycogen-replete state. However, it might be possible to use carbohydrate restriction to augment mitochondrial adaptations to exercise but offset those negative effects on muscle protein turnover by supplementing with dietary protein. Recent evidence has demonstrated that consuming dietary protein during or immediately following aerobic exercise increases mixed muscle protein synthesis, resulting in positive net protein balance (17,18). Furthermore, increasing extracellular amino acid levels upregulates mitochondrial protein synthesis (62), suggesting that protein supplementation with aerobic exercise during carbohydrate restriction may not only maintain skeletal muscle protein balance but may also contribute to mitochondrial adaptations to aerobic exercise.

The mechanism by which dietary protein modulates skeletal muscle protein synthesis through the mammalian target of rapamycin complex 1 (mTORC1) is well described (63,64). Activation of the mTORC1 complex triggers downstream signaling through p70 S6 kinase (p70 S6K1), ribosomal protein S6 (rpS6), eukaryotic elongation factor 2 kinase (eEF2), and eukaryotic initiation factor 4E-binding protein (4E-BP1) that increases mRNA translational efficiency and ultimately muscle protein synthesis (65). Although it was generally accepted that activation of the mTORC1 and AMPK–PGC-1α signaling pathways require different stimuli, with mTORC1 activated by primarily resistance exercise and AMPK–PGC-1α activated by mainly by aerobic exercise (43), recent investigations indicate potential interactions between the pathways (Fig. 2) (66–68). For example, p38 MAPK phosphorylation can inhibit eEF2 kinase (eEF2K), thereby activating eEF2 and stimulating muscle protein synthesis (66). Also, p38 MAPK phosphorylation activates mitogen and stress activated kinase (MNK), which catalyzes the phosphorylation eukaryotic initiation factor 4E (eIF4E), an important regulator of translation initiation (67). Additionally, it has been reported that the amino acid leucine, a potent stimulator of mTORC1 signaling, may increase mitochondria size via SIRT1 and subsequent activation of PGC-1α (69). The interaction of these regulatory pathways also operates in the other direction. Inhibition of mTOR decreases activation of PGC-1α, resulting in decreased expression of mitochondrial genes and mitochondrial DNA via an inhibition of yin yang 1 (YY1) (68).
This finding suggests a potential mechanism of crosstalk between intracellular pathways such that mTOR balances anabolic activity and energy metabolism through transcriptional control of mitochondrial biogenesis (68).

In addition to the observed overlap in signaling of muscle protein synthesis and mitochondrial biogenesis, similar up-regulation in mTOR and AMPK-PGC-1α signaling cascades can be achieved in response to resistance and aerobic exercise, particularly when supplemental protein is consumed (70–72). Camera et al. (70) reported that phosphorylation of protein kinase B (Akt) and mTOR in the fasted state are similar with aerobic and resistance-type exercise. However, AMPK was phosphorylated only in response to aerobic exercise. On the other hand, when participants consume a mixed-meal containing 20 g of high-quality protein before, during, and after exercise, phosphorylation of Akt, mTOR, p70S6K, and AMPK were all similar in response to aerobic exercise (71). Concomitant phosphorylation of AMPK and mTOR suggests both cellular growth and mitochondrial biogenesis may occur in response to combined training.

Several studies have observed that consumption of supplemental protein following aerobic exercise stimulates mitochondrial protein synthesis (72,73). However, studies have reported no differences in postaerobic exercise mitochondrial protein synthesis when volunteers consumed a combined carbohydrate and protein supplement compared with a noncaloric placebo (74) or carbohydrate alone (75), nor was there a difference in the phosphorylation of AMPK or PGC-1α mRNA expression immediately and 3 h postexercise (76). Furthermore, dietary leucine may also suppress phosphorylation of AMPK (77). Conversely, Hill et al. (78) reported greater PGC-1α mRNA expression when participants consumed a carbohydrate-protein supplement compared with carbohydrate alone 6 h postexercise.

Despite the conflicting results, protein supplementation does not appear to further enhance aerobic exercise–induced mitochondrial biogenesis when carbohydrate is restricted. However, it is important to recognize that protein supplementation does not hinder the activation of intracellular signaling proteins associated with mitochondrial biogenesis, nor does protein supplementation impede mitochondrial protein synthesis. Additionally, protein supplementation increased myofibrillar protein synthesis and phosphorylation of mTOR, p70S6K, and rpS6 following aerobic exercise (74,75). Thus, although protein supplementation may not elevate mitochondrial biogenesis per se, consuming high-quality protein during or after aerobic exercise promotes skeletal muscle recovery, especially when aerobic exercise is performed with concomitant carbohydrate restriction.

In conclusion, mitochondrial biogenesis is a critical metabolic adaptation to aerobic exercise training. The activity of PGC-1α appears central to aerobic training-induced mitochondrial adaptations. Emerging evidence suggests that the mitochondrial adaptive response to aerobic exercise can be further potentiated by restricting carbohydrate availability, although the underlying mechanism has not been determined. The synergistic effect of carbohydrate restriction with aerobic exercise training may elicit greater aerobic exercise–induced adaptations, thereby delaying the onset of muscle fatigue and improving aerobic performance.
Additionally, consuming supplemental protein during or in recovery from aerobic exercise, especially during periods of carbohydrate restriction, may facilitate the maintenance of skeletal muscle integrity and support mitochondrial biogenesis, although standardized dietary carbohydrate and protein recommendations are not possible at this time. Further study is warranted to determine dietary recommendations by assessing the isolated effects of supplemental protein on mitochondrial biogenesis following aerobic exercise and whether habitual dietary carbohydrate and protein intake modulates skeletal muscle mitochondrial adaptive response to chronic aerobic training.

Acknowledgments
The authors thank Dr. Andrew J. Young for his critical review in the development of this manuscript. Both authors read and approved the final manuscript.

Literature Cited


