



NUTRITIONAL STRATEGIES TO IMPROVE MUSCLE MITOCHONDRIAL CONTENT AND FUNCTION

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KEY POINTS

- Training-induced increases in mitochondrial content improve exercise tolerance by attenuating rises in cytosolic free adenosine diphosphate (ADP) concentrations.
- Nutritional approaches to improve training-induced mitochondrial biogenesis are limited, partially because of a lack of understanding of the initiating molecular signals regulating this process.
- The recent revelation that mitochondrial derived reactive oxygen species (ROS) can induce mitochondrial biogenesis may result in novel training approaches.
- Training in a low-carbohydrate environment has been shown to increase mitochondrial content, although the mechanism(s) responsible for this adaptation remain debatable.
- Beetroot juice (nitrate) ingestion does not alter mitochondrial-coupling efficiency, but does increase mitochondrial ROS emission rates, although the biological relevance of this observation remains unknown.
- The intrinsic response of mitochondria to ADP is influenced by acute and chronic exercise, as well as the consumption of polyunsaturated fatty acids, and therefore mitochondrial ADP sensitivity can be altered independently from mitochondrial content.

INTRODUCTION

Strenuous exercise can increase the energetic demands of skeletal muscle by 100-fold over resting requirements, placing an enormous challenge on bioenergetic pathways to maintain concentrations of adenosine triphosphate (ATP), the basic unit of energy within muscle. Exercise performance is influenced by several factors, including blood flow, diffusion of metabolic substrates, metabolism within skeletal muscle, and the ability to generate optimal/desired mechanical force. While skeletal muscle is equipped with an intricate series of enzymatic reactions that resynthesize ATP to ensure cellular survival during these conditions, mitochondria are thought to represent a key organelle influencing metabolic homeostasis within muscle. The transport of adenosine diphosphate (ADP) from the cytosol into the mitochondrial matrix can indirectly influence glycolytic flux (ADP is an allosteric activator of rate-limiting enzymes) and directly affect oxidative phosphorylation rates. As a result, the improvement in exercise performance, muscle glycogen sparing, attenuated production of lactate, and the increased reliance on aerobic metabolism following training have been attributed to an improvement in mitochondrial ADP sensitivity due to the increase in mitochondrial content (Holloszy & Coyle, 1984). Historically, this response has been entirely accredited to the induction of mitochondrial biogenesis and increased mitochondrial content (Holloszy & Coyle, 1984); however, external regulation on the proteins involved in mitochondrial ADP (changes in “efficiency”) transport likely also exists. This Sports Science Exchange article will focus on discussing potential strategies to increase either 1) mitochondrial content or 2) mitochondrial efficiency. The biological consequence of increasing either mitochondrial content or function is an

improvement in ADP sensitivity (as discussed below), and therefore, this review will also discuss 3) nutritional and training strategies to directly improve mitochondrial ADP sensitivity.

MITOCHONDRIAL BIOGENESIS

It has been known for almost a century that elite athletes have a higher maximal rate of oxygen consumption (VO_{2peak}) and higher maximal mitochondrial enzymatic activities, ultimately contributing to elite performances. While originally attributed to genetics, Holloszy's landmark research in 1967 demonstrated the remarkable plasticity of skeletal muscle to increase mitochondrial content and improve exercise capacity (Holloszy, 1967). This seminal paper described the basic observation that overload training increases mitochondrial content, but does not alter the intrinsic function of mitochondria. As a result, research in the subsequent 50 years has focused on elucidating the mechanisms responsible for the induction of mitochondrial biogenesis. This review will not focus on a detailed description of the processes resulting in the induction of mitochondrial biogenesis, but a brief description is required to provide a basic framework for discussions on strategies aimed at optimizing this response.

The mitochondrial proteome consists of ~1,600 proteins, the vast majority of which are encoded within the nucleus, as the mitochondrial DNA (mtDNA) only transcribes for 13 protein subunits of the electron transport chain and proteins required for mRNA translation within this organelle (for a review, see Bartlett et al., 2015). The induction of mitochondrial biogenesis therefore involves a coordinated signaling response that stimulates both genomes. The identification of peroxisome

proliferator activated receptor γ co-activator 1 α (PGC-1 α) protein as a transcriptional co-activator synchronizing this process was a major advancement in our understanding of the molecular mechanisms regulating mitochondrial content. Cytosolic calcium-induced activation of Ca²⁺/calmodulin-dependent protein kinase (CaMK), activation of adenosine monophosphate kinase (AMPK) by energy turnover, and increased reactive oxygen species (ROS) production have all been implicated as primary mechanisms in the induction of mitochondrial biogenesis (Bartlett et al., 2015). However, while research continues to improve our understanding of the processes involved in expanding mitochondrial volume, our knowledge on the signals initiating mitochondrial biogenesis remains poorly defined, limiting our ability to design optimal training interventions.

Despite the limitation in our molecular understanding of mitochondrial biogenesis, training strategies that augment these responses have been identified. Of particular interest is the notion of periodized training in a low carbohydrate environment, an approach that has been shown to 1) activate molecular pathways associated with mitochondrial biogenesis, 2) increase the oxidative capacity of muscle, and in some situations 3) improve exercise capacity (Bartlett et al., 2015). The seminal work by Pilegaard and colleagues was instrumental in highlighting that low-glycogen availability during and after exercise amplifies the normal exercise-induced expression of mitochondrial genes (Pilegaard et al., 2002; 2005). Conversely, others have shown that acute exercise in the presence of high carbohydrate availability attenuates signals associated with mitochondrial biogenesis (Bartlett et al., 2013). Importantly, these findings of transient acute signals in untrained individuals appear to translate to athletes, as periodized training in a low-glycogen state similarly increases mitochondrial content in highly trained individuals. Specifically, Hawley's group has demonstrated that training twice a day every other day in already trained individuals increases muscle glycogen content, markers of mitochondrial content, and rates of fat oxidation, while similar amounts of work separated over single exercise sessions on consecutive days does not (Yeo et al., 2008). In addition, withholding carbohydrate after an evening training bout has been shown to improve 10 km run times (Marquet et al., 2016), suggesting possible performance benefits are associated with the observed molecular responses. The observed beneficial adaptations to periodized training in a transient low-carbohydrate environment have been attributed to AMPK activation (Yeo et al., 2008), as previous work highlighted a glycogen-binding domain on the β -subunit of AMPK and activation in the presence of low glycogen content within muscle (McBride et al., 2009; Wojtaszewski et al., 2003). However, while a large body of literature has been devoted to studying the role of energy turnover and activation of AMPK as a key signal to induce mitochondrial biogenesis (reviewed in Marcinko & Steinberg, 2014), genetic models that render AMPK activity substantially impaired have been confounded by potential impairments in cardiovascular performance during exercise, making interpretations difficult. In contrast, ablating liver kinase B1 (LKB1), an upstream activator of AMPK in rodent muscle, impairs exercise capacity and reduces mitochondrial content in sedentary animals, but does not affect exercise training

responses (Tanner et al., 2013). This suggests that activation of AMPK is not required for the induction of mitochondrial biogenesis. Therefore, while training in a low-carbohydrate environment appears to increase mitochondrial content, the molecular mechanisms remain debatable.

ROS has also been considered a signal to induce mitochondrial biogenesis, but clear evidence for a mechanistic role for ROS had not been previously established. However, a theoretical argument has been made based on observations that exercise increases oxidative damage of muscle (Davies et al., 1982). However, several lines of evidence have recently been established to implicate ROS, and specifically mitochondrial-derived ROS, in the induction of mitochondrial biogenesis. Specifically, consumption of a high-fat diet has been shown to increase mitochondrial content (Jain et al., 2014), mitochondrial ROS emissions, redox alterations of the calcium release channel ryanodine receptors (RyR), and activation of calcium signaling (CaMKII) (Jain et al., 2014). These responses were entirely prevented with the consumption of a mitochondrial-targeted antioxidant (SkQ) (Jain et al., 2014). In addition, a single bout of high-intensity interval training has been shown to increase ROS-mediated fragmentation of the RyR in association with the induction of mitochondrial biogenesis (Place et al., 2015). These responses were attenuated after chronic training, potentially accounting for the diminished returns of exercise training with respect to continued expansion in mitochondrial volume (Place et al., 2015). Altogether, these data suggest that mitochondrial-derived ROS is a key molecular signal for the induction of mitochondrial biogenesis, a process which may require redox modifications of the RyR and calcium-mediated signaling. These data help explain the lack of mitochondrial biogenesis observed in humans consuming high quantities of certain antioxidants while exercise training (Paulsen et al., 2014). In this manner, increasing the redox stress during exercise training/recovery may increase mitochondrial biogenesis. While speculative, training in a low-carbohydrate environment may promote redox signaling, as fatty acids have a high propensity to produce mitochondrial-derived ROS. Clearly, future research is required to fully delineate the role of redox signaling in the induction of mitochondrial biogenesis, and to establish novel training paradigms to maximize these processes in athletes.

MITOCHONDRIAL ADP SENSITIVITY

The induction of mitochondrial biogenesis, and the subsequent improvement in mitochondrial ADP sensitivity has become synonymous with exercise training adaptations. While several processes could influence free ADP concentrations in vivo, direct assessments of mitochondrial respiration using permeabilized muscle fibers have consistently shown 1) an improvement in respiration at a submaximal ADP concentration following training (see Ludzki et al., 2015 for example), or 2) conversely a reduction in the amount of ADP required to maintain a given aerobic flux. These findings suggest that mitochondrial changes contribute to the improvement in ADP sensitivity following training (Fig. 1A, B). This classical working model is premised on the belief that mitochondrial "function" remains unaltered following a

chronic training intervention. However, evidence is accumulating to suggest that mitochondrial ADP transport is a regulated process, raising the possibility that lifestyle interventions can influence mitochondrial ADP sensitivity in the absence of increased mitochondrial content.

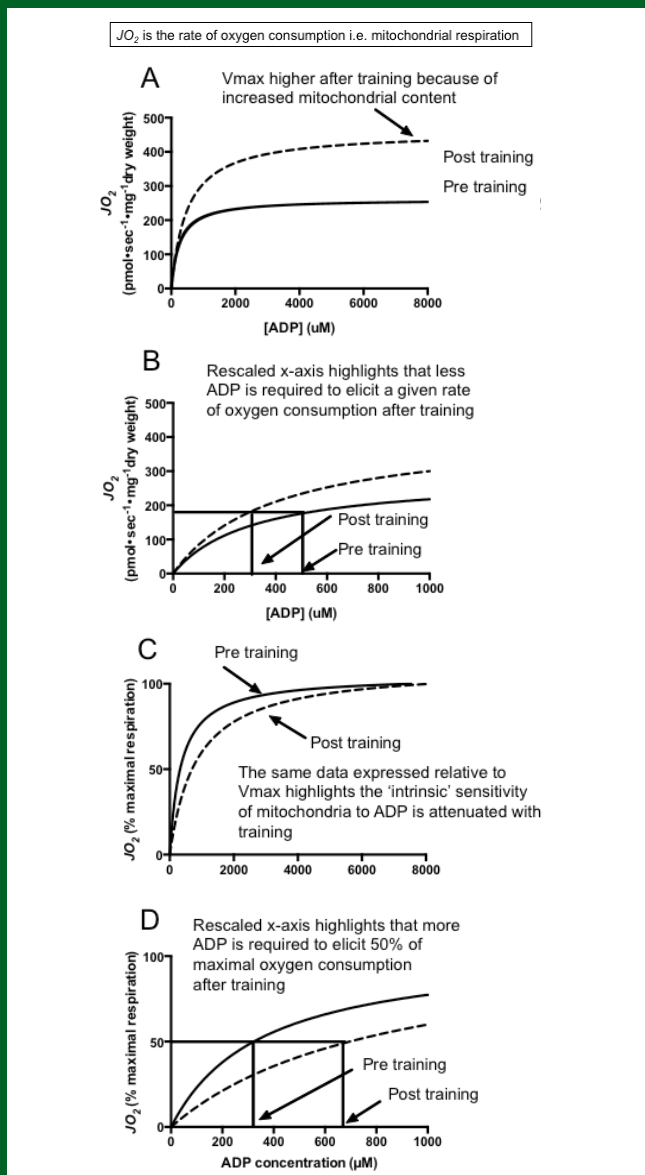


Figure 1. These schematic figures illustrate the response of adenosine diphosphate (ADP) respiratory kinetics in permeabilized muscle fibers before and after training. In these experiments saturating pyruvate (+malate) were provided, and then subsequently ADP is titrated into a sealed system to measure the respiratory drive. If these values are expressed as absolute values (normalized to dry weight as in A and B) the increase in maximal respiration reflects mitochondrial biogenesis and an increase in mitochondrial content. As a result, the amount of ADP required to achieve a specific rate of oxygen consumption was lower after training (B). However, if mitochondrial content is negated by expressing respiration as a percentage of maximal oxygen consumption (C and D) a different observation was apparent. Specifically, after training a higher concentration of ADP was required to achieve 50% of maximal respiration (termed the apparent ADP Km). These later analyses suggested the intrinsic sensitivity of mitochondria was actually decreased with training. J_{O_2} , rate of oxygen consumption or respiration; V_{max} , maximal activity rate.

Indeed, paradoxically, the concentration of ADP required to illicit half-maximal respiration (K_m), termed the apparent ADP K_m , is reduced following training (Fig. 1C, D), suggesting the intrinsic sensitivity of a given mitochondrion to ADP is attenuated with training. While this biochemical definition does not have direct biological relevance, it does demonstrate that ADP sensitivity can be externally regulated, and further understanding of the regulation of this process may yield insight into novel training programs.

While adenine nucleotide translocase (ANT) is required for ADP/ATP exchange (Fig. 2), mitochondrial creatine kinase (mi-CK) is thought to concentrate ADP within the intermembrane space to optimize diffusion of ADP into the mitochondria, while phosphocreatine (PCr)/Creatine (Cr) is estimated to diffuse ~2,000 times faster across the outer mitochondrial membrane/through the cytosol (Wallimann et al., 2011). Therefore, phosphate transfer via creatine kinase reactions is believed to contribute substantially to metabolic homeostasis, particularly during muscle contraction where ATP requirements can increase ~100-fold (Saks et al., 1985). However, ANT is required for both Cr dependent and independent transport of ADP/ATP across the inner mitochondrial membrane, and external regulation of ANT exists as an acute bout of high-intensity interval exercise has been shown to acutely improve mitochondrial ADP sensitivity (Ydfors et al., 2016), while steady-state exercise (~60% VO_{2peak} for 2 hr) attenuated mitochondrial ADP sensitivity in the absence of Cr (Perry et al., 2012). These data suggest the regulation of ANT is highly complex and exercise intensity dependent (Fig. 2). While our understanding of the regulation of mitochondrial ADP sensitivity is incomplete, the fatty acid palmitoyl-CoA is known to interact with ANT to inhibit ADP/ATP exchange, a process attenuated by chronic exercise training (Ludzki et al., 2015), which in theory could contribute to "buffering rises in cytosolic free ADP" during exercise after

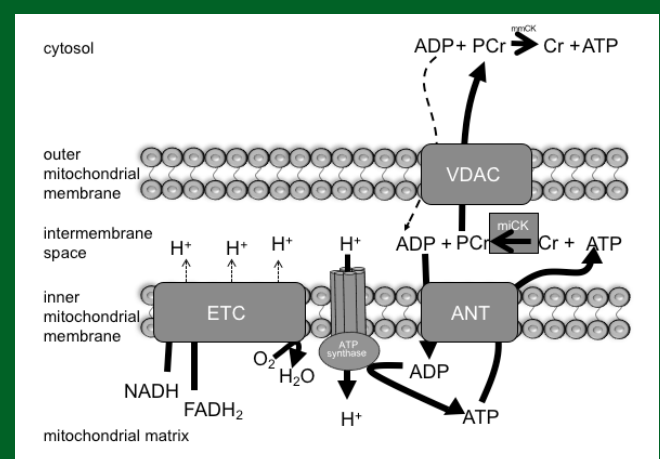


Figure 2. Schematic diagram of energy production in mitochondria and energy transfer across the mitochondrial membranes. PCr, phosphocreatine; Cr, creatine; ATP, adenosine triphosphate; ADP, adenosine diphosphate; NADH, nicotinamide adenine dinucleotide reduced; FADH₂, Flavin adenine dinucleotide reduced; ETC, electron transfer chain; ANT, adenine nucleotide translocase; VDAC, voltage dependent anion channel; miCK, mitochondrial creatine kinase; mmCK, cytosolic creatine kinase.

training. Clearly, evidence is mounting to suggest that mitochondrial ADP sensitivity is regulated extensively during acute exercise and is influenced by chronic training (Fig. 2). Combined, these data highlight the possibility of altering mitochondrial ADP sensitivity in the absence of mitochondrial biogenesis, challenging the long-held dogma that increases in mitochondrial content are required for fuel-shifts and performance improvements.

POLYUNSATURATED FATTY ACIDS AND MITOCHONDRIAL BIOENERGETICS

Given the relative novelty of identifying mitochondrial ADP transport as a regulated process, very little evidence has been generated with respect to nutritional approaches to augment this process. However, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) supplementation has been shown to alter the lipid composition of mitochondrial membranes in association with an increase in mitochondrial ADP sensitivity (Herbst et al., 2014). Specifically, a 12-week daily supplementation of 2 g EPA and 1 g DHA in healthy individuals (mean age was 22) improved the sensitivity of mitochondria in permeabilized muscle fibers to ADP by ~30% in the absence of changes in mitochondrial content (Herbst et al., 2014). Intriguingly, EPA/DHA feeding also increased mitochondrial ROS emission rates (Herbst et al., 2014), and while this did not result in the induction of mitochondrial biogenesis in sedentary individuals, it raises the potential for this nutritional approach to improve exercise-induced mitochondrial biogenesis. However, since EPA/DHA supplementation has been linked to improvements in protein synthesis, cognitive performance, immune function, bone integrity, cardiovascular function and the expression of genes associated with lipid oxidation in several tissues (reviewed in Jeromson et al., 2015), it is tempting to speculate that EPA/DHA supplementation could improve exercise performance. However, there is a paucity of literature surrounding the ability of EPA/DHA to improve metabolic responses during exercise in human skeletal muscle.

NITRATE AND MITOCHONDRIAL BIOENERGETICS

Traditional dogma stipulates that mitochondrial function is not externally regulated beyond the provision of substrates required for oxidative phosphorylation. This belief extends from Holloszy's original observation that *in vitro* assessments of mitochondrial stoichiometry (P/O ratios: ADP consumed per oxygen atom) remain constant following chronic training (Holloszy, 1967). However, the seminal finding by Larsen and colleagues showing that three days of dietary sodium nitrate ingestion (daily consumption of ~7 mmol sodium nitrate) improved mitochondrial coupling efficiency, maximal rates of ATP production, and reduced whole body oxygen consumption in humans (Larsen et al., 2011) indicated that this notion needed to be reconsidered. Intriguingly, the oral consumption of beetroot juice also decreases the oxygen cost of submaximal exercise in humans, suggesting that oral nitrate sources universally improve mitochondrial respiratory efficiency. However, in contrast to sodium nitrate, consuming a higher amount of oral nitrate in

the form of beetroot juice (~26 mmol of daily nitrate) over seven days did not alter isolated mitochondria coupling ratios, leak respiration, mitochondrial membrane potential or mitochondrial ADP sensitivity in permeabilized muscle fibers (Whitfield et al., 2015), indicating that beetroot juice does not alter mitochondrial coupling efficiency. These "in vitro" observations are supported by the finding that PCr resynthesis rates *in vivo*, an estimate of mitochondrial oxidative metabolism, were not altered following six days of beetroot juice consumption, but instead, rates of ATP hydrolysis were decreased (Bailey et al., 2010). Combined, these data suggest the mechanism-of-action of beetroot juice does not involve an improvement in rates of mitochondrial efficiency, but rather an improvement in mechanical efficiency. Moreover, the provision of sodium nitrate reduced ANT protein content (Larsen et al., 2011), and while this may improve mitochondrial-coupling ratios, it would be expected to decrease mitochondrial ADP sensitivity, which would be counterproductive to training responses (Fig. 2).

While beetroot juice does not appear to influence mitochondrial oxidative metabolism, it has been shown to increase mitochondrial ROS emission rates (Whitfield et al., 2015), which may contribute to the apparent improvement in exercise efficiency following beetroot juice consumption. While a direct cause-and-effect relationship between mitochondrial ROS and an improvement in the mechanical efficiency of muscle remains to be determined, mechanisms pertaining to redox modifications within sarcomeres (e.g., troponin I) and calcium handling (e.g., RyR and sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) remain likely targets (Hernandez et al., 2012). The necessity of mitochondrial ROS in mediating beetroot juice improvements in exercise performance is an attractive model, given the known reduction in mitochondrial ROS following training (Place et al., 2015), and the apparent resistance/attenuated response to beetroot juice in elite endurance athletes (Boorsma et al., 2014). It is also tempting to speculate that consumption of beetroot juice during a chronic training program would increase mitochondrial biogenesis as a result of an increase in mitochondrial ROS-mediated gene transcription; however, this possibility awaits direct scientific support. It appears that beetroot juice does not alter mitochondrial-coupling efficiency (Bailey et al., 2010; Whitfield et al., 2015). The seminal finding that sodium nitrate improves mitochondrial coupling efficiency raises the potential that future nutritional targets will be identified with similar biological effects (Larsen et al., 2011).

SUMMARY AND PRACTICAL APPLICATIONS

Improving mitochondrial content and/or function would be advantageous for exercise performance, and therefore a basic understanding of the regulation of mitochondria is required to elucidate novel approaches to alter this dynamic organelle. The novel mechanistic link between mitochondrial ROS emission and mitochondrial biogenesis raises the potential for numerous nutritional approaches to be tested in concert with a training program in athletes. In particular, the consumption of EPA/DHA and beetroot juice has been shown to increase mitochondrial ROS emission rates. The biological relevance of this observation remains

unknown, but it may contribute to the known improvement in mechanical efficiency and reduction in oxygen consumption observed with beetroot juice supplementation. There is clear evidence that training in a low-carbohydrate situation increases mitochondrial content, and it would be intriguing to determine if beetroot juice consumption could augment this response. A major benefit of beetroot juice is the rapid nature of this supplement (i.e., hours and days), while in contrast a limitation of EPA/DHA is the requirement for chronic consumption of these lipids (i.e., weeks and months), which have also been shown to attenuate signals associated with protein synthesis.

The biological effect of increasing mitochondrial content is an improvement in mitochondrial ADP sensitivity. Intriguingly, high-intensity intermittent exercise has been shown to acutely improve mitochondrial ADP sensitivity, raising the possibility that brief high-intensity bouts of exercise in a “warm-up” period may improve metabolic control in a subsequent bout of exercise. This may be particularly beneficial for athletes, as training decreases the intrinsic mitochondrial ADP sensitivity (see Figures 1C, D). However, the minimal time and intensity to achieve an improvement in ADP sensitivity need to be determined to ensure depletions of muscle glycogen do not occur immediately before competition, as this would be counterproductive. The recent advancement in our understanding of the regulation of mitochondrial ADP transport has elucidated gaps in our working models that need to be addressed. However, the awareness of these knowledge gaps creates the possibility for unique experimental approaches to be designed with the aim to improve exercise performance in the future.

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