High-Intensity Exercise and Mitochondrial Biogenesis: Current Controversies and Future Research Directions

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Abstract

It is well established that different types of exercise can provide a powerful stimulus for mitochondrial biogenesis. However, there are conflicting findings in the literature and a consensus has not been reached regarding the efficacy of high-intensity exercise to promote mitochondrial biogenesis. The purpose of this review is to examine current controversies in the field and to highlight some important methodological issues that need to be addressed in order to resolve existing conflicts.

1. **Introduction**

Mitochondria are double-membrane organelles that generate cellular energy via oxidative phosphorylation (OXPHOS). In skeletal muscle, they range in size from 0.1 to 5.0 µm in diameter and form a reticulum that provides a pathway for energy distribution along the cell (36, 102) (Figure 1). Mitochondria contain their own genome, the mitochondrial DNA (mtDNA), which encodes for 37 proteins - of which 13 are essential polypeptides of the electron transport chain (ETC). However, the vast majority of the mitochondrial proteome (~ 1100 proteins) consists of nuclear-encoded proteins that are imported into the mitochondria.

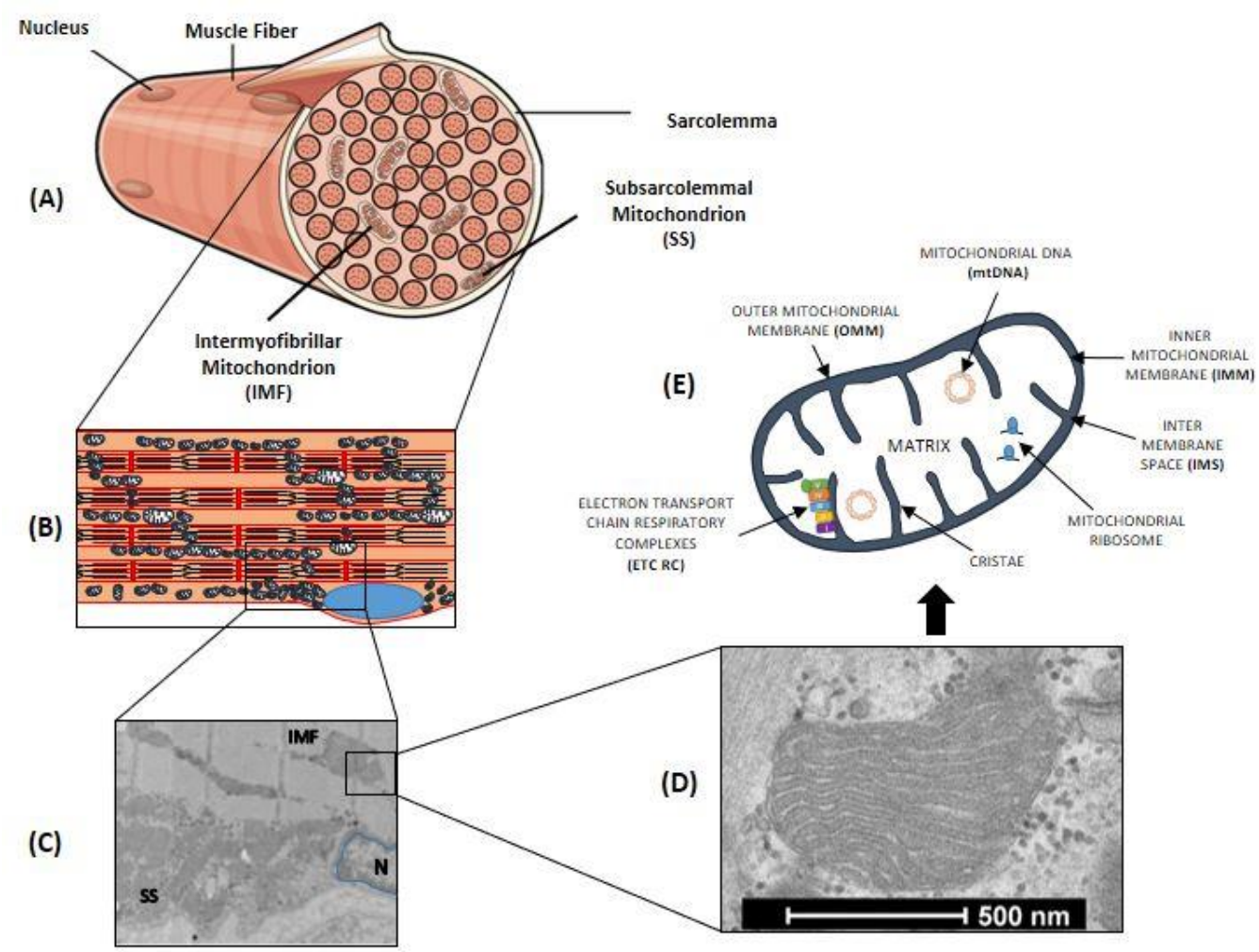

Figure 1. (A) Structure of a skeletal muscle fiber. Two sub-populations of mitochondria can be found: subsarcolemmal mitochondria (SS) and intermyofibrillar mitochondria (IMF). (B) Transversal illustration of a skeletal muscle fiber. Mitochondria create a reticulum that connects SS and IMF mitochondria for optimal energy distribution. (C) Transmission electron microscopy transversal image from a human skeletal muscle biopsy showing the nucleus (N), and the SS and IMF mitochondria. (D) Image of a mitochondrion. High-resolution imaging allows visualization of the densely-packed cristae within a mitochondrion. (E) Image of how a mitochondrion is usually illustrated in textbooks or research articles. Notice how the mitochondrial DNA (mtDNA) and the mitochondrial ribosomes are found in the matrix. The electron transport chain respiratory complexes are located on the IMM, and for the most part in the cristae.

As mitochondria are involved in many essential cell functions related to cellular metabolism and homeostasis (120), it is not surprising that sub-optimal mitochondria characteristics have been related to an increasing number of diseases and medical conditions (99, 107) (Figure 2). Mitochondria are also thought to be important for endurance performance, as their content and respiratory function have been correlated with maximal oxygen consumption ($\dot{V}O_{2max}$) (58, 127), time−trial performance (45, 64), and the lactate threshold (LT) (61). Hence, a better understanding of how mitochondria adapt to exercise has implications for both health and endurance performance.

It is well established that different types of exercise can provide a powerful stimulus for mitochondrial biogenesis (55, 76, 85, 114). However, there are conflicting findings in the literature, and a consensus has not been reached, regarding the efficacy of high-intensity exercise to promote mitochondrial biogenesis. The purpose of this review is to examine current controversies in the field and to highlight some important methodological issues that need to be addressed to resolve existing conflicts.

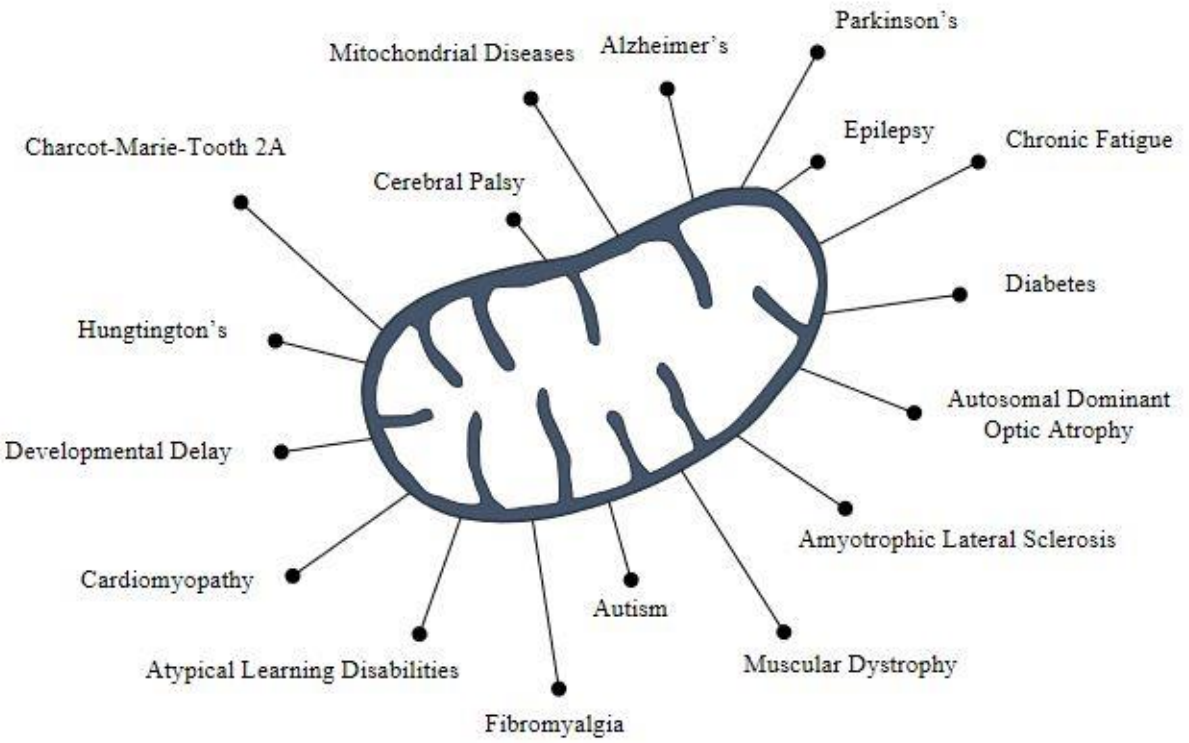

Figure 2. Summary of diseases or medical conditions that have been linked to sub-optimal mitochondrial characteristics.

2. Mitochondrial Biogenesis

Despite its widespread use in the literature, there is currently no widely-accepted definition of "mitochondrial biogenesis" (87, 117) (a Google Scholar search for "mitochondrial biogenesis" returns > 60 000 hits) and this has contributed to confusion and conflicting interpretations about the effects of exercise on mitochondrial biogenesis. Given its etymological meaning (i.e., the synthesis of new mitochondrial components), it has been suggested that mitochondrial biogenesis can best be assessed by measuring the rate of mitochondrial protein synthesis (mitoPS) (1, 87). However, a relationship between changes in mitoPS and subsequent changes in mitochondrial content (and/or respiratory function) remains to be established; this relationship cannot be assumed, especially as increases in muscle protein synthesis (MPS) following a single session of resistance exercise do not correlate with subsequent changes in muscle size in response to repeated resistance exercise sessions (i.e., exercise training) (80, 89). Further research is needed to establish whether exercise-induced changes in mitoPS can provide quantitative or qualitative information about subsequent training-induced changes in mitochondrial content and/or respiratory function before mitoPS can be adopted as the best measure of mitochondrial biogenesis.

Changes in mitoPS occur in conjunction with the processes of mitochondrial remodeling (mitochondrial fusion and fission) (28), as well as catabolic events such as mitochondrial protein breakdown (mitoPB), mitophagy (19), and apoptosis (131) (Figure 3). Thus, measuring only the global synthesis rate of mitochondrial proteins does not provide information about mitochondria remodeling, or changes in mitochondrial content (the net outcome of mitoPS and mitoPB), mitochondrial respiratory function, or other aspects of mitochondrial quality such as cristae density or supercomplex formation (84, 117). Therefore, while it is of value to assess exercise-induced changes in mitoPS, which indicates that mitochondrial biogenesis has been activated, researchers should consider including a comprehensive assessment of training-induced changes in mitochondrial content, structure, quality, and respiratory function, to put the results of mitochondrial biogenesis in context (12, 117). Furthermore, to expand the knowledge of the mechanisms leading to exercise-induced mitochondrial biogenesis, measurement of changes in gene expression, as well as proteins and transcription factors mediating these molecular processes, should be included where possible.

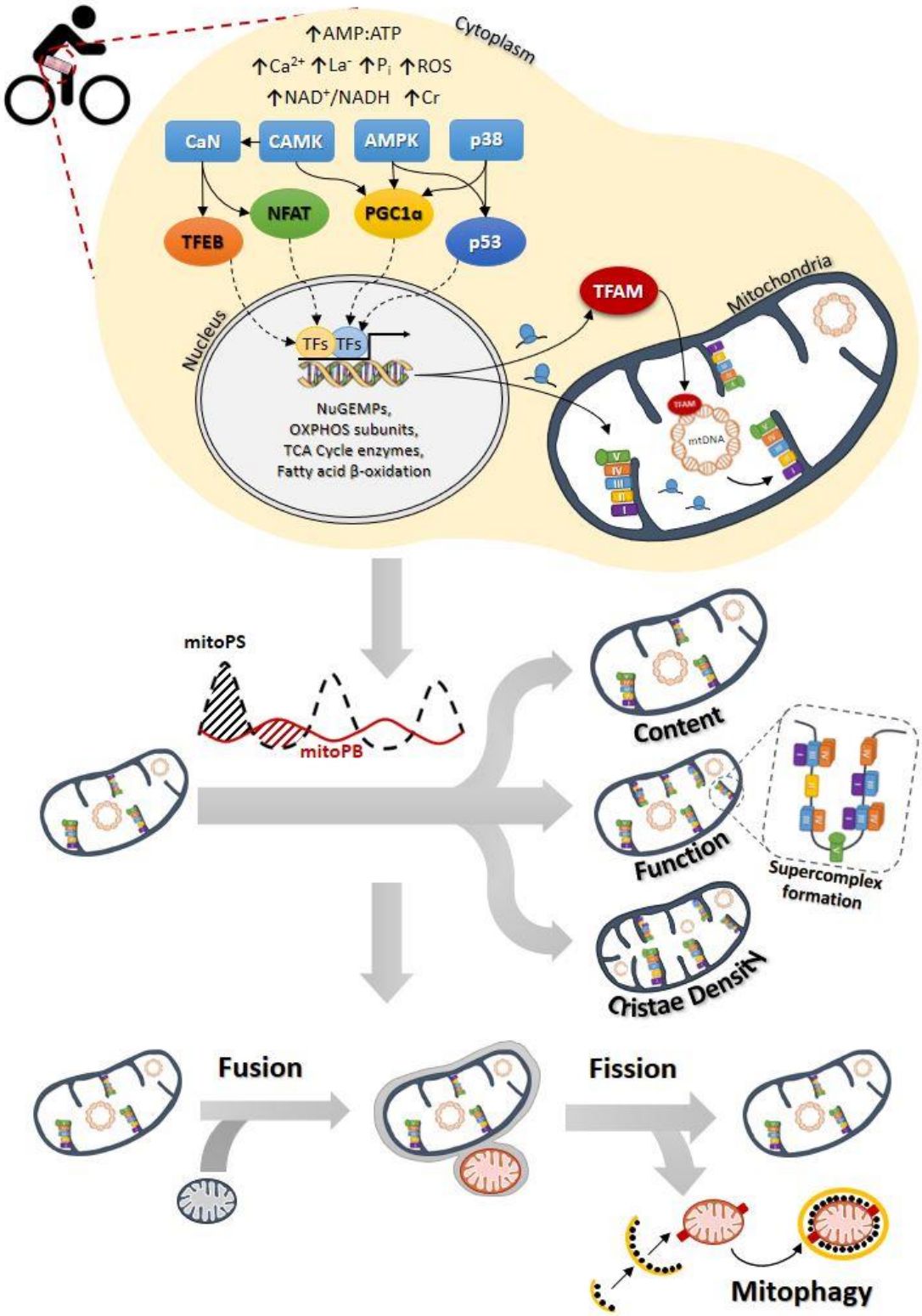

Figure 3. Schematic representation of the effects of high-intensity exercise and training on mitochondrial adaptations. AMP: *Adenosine monophosphate*, ATP: *Adenosine triphosphate*, Ca^{2+}: *Calcium*, La^{-}: *Lactate*, P_i: *Inorganic phosphate*, ROS: *Reactive oxygen species*, NAD: *Nicotinamide adenine dinucleotide*, CaN: *Calcineurin*, CAMK: *Ca^{2+}/calmodulin-dependent protein kinase*, AMPK: *AMP-activated protein kinase*, p38: *p38 mitogen-activated protein kinase*, TFEB: *Transcription factor EB*, NFAT: *Nuclear factor of activated T-cells*, PGC-1α: *Peroxisome proliferator-activated receptor gamma co-activator-1a*, p53: *Tumor protein 53*, TFs: *Transcription factors*, NUGEMPs: *Nuclear genes encoding mitochondrial proteins*, OXPHOS: *Oxidative phosphorylation*, TCA cycle: *Tricarboxylic acid cycle*, TFAM: *Mitochondrial transcription factor A*, mtDNA: *Mitochondrial DNA*, mitoPS: *Mitochondrial protein synthesis*, mitoPB: *Mitochondrial protein breakdown*.

3. High-intensity exercise and mitochondrial biogenesis

a) *Defining high-intensity exercise*

While others have attempted to provide standardized definitions of endurance exercise intensity (133), a consensus has not been reached. For the purpose of this review, we have defined high-intensity exercise as including intervals performed above 75% of the maximal power ($\dot{W}_{max}$) achieved during an 8- to 12-minute graded exercise test (GXT)[1]. Consequently, this includes both high-intensity interval exercise and training (HIIE and HIIT, respectively) and sprint-interval exercise and training (SIE and SIT, respectively). Moderate-intensity continuous exercise or training (MICE or MICT, respectively) has been defined as consisting of continuous exercise performed at an intensity below 75% $\dot{W}_{max}$ (Figure 4). We acknowledge this classification is imperfect, and, as we discuss later, this lack of consensus on how to define high-intensity exercise is a major hurdle to reconciling some of the conflicting findings in the literature.

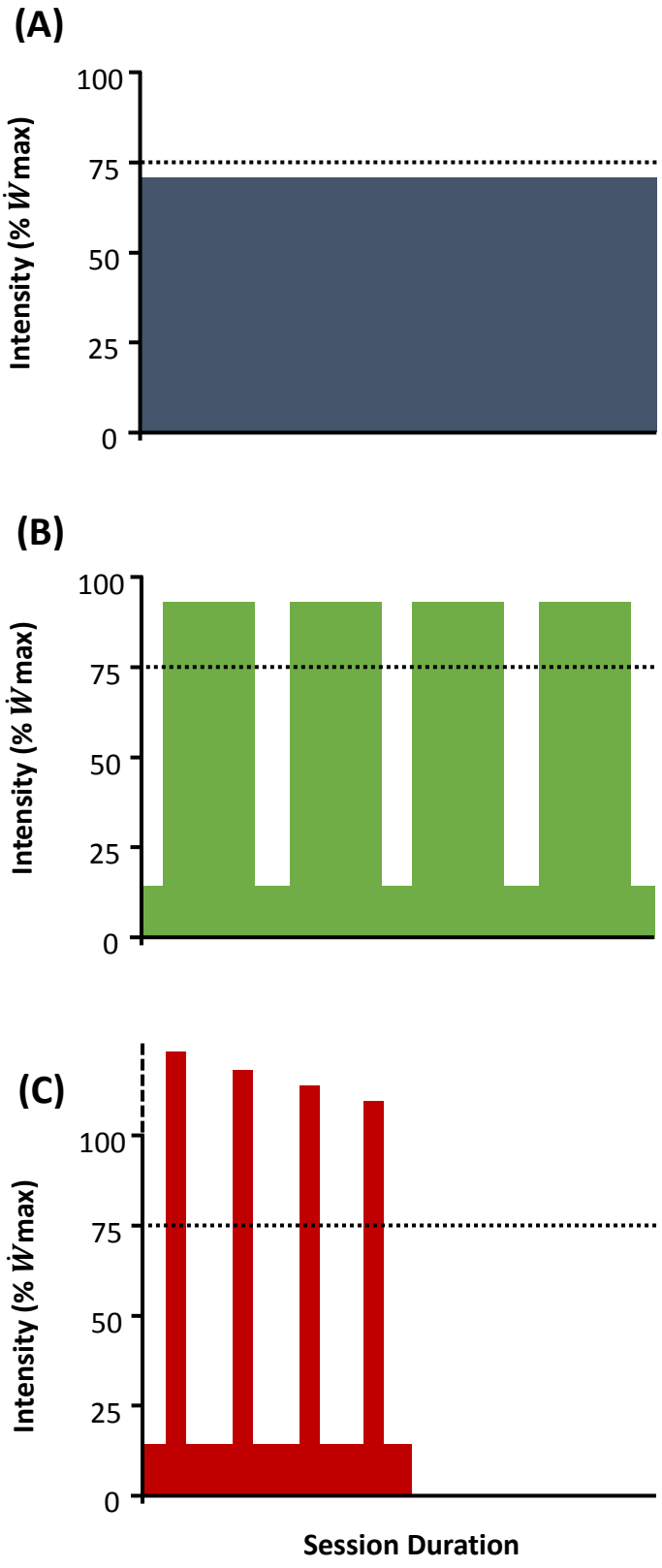

Figure 4. Schematic representation of the definitions used in this review to categorize (A) moderate-intensity continuous exercise or training (< 75% $\dot{W}_{max}$), and high-intensity exercise or training, which includes both (B) high-intensity, and (C) sprint-intervals (> 75% $\dot{W}_{max}$). Adapted from MacInnis & Gibala (75).

[1] Longer GXTs will underestimate $\dot{W}_{max}$ and the final power is typically referred to as peak power ($\dot{W}_{peak}$) (9).

b) Genes and proteins associated with mitochondrial biogenesis

The measurement of exercise-induced changes in genes and proteins is not sufficient by itself as a measurement of mitochondrial biogenesis (i.e., the synthesis of new mitochondrial components) (87); nonetheless, these changes can provide an indication that mitochondrial biogenesis has been activated. In this regard, many studies have reported exercise-induced changes in the mRNA or the sub-cellular localization of peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α, encoded by the PPARGC1A gene) (3, 21, 34, 42, 44, 73) - often described as the "master regulator" of mitochondrial biogenesis (67, 103, 104). However, while there is evidence that exercise-induced changes in both PGC-1α mRNA and protein are intensity-dependent (29, 44, 98), the initiation of mitochondrial biogenesis is complex and the assessment of exercise-induced changes in other genes and proteins is important to better explain some of the purported intensity-dependent effects of exercise on mitochondrial biogenesis (67, 71). Potential targets that warrant further investigation include p53 (44, 45), transcription factor EB (TFEB) (56, 79), nuclear factor of activated T-cells (NFAT) (18), nuclear respiratory factor (NRF-1) (3, 7, 67), and mitochondrial transcription factor A (Tfam) (67). However, to advance the field, researchers need to go beyond examining just a small fraction of the potential total number of genes and proteins that are altered by high-intensity exercise. The reduced costs and the increased accessibility and sensitivity of several "omics" techniques (e.g., transcriptomics, proteomics) provide an opportunity to obtain an in-depth map of all the genes and proteins associated with mitochondrial biogenesis that change in response to high-intensity exercise (5, 54, 72). More research is also needed to better understand how high-intensity exercise affects protein abundance in different sub-cellular locations (e.g., in the cytosol, the nucleus, and the mitochondria) and protein-protein interactions (e.g., using techniques such as quantitative LC-MS/MS and co-immunoprecipitation, respectively).

c) Mitochondrial protein synthesis

Even though it has been proposed that assessing mitoPS may best reflect exercise-induced mitochondrial biogenesis (87), few studies have directly assessed changes in mitoPS in response to high-intensity endurance exercise. In one of these studies, a single session of unilateral cycling (45 min, 75% $\dot{V}O_{2max}$) was reported to elicit similar increases in mitoPS 4 h post-exercise (~2.0 to 2.5-fold) in both untrained

and trained participants (136). High-intensity cycling has also been shown to induce greater mitoPS compared to higher volume (~1.5-fold greater following 10 x 1 min at ~86% $\dot{W}_{max}$ vs 30 min at ~59% $\dot{W}_{max}$) (6) or work-matched (~2.5-fold greater with 30 min at 60% $\dot{W}_{max}$ vs 60 min at 30% $\dot{W}_{max}$) (27) moderate-intensity cycling. Even though the "*high*" intensity protocol in this latter study was less intense than what is typically defined as high-intensity exercise, these studies indicate high-intensity exercise may provide a more potent stimulus to increase mitoPS than moderate-intensity exercise; however, further research is clearly required to resolve this controversy.

There are some methodological issues that likely contribute to existing conflicts. For example, changes in mitoPS are sometimes inferred from changes in protein synthesis in the sarcoplasmic fraction (*sarcoPS*) (6). This is based on the notion that during fractionation the majority of skeletal muscle mitochondria is retained in the sarcoplasmic fraction and changes in sarcoPS should indirectly reflect changes to mitoPS (a notion supported by unpublished data from our laboratory). However, while the sarcoplasmic fraction may be enriched with mitochondria, it will also contain other cellular components; thus, quantifying protein synthesis rates within this fraction is likely to provide only a crude estimate of mitoPS. Where tissue sample sizes permit, researchers should aim to directly measure mitoPS. Future research would also benefit from incorporating additional analyses to verify the purity of each analyzed sub-fraction.

The fractional protein synthesis rates of mixed muscle protein, and its sub-fractions, are typically quantified via the infusion of stable isotope-labelled amino acid tracers. However, while this technique has enabled researchers to unravel the complexities of protein synthesis, there are limitations to this approach (for a comprehensive critique, see (17, 83, 134)). These limitations include that measurements of protein synthesis are restricted to short durations, in laboratory-controlled settings, and often with feeding strategies that don't reflect real-world practices. An alternative technique, involving the oral administration of the stable isotope deuterium oxide (D_2O, "*heavy water*") (6, 17, 83, 134, 135), has been validated for assessing protein synthesis over extended time-frames and in free-living conditions; more research investigating exercise-induced changes in mitoPS with this method are warranted. Although methodologically challenging, exercise-induced changes in mitochondrial protein breakdown also contribute to the regulation of mitochondria and warrant further research.

d) Other methodological considerations

i. Biopsy timing

High-intensity exercise is a powerful stimulus affecting the content, location and/or activity of nuclear proteins, and genes encoding mitochondrial proteins, leading to an increase in mitoPS (5, 30, 44, 49, 54). However, these changes are transient and research has shown that the timing of muscle sampling is a critical methodological issue contributing to current controversies regarding the molecular response to exercise (8, 44, 45, 69, 73, 74, 82)（Figure 5）. Despite this, most studies continue to assess changes in proteins, genes, and mitoPS, only at convenient, and often arbitrary, time points. This has limited our current "understanding" of the molecular response to high-intensity exercise and, consequently, much of the knowledge in this area remains incomplete (and some is likely to be incorrect) (59, 60). Further research is required to establish a comprehensive time course for changes in the content, location and/or activity of proteins and genes, and mitoPS, in response to high-intensity exercise (and other types of exercise); this time course should also be investigated in different human populations (e.g., men, women, young, elderly). This fundamental issue needs to be resolved before addressing some of the more complex issues concerning the effects of high-intensity exercise on mitochondrial biogenesis.

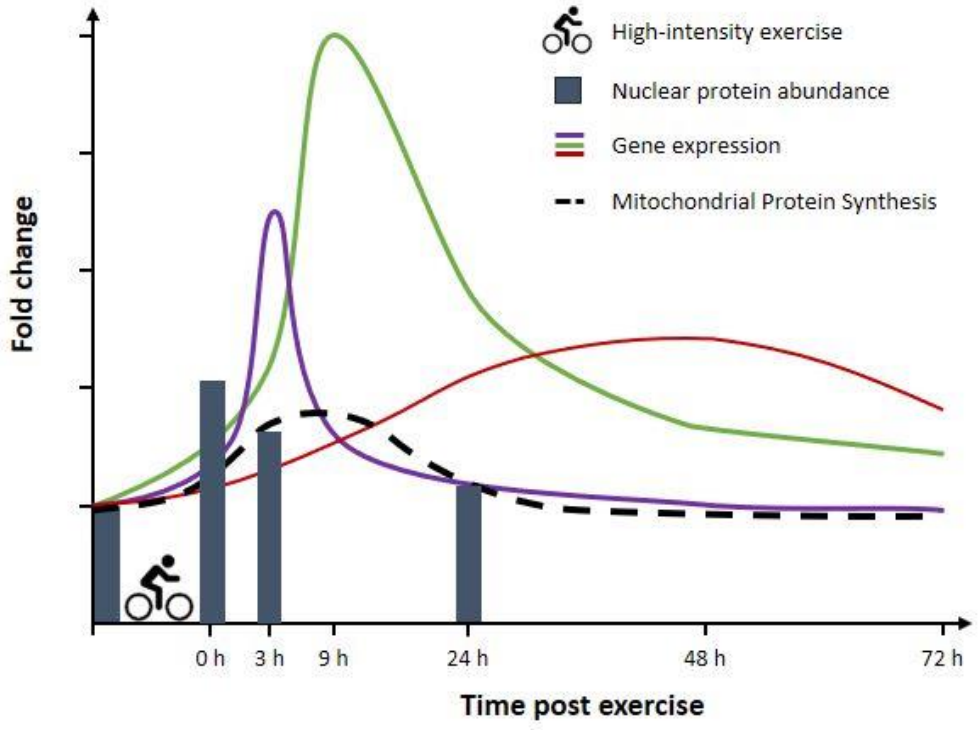

Figure 5. Schematic representation of the time course and magnitude of changes in the abundance of nuclear proteins (*blue bars*), the expression of different genes associated with mitochondrial biogenesis (*colored lines*), and mitochondrial protein synthesis (*dotted black line*) following high-intensity exercise.

ii. Fiber-specific effects

Most research has investigated exercise-induced changes in factors associated with mitochondrial biogenesis in whole-muscle samples. However, mammalian skeletal muscle is comprised of different fiber types (for a comprehensive review, please see (109)) and fiber-specific changes may contribute to some of the controversies concerning the effects of high-intensity exercise on mitochondrial biogenesis. The widely-known *size principle* states that slow-twitch fibers (type I) have a lower threshold of activation and will be utilized at lower exercise intensities, while fast-twitch fibers (type IIa and IIx) have a higher threshold and will be increasingly activated as exercise intensity increases (26, 50) (Figure 6). Based on the size principle, it has been hypothesized that different exercise intensities may induce fiber-specific changes (68). This hypothesis is supported by the observation that most measured proteins were equally affected in type I and II fibers following moderate-intensity exercise (70% $\dot{W}_{max}$ for 30 min), while there was a fiber-specific regulation of some proteins (e.g., AMPK phosphorylation) following high-intensity exercise (6 x 1.5 min at ~ 100% $\dot{W}_{max}$ with 2.5 min of active rest between bouts) (68). However, two other studies were not able to detect any significant difference in exercise-induced glycogen utilization or cell signaling (PGC-1α and PDK4 gene expression) between muscle fiber types after moderate-intensity (30 to 90 min at 60 to 65% $\dot{W}_{max}$) and high-intensity exercise (8 x [20 s at 170% of $\dot{W}_{max}$:10 s of rest] or 180 x [12 s at 150% of $\dot{W}_{max}$:18 s at 40% of $\dot{W}_{max}$]) (110, 130). In one of these studies, this was attributed to the absence of fiber-specific changes in muscle glycogen content between the two exercise conditions (110). Further research is required to clarify the role of relative exercise intensity on fiber recruitment and fiber-specific muscle glycogen depletion, and to investigate if fiber-specific changes in mitochondrial biogenesis contribute to some of the purported benefits of high-intensity exercise compared with other types of exercise.

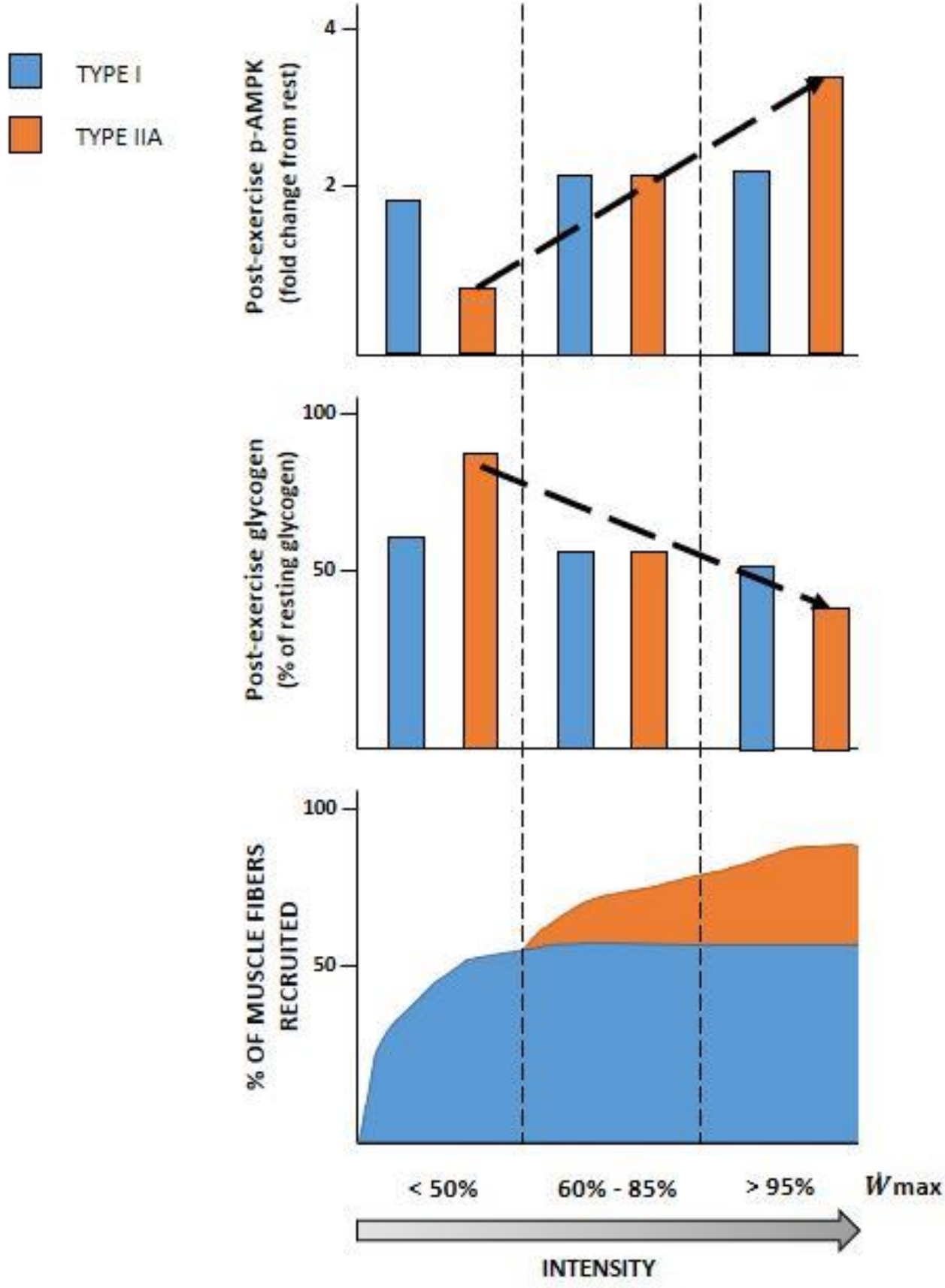

Figure 6. Illustration of the size principle and how it may influence fiber-type-specific glycogen depletion (37-39, 68, 121) and cell signaling (68) in response to different exercise intensities.

iii. Influence of sex

Even though there are roughly as many women in the world as men, women are notably under-represented as participants in the exercise-science literature (women represent less than 40% of the participants in published studies (23)). Most of our understanding of mitochondrial biogenesis in response to high-intensity exercise has consequently been gained using men, with the ensuing results assumed to be similar for women. However, women have been reported to have fiber-type differences to men, with a greater type I and a lower type II cross-sectional area (78). Additionally, women oxidize more lipids, and less carbohydrates and proteins, compared with men during endurance exercise (123). While these differences are unlikely to be due to differences in testosterone levels (132), 17-β estradiol potentially plays a role and may also influence the function of mitochondria (32, 77, 88). It has therefore been hypothesized that there may be sex differences associated with exercise-induced mitochondrial biogenesis (92, 105), although few studies have directly assessed this.

At rest men and women have a similar abundance of mitochondrial proteins, including those involved in the transcriptional regulation of mitochondrial biogenesis (88). It has also been reported that exercise-induced phosphorylation of AMPK, ERK1/2, and p38 MAPK was not different between men and women (33). Consistent with this, a similar expression of genes associated with mitochondrial biogenesis has been observed following a single session of high-intensity exercise (three 30-s "all-out" cycling bouts) in men and women (118). However, one study has reported that sex differences for exercise-induced changes in PGC-1α gene expression depended on the menstrual cycle phase (32). The question of whether there are sex differences for changes in protein abundance, gene expression, and mitoPS, in response to high-intensity exercise (and other types of exercise) warrants further research.

An unresolved methodological issue that may be contributing to existing conflicts, is how best to match men and women. It has been suggested that matching on the basis of $\dot{V}O_{2max}$ per kilogram of fat-free mass is the most appropriate way to match men and women (118, 123). However, women typically have lower aerobic power, less muscular strength, lighter body mass (116), lower power output during "all-out" (sprint) exercise (35, 108), and greater fat metabolism during sub-maximal exercise (123). Thus, more research is also required to establish how best to match the metabolic and mechanical stress of exercise between men and women. This issue is discussed further in the next section.

iv. Relative exercise intensity and the 'first bout' effect

When investigating the effects of high-intensity exercise on mitochondrial biogenesis, an important methodological issue to considere is the reference point used to calculate relative exercise intensity (Figure 7a). We have previously observed that the same relative exercise intensity (expressed as a percent of $\dot{W}_{max}$ or $\dot{W}_{peak}$) can differ by more than 150 W depending on the stage duration of the GXT (41). Thus, caution is required when comparing the exercise-induced changes in protein abundance, gene expression, or mitoPS, reported in different studies when exercise intensities are based on values determined from different GXT protocols. It has been suggested that if the aim is to compare exercise-induced changes in individuals under similar physiological conditions, then exercise intensity should not be determined relative to $\dot{W}_{peak}$ (or $\dot{W}_{max}$) but should be calculated relative to the LT (4). These authors observed that markers of metabolic stress were greater in untrained compared with trained participants when exercise was performed at 70% $\dot{W}_{peak}$, but were similar during exercise performed at 95% LT. Consistent with this, when cycling at the same percentage of $\dot{W}_{peak}$ glycogen utilization is greater in trained individuals with a similar $\dot{W}_{peak}$ but a lower LT (25). Exercise-induced changes in mitochondrial biogenesis in response to high-intensity exercise may therefore be more strongly associated with exercise intensity expressed relative to the LT than relative to $\dot{W}_{peak}$ or $\dot{W}_{max}$. The observation that the LT is dependent on both the GXT protocol and the calculation method further complicates this issue (10, 11, 66). How the determination of relative intensity influences factors associated with exercise-induced mitochondrial biogenesis has not been adequately studied; this issue needs to be addressed to resolve existing conflicts about the effects of different exercise intensities, in different populations, on mitochondrial biogenesis.

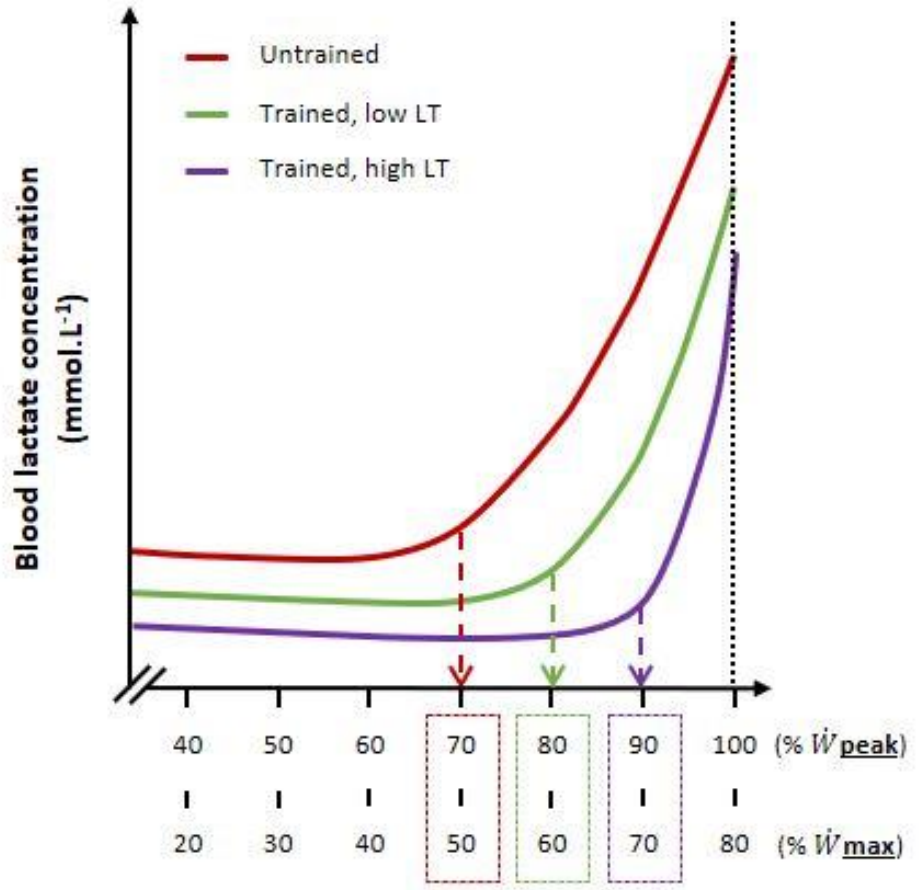

Figure 7a. Schematic representation of a typical lactate curve from an untrained individual (red), and trained individuals with either a low (green) or a high (purple) lactate threshold (LT), respectively. Exercise intensity can be determined relative to the LT (dashed arrows), the maximal power ($\dot{W}_{max}$) achieved during a graded exercise test (GXT) that consists of short (e.g., 1-minute) stages of progressive increases in power (or velocity for running and swimming protocols), or peak power ($\dot{W}_{peak}$) achieved during a GXT that consists of longer (e.g., 4-minute) stages of progressive increases in power or velocity (22, 97, 139). This schematic highlights how the relative exercise intensity depends on the GXT design, the anchor point chosen, and the training status. Note that in this example, the lactate threshold could range from a value of 50 to 90% depending on whether it is expressed relative to $\dot{W}_{max}$ or $\dot{W}_{peak}$ and depending on training status.

A second important methodological issue is the impact of recruiting individuals who are naïve to performing high-intensity exercise (Figure 7b). It has been reported that the transcriptional response after the first session of resistance exercise is reflective of muscle damage and differs substantially from a second resistance exercise session performed 48 h later (95). Research has also shown that increases in PGC-1α mRNA in response to high-intensity exercise are reduced with every subsequent session, even when the exercise intensity is maintained (100). Similarly, rates of MPS have been reported to decrease by ~ 40% in response to the same resistance session performed every second day during an eight-day resistance training period (135). While it has not been investigated, exercise-induced changes in mitoPS are probably also similarly decreased when high-intensity exercise is repeated. These observations suggest that changes in proteins, genes, and mitoPS, in individuals who are naïve to performing high-intensity exercise may represent an atypical response. Familiarizing participants to the high-intensity exercise to be performed, before conducting the experimental (i.e., biopsy) trials, may help to resolve some of the conflicting findings reported in the literature.

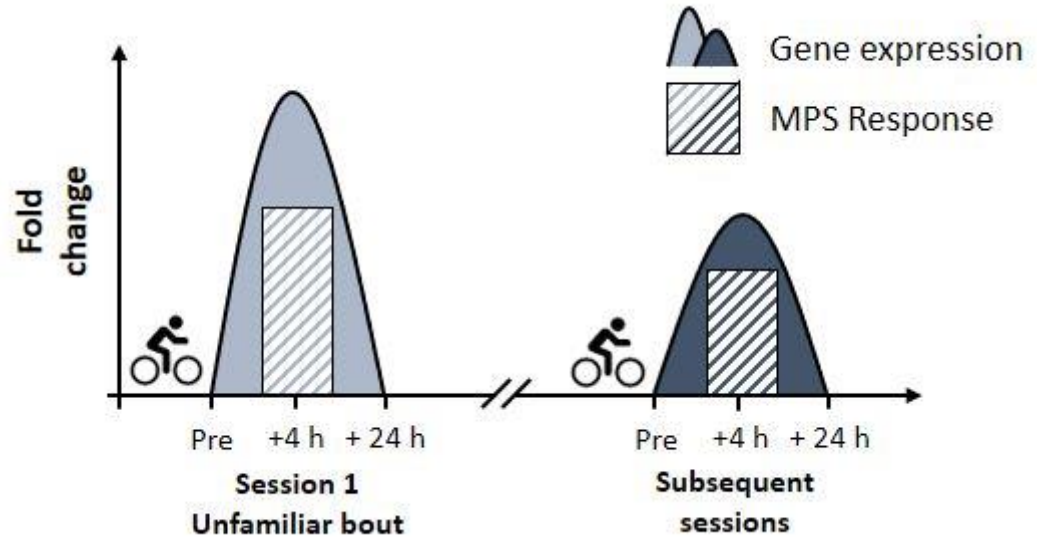

Figure 7b. Schematic representation depicting how the changes in gene expression (*curved lines*) and protein synthesis (*hatched boxes*) following an initial bout of high-intensity exercise diminish in response to subsequent sessions of the relative same stimulus.

4. Outcomes of mitochondrial biogenesis

a) *Mitochondrial content versus respiratory function*

If mitochondrial biogenesis is defined as "the making of new components of the mitochondrial reticulum", it follows that repeated exercise sessions (i.e., exercise training) should lead to increases in mitochondrial content and/or respiratory function (and possibly other changes, such as increased cristae density (96) or supercomplex assembly (46)). However, although it is sometimes assumed that mitochondrial content and respiratory function increase in parallel (62), it is clear that this is not always the case (42, 43). Training-induced changes in mitochondrial respiratory function have been reported without concomitant changes in mitochondrial content (45, 63) and changes in mitochondrial content are not always accompanied by an increase in mitochondrial respiratory function (90, 139). It has subsequently been suggested that training-induced changes in mitochondrial content and respiratory function may be regulated by different types of exercise, which highlights the need to assess both when conducting training studies (42).

b) *Mitochondrial Content*

Since the pioneering work of John Holloszy in the 1960's, it has been known that exercise training can increase mitochondrial content (as assessed by total protein content of the mitochondria) (84). Subsequent research using transmission electron microscopy (TEM), considered the 'gold standard' for the measurement of mitochondrial content (102), has confirmed these results in humans (40, 52, 57, 81, 90, 91, 106, 113, 124, 126). However, while TEM can provide measures of mitochondrial content, and also size and shape, it requires specialized equipment and expertise not available in all laboratories. Therefore, biochemical measurements are often used as an indirect measure of mitochondrial content. The most widely-used biomarker for mitochondrial content in skeletal muscle is citrate synthase (CS) activity, and a strong correlation has been reported between resting CS activity and resting mitochondrial content - as measured by TEM (70). Given that many studies have assessed training-induced changes in CS activity, we recently pooled the results of the published research and concluded that training volume is an important determinant of changes in mitochondrial content (42). However, others have reported that high-intensity training (i.e., sprint interval training) increases mitochondrial content to a similar extent to MICT (76), despite a reduced exercise volume, and further research is required to resolve these conflicting conclusions.

Despite the wide-spread use of CS activity as an indirect biomarker for changes in mitochondrial content, there are some limitations with this approach. One limitation is that it is difficult to compare values between studies, as different laboratories use different methods and different units of measurement (e.g., μmol · min^{-1} (13, 125), μmol·min^{-1}·g of $tissue^{-1}$ (70), μmol·min^{-1}·g $protein^{-1}$ (137), μmol·min^{-1}·μg $protein^{-1}$ (31), or mIU· mg $protein^{-1}$ (85)). Even when reported in the same units, CS activity values can be very different between studies (sometimes by a factor of 10^3 (128)). Another unresolved issue is how well training-induced changes in CS activity correlate with training-induced changes in mitochondrial content (85). The field would benefit from the adoption of a standard analysis method and reporting unit for CS activity, as well as studies to determine whether training-induced changes in CS activity provide a valid estimate of changes in mitochondrial content.

c) Mitochondrial Respiratory *Function*

Exercise training can be a potent stimulus to improve mitochondrial respiratory function (76). In a recent review, it was reported that exercising at higher intensities provides a greater stimulus to increase mitochondrial respiratory function (42). However, few studies have directly compared the effects of training at different exercise intensities and more research is required.

The assessment of skeletal muscle mitochondrial respiratory function is typically conducted on either isolated mitochondria or permeabilized muscle fibers obtained from muscle biopsy samples. The use of permeabilized muscle fibers is considered to be the 'gold standard', as it allows for the evaluation of individual complexes of the electron transport system in response to the addition of different metabolic substrates *ex vivo* (101). However, mitochondrial respiration measured with this technique is quite variable (coefficient of variation [CV] of 15.2% (20), a value consistent with our unpublished CV of 13%), which raises questions about the ability of this technique to detect meaningful differences in response to different types of training (85). It is likely that this variability has contributed to some of the conflicting findings reported in the literature, and more attention needs to be directed towards ways to improving the reliability of this technique.

Further research is also required to establish the physiological relevance of training-induced changes in mitochondrial respiratory function. Supra-physiological substrate concentrations are often used with this technique, and it is not clear how representative these are of changes to *in vivo* mitochondrial respiration. It has also been reported that mitochondrial respiratory function exceeds oxygen delivery during maximal exercise (15), which raises further questions about the physiological relevance of training-induced changes in maximal, ADP-stimulated mitochondrial respiration. The use of sub-maximal substrate concentrations and substrate titrations, as is sometimes carried out with ADP, may provide more physiologically-relevant results. An increase in ADP-sensitivity, as opposed to an increase in maximal oxidative capacity, is thought to provide a more relevant indicator of training-induced improvements in the mitochondria (88, 119). Further research is required to establish which, if any, measurements of mitochondrial respiratory function are most important for human health and/or performance.

d) Other considerations

i. How best to organize training to promote mitochondrial adaptations?

Most of the research to date on mitochondrial adaptations to training has assigned groups to one type of training – either MICT, HIIT, or SIT. However, this mode of training contrasts with that of endurance athletes who typically distribute their training across various training zones (112). Some of the largest increases in aerobic fitness (53) and mitochondrial respiration (for review see (42)) have been reported following a combination of moderate-intensity and high-intensity exercise training. There is also evidence that training twice every day (24) or twice every second day (47, 138) may be superior to daily training to increase CS activity. There is, therefore, a need to move beyond studies that investigate the effects of just one type of training (typically repeated 3 times/week) and to investigate how best to distribute different types of training across days, weeks, and months, to optimize mitochondrial adaptations to training.

ii. Longer-duration and time-course studies

The results of short-duration (< 10 weeks) training studies suggest that high-intensity exercise training is more effective to increase mitochondrial respiratory function than moderate-intensity training (even when training volume is matched) (42). However, the duration of most of these studies was only 4 to 6 weeks and the maximum duration of any published study is only 10 weeks. There is a need to investigate the effects of longer periods of high-intensity training on mitochondrial adaptations, and to also determine if the greater increases in mitochondrial respiration in response to high-intensity compared with moderate-intensity exercise training are maintained when training is continued beyond 10 weeks. It is also important to better understand the time course of mitochondrial adaptations to exercise training. Only two weeks of high-intensity training has been reported to increase mitochondrial respiration by 22% in healthy, untrained males (76); if this rate of change was to continue, only three to four months would be required to achieve values recorded by well-trained to elite athletes (63, 86). It seems more likely the rate of increase in mitochondrial respiratory function slows as training duration lengthens, but time-course studies (i.e., multiple biopsies at different times during a training program) are required to confirm this hypothesis.

iii. Influence of fiber type?

Research investigating the effects of training on fiber-specific mitochondrial adaptations is scarce. Henriksson and Reitman (51) first explored this issue, reporting that 7 to 8 weeks of high-intensity training (5 x 4 min at 100% $\dot{W}_{max}$ with 2 min rest between bouts) increased succinate dehydrogenase activity (SDH) in type II fibers, whereas there was an increase in SDH activity in type I fibers following 7 to 8 weeks of continuous training (27 min at 80% of $\dot{W}_{max}$). In another study, 6 weeks of moderate-intensity training (30 min at 72% $\dot{W}_{max}$) resulted in a similar increase in mitochondrial volume density in both type I and II fibers (57). Two of the participants from this study continued training for 24 weeks and showed further increases of mitochondrial volume density in type I but not type II fibers. More recent studies have not observed fiber-type-specific mitochondrial adaptations following either moderate- or high-intensity endurance training (76, 110, 122). The differences between exercise volume and intensity among studies make it difficult to rule out differences in fiber-type adaptations following endurance training. More

research is needed to determine the effects of training, and the role of relative exercise intensity, on fiber-specific, training-induced, mitochondrial adaptations. It is also important to move beyond the measurement of just a few targeted proteins and to take advantage of recent advances in single muscle fiber proteomics techniques (93, 94) to measure training-induced changes in hundreds to thousands of proteins in single muscle fibers.

iv. Sex differences

Few studies have investigated possible sex-specific mitochondrial adaptations to training and the studies published to date have produced conflicting results. Men and women have been reported to have similar increases in mitochondrial area and CS activity in response to moderate-intensity training (124), and in CS activity (35, 108, 124) following high-intensity exercise training. However, increases in β-hydroxy acyl CoA dehydrogenase (β-HAD) activity were only observed in men after training, although the higher basal levels of women in this study may have contributed to this finding (35). Another study reported that women had greater COX IV protein content at both baseline and after three weeks of SIT, while muscle protein synthesis was higher in men in comparison to women (108). More research is clearly required to clarify if there are sex differences for training-induced mitochondrial adaptations.

v. Mitochondrial Memory?

There is emerging evidence of what has been termed "skeletal muscle memory" – i.e., the ability of skeletal muscle to respond differently to an environmental stimulus (e.g., exercise) if the stimulus has previously been encountered (115). For example, healthy men had a significantly greater increase in lower-limb lean mass following seven weeks of resistance training (3 d/wk) if they had previously (7 weeks earlier) completed a similar resistance training program (111). It was further suggested the mechanistic underpinnings of this muscle memory could be related to epigenetics – modifications of gene expression as a result of structural modifications of DNA, without altering the underlying DNA sequence (for an extensive review of epigenetic mechanisms in muscle, please refer to (81)). One important example of an epigenetic modification is DNA methylation, which usually leads to the suppression of gene expression (48). Conversely, hypomethylation (reduced DNA methylation) generally leads to enhanced gene expression (14). Both a single session of aerobic exercise and regular aerobic exercise training have been reported to decrease the DNA methylation of genes associated with mitochondrial

biogenesis (e.g., PGC-1α) (129). This suggests there might be an epigenetic memory of previous exercise-induced mitochondrial biogenesis, and that training-induced mitochondrial adaptations may be enhanced in individuals who have previously performed aerobic exercise training. However, in the only study to date, increases in CS activity after three months of moderate-intensity endurance training (45 min, 4d/wk) were similar to the changes observed when the same participants previously completed (9 months earlier) the same endurance training program (72). Further research is required to investigate if there is a skeletal muscle memory for other mitochondrial adaptations and to also determine how long any changes in methylation are retained. It is also interesting to note that one study reported that only high-intensity (80% $\dot{W}_{peak}$), and not low-intensity (40% $\dot{W}_{peak}$), was able to alter the methylation of some of the investigated genes (5). Further research is therefore required to investigate the effects of different types of exercise on epigenetic modifications and whether high-intensity exercise is associated with a greater or longer epigenetic memory of training-induced mitochondrial adaptations than lower-intensity exercise.

5. Conclusions

In the last 50 years, there has been much research, and improved understanding, of mitochondrial adaptations to training. Despite this, there are many conflicting findings in the literature and a consensus has not been reached regarding the role of high-intensity exercise to promote mitochondrial biogenesis. This often leads to calls for more research and the use of more advanced methodologies (as we have done). However, in this review we have also highlighted that many fundamental questions require attention before we can inch closer to solving some of the more complex issues. For example, how should we define mitochondrial biogenesis, when should we take muscle biopsies to best capture the molecular events associated with mitochondrial biogenesis, and which mitochondrial characteristics are most important for human health and/or performance? There is also a pressing need for a consensus on how to define high-intensity exercise (especially in terms of the most physiologically-relevant anchor points). Finally, although space restrictions did not allow us to discuss the influence of nutrition (e.g., carbohydrate and protein ingestion) (2) and training manipulations (e.g., hypoxia, blood-flow restriction etc.) (16, 21, 65) on mitochondrial adaptations to high-intensity exercise, more research is also required on these aspects.

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