REVIEW ARTICLE

Unravelling the mechanisms regulating muscle mitochondrial biogenesis

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Skeletal muscle is a tissue with a low mitochondrial content under basal conditions, but it is responsive to acute increases in contractile activity patterns (i.e. exercise) which initiate the signalling of a compensatory response, leading to the biogenesis of mitochondria and improved organelle function. Exercise also promotes the degradation of poorly functioning mitochondria (i.e. mitophagy), thereby accelerating mitochondrial turnover, and preserving a pool of healthy organelles. In contrast, muscle disuse, as well as the aging process, are associated with reduced mitochondrial quality and quantity in muscle. This has strong negative implications for whole-body metabolic health and the preservation of muscle mass. A number of traditional, as well as novel regulatory pathways exist in muscle that control both biogenesis and mitophagy. Interestingly, although the ablation of single regulatory transcription factors within these pathways often leads to a reduction in the basal mitochondrial content of muscle, this can invariably be overcome with exercise, signifying that exercise activates a multitude of pathways which can respond to restore mitochondrial health. This knowledge, along with growing realization that pharmacological agents can also promote mitochondrial health independently of exercise, leads to an optimistic outlook in which the maintenance of mitochondrial and whole-body metabolic health can be achieved by taking advantage of the broad benefits of exercise, along with the potential specificity of drug action.

Key words: aging, calcium signalling, exercise, exercise training, mitochondrial protein import, mitochondrial reticulum, mitophagy, mtDNA, muscle disuse, p53, PPARγ co-activator-1α (PGC-1α), reactive oxygen species, Tfam, Tfeb.

INTRODUCTION

The mitochondrial content of any tissue is one of the most highly variable phenotypic features of a cell type. In skeletal muscle, mitochondrial content depends on the fibre type, and their recruitment patterns during contractile activity. Although species differences exist, in human muscle mitochondria tend to be abundant in slow-twitch, type I fibres that are readily recruited during posture and locomotion, whereas the concentration of mitochondria is considerably less in fast-twitch, type II fibres that are only used periodically for explosive movements. Based on this, it stands to reason that the cellular energy demand must be an important determinant in the regulation of mitochondrial content. This is certainly why the heart, which beats continuously, has the highest organelle content of any tissue (30–35 % of the cell by volume), whereas fast-twitch white fibres, which only contract very infrequently, have a very low (2–3 %) mitochondrial concentration [1]. A consistent change in energy demand is the likely cause of why a previously non-exercised muscle, which undergoes a period of active contractile activity in the form of regular exercise, increases its mitochondrial content to support the increase in energy demand. Although the increase in mitochondrial content following exercise training is a well-documented phenomenon, the specific signalling pathways leading from changes in cellular energy demand to an increase in organelle content still remain to be completely defined. Having this knowledge in hand would be valuable from clinical, pharmacological and therapeutic perspectives, because it would allow for the targeting of specific signalling pathways, which could serve to augment mitochondrial content and/or function. Decrements in volume and function in various tissues are frequently observed in multiple clinically-relevant conditions, including type 2 diabetes, obesity, neurodegenerative disorders, along with aging and immobilization [2–6]. Thus, research designed to investigate how to best ameliorate organelle content and function is warranted, and recognized to be a highly fundable pursuit.

The steady-state content of a molecule is the product of both the rate of synthesis and the degree of breakdown. Naturally, this concept holds true for organelles as well. Thus, steady-state mitochondrial content is a net result of organelle biogenesis (synthesis) and mitophagy (breakdown). Considerable effort has been devoted to the understanding of biogenesis over the last three decades. Indeed, molecules have been identified which appear critical for the maintenance of organelle content in a tissue-specific manner, such as peroxisome proliferator-activated receptor γ (PPARγ) co-activator-1 (PGC-1) family members, for example. On the other hand, our knowledge of the regulation of mitophagy in the context of cellular energetic disturbances remains in its infancy. However, as discussed below, a greater knowledge of transcription factor EB (TFEB), the protein widely considered to be the most important regulator of autophagy and lysosomal biogenesis, would shed considerable light on the regulation of mitophagy in various cell types.

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMPK, AMP-activated protein kinase; COX, cytochrome *c* oxidase; CS, citrate synthase; EM, electron microscopy; IMF, intermyofibrillar; LC3, light chain 3; MitoPS, mitochondrial protein synthesis; MTS, mitochondrial targeting sequences; NuGEMP, nuclear genes encoding mitochondrial protein; PGC-1, PPARγ co-activator-1; PIM, protein import machinery; PINK1, PTEN-induced putative kinase 1; PPARγ, peroxisome proliferator-activated receptor γ; ROS, reactive oxygen species; SDH, succinate dehydrogenase; SIRT1, sirtuin 1; SS, subsarcolemmal; TFAM, mitochondrial transcription factor A; TFEB, transcription factor EB; TIM, translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane; ULK1, unc-51-like kinase 1; VDAC1, voltage-dependent anion channel 1.

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In skeletal muscle, a number of proteins have been identified that appear to be important in maintaining basal levels of mitochondrial content and function. These include PGC-1α, p53, mechanistic target of rapamycin (mTOR) and sirtuin 1 (SIRT1). Genetic inactivation of the genes encoding these proteins leads to impaired mitochondrial respiration, and reduced basal mitochondrial concentration in muscle [7–10]. Interestingly, their absence becomes much less important during adaptations to chronically imposed muscle contractions in animal and cell culture models, as this treatment effectively reverses the functional and biochemical defects observed under basal conditions [7–10]. To date, the absence of a single protein does not appear to abrogate the beneficial effects that chronic muscle contractile activity, such as that achieved in an exercise training programme, can bring to bear on mitochondrial content and function. This appears to be mainly due to the ability of exercise to activate a number of overlapping intracellular signalling pathways towards mitochondrial biogenesis in skeletal muscle.

METHODS USED TO EXPLORE CHANGES IN MITOCHONDRIAL CONTENT AND FUNCTION

Numerous experimental models exist to modulate mitochondrial content physiologically both *in vitro* and *in vivo*. Exercise training, either of the endurance or intermittent high intensity variety, are classic and powerful tools to provoke skeletal mitochondrial biogenesis in skeletal muscle [11]. This can be achieved in human and rodent models using a variety of paradigms appropriate to the species being investigated, including voluntary wheel running, regimented treadmill running, cycling or swimming. Further, techniques involving chronic electrical stimulation-induced contractile activity have effectively evolved to simulate the mitochondrial adaptations in rodent muscle to an exercise training programme [12,13]. In addition, we [10,14–16] and others [17–20] have developed "exercise-in-a-dish" models in cell culture, using depolarizing electrical currents on skeletal muscle myotubes to effectively mimic the changes which occur in skeletal muscle with either acute or chronic exercise [10,14]. Pharmacological treatments have also been developed to promote mitochondrial biogenesis, the most common of which are highlighted below. On the other hand, reductions in mitochondrial content are observed in models of muscle inactivity, such as immobilization and denervation [3], as well as during chronological aging [5]. Regardless of the technique employed, models such as these have allowed for close examination of the changes in organelle quantity and quality that arise, and permit the identification of signalling mechanisms which control these alterations.

A number of time-honoured and more recently developed methods exist for the study of mitochondrial content and function. Organelle content can be evaluated most directly using fluorescence imaging or electron microscopy (EM). In cell culture, incubation of cells with fluorescent molecular probes such as MitoTracker green can provide a relative index of mitochondrial content when compared among treatments. cDNAs encoding mitochondrially-targeted proteins such as Mito-DsRed2 can be transfected into cells and are very useful tools to quantify mitochondrial morphology, movement dynamics and content within single cells [16,21,22]. Alternatively, isolated single muscle myofibres can be immunohistochemically stained *ex vivo* to identify mitochondrially-localized proteins, again providing valuable insight into the shape of the mitochondrial network [23]. EM is better suited to the detailed analysis of fixed tissues, as it permits observations on the subdivisions of mitochondria that exist in muscle. These pools of mitochondria are named after their distinct geographical localization within muscle, such as the subsarcolemmal (SS) or intermyofibrillar (IMF) organelles [24,25] which reside close to the plasma membrane, or between the myofibrils respectively. Recent work by Picard et al. [26] and others [27], using both scanning and transmission EM techniques, has advanced this methodology to allow snapshot visualizations of mitochondrial morphology and connectivity in three dimensions.

For many years, organelle content has been approximated by determining the activity of enzymes involved in mitochondrial oxidative metabolism, such as succinate dehydrogenase (SDH), cytochrome *c* oxidase (COX) or citrate synthase (CS). As enzymes within same metabolic pathway are in 'constant proportion' with each other [28], these enzymes are often also employed as dependable indices of mitochondrial content. Naturally, because of the independent turnover of individual proteins, single protein markers are generally considered to be approximations of mitochondrial content. CS and SDH are nuclear-encoded enzymes that form part of Krebs' cycle and Complex II, respectively, whereas COX is a holoenzyme composed of subunits derived from both the nuclear and mitochondrial genomes. Thus, COX activity may be more representative of the coordinated stoichiometric expression of the two genomes required for holoenzyme function. The use of these marker enzymes, or of subunits therein, often parallels morphometric estimates of mitochondrial volume derived using EM [29], and they provide the advantage of being less time-consuming measures, with less subjective involvement.

mtDNA content has often been used as a surrogate marker of organelle content as well, however its capacity to replicate autonomously suggests that linearity between changes in tissue oxidative capacity and mtDNA content should be assessed, as done previously [30]. Another marker which has gained some usage is the inner-membrane phospholipid cardiolipin [31]. Localized uniquely in mitochondria, this phospholipid can be measured using methods such as thin-layer chromatography or high performance liquid chromatography. Previous work has shown it to be a sensitive indicator of membrane biogenesis in muscle under conditions of chronic use [32] or disuse [33,34].

Mitochondrial protein synthesis (MitoPS) is another measurement of organelle biogenesis that can be utilized. Mitochondria manufacture 13 mtDNA gene products vital for electron transport chain function. MitoPS can be measured by assessing the extent and rate of incorporation of radiolabelled amino acids into protein in isolated mitochondria [35,36]. This method has been used to shed light on the effect of contractile activity, as both acute exercise and chronic training appear to augment overall MitoPS [37,38], whereas MitoPS in specific mitochondrial subfractions appears to be both time- and stimulus-sensitive [36]. Recent studies have also illustrated the utility of using heavy water (^2H_2O) labelling to measure MitoPS [39]. This method will certainly be valuable for improving our understanding of the mitochondrial translation system, the study of which has been underdeveloped in mammalian cells and in whole-body experimental models.

In addition to measuring mitochondrial biogenesis, determining changes in mitochondrial function can reveal qualitative alterations within the organelle that are not observed when their quantity is being assessed. Mitochondrial function has historically been documented using organelles isolated by differential centrifugation. Functional measures usually include indicators of basal and active respiration, reactive oxygen species (ROS) emission, apoptotic protein release and/or calcium uptake. Isolated organelles are also useful for understanding processes of mitophagy, as described below. There is no doubt that the removal of any organelle from its native cellular environment

disrupts its *in vivo* morphology, and thus runs the risk of producing organelle damage. In the case of mitochondria, several quality control checks can be used to verify the intactness and quality of the organelles [40]. Further, comparisons among treatments (e.g. trained and untrained muscle) are usually valid if the same isolation procedure has been used for both. A popular method emerging in the literature is the use of permeabilized muscle fibres in respirometry measurements. The main advantages are that the technique is very sensitive, requiring only a few milligrams of tissue, and there is little chance of damaging the mitochondria in the preparation of the tissue for respirometry. A more complete review of the literature in this area has been summarized by Perry et al. [41].

In contrast with biogenesis, mitochondrial degradation can be assessed most simply by the decline in the organelle markers described above, expressed per gram of tissue weight. Alterations in mitochondrial composition can also be documented by making the same enzyme or protein measurements on isolated, purified organelles. The specific degradation of mitochondria, termed mitophagy, requires more sophisticated analyses, including the localization of autophagy adapter proteins, such as the lipidated form of microtubule-associated protein light chain 3 (LC3-II) or p62, on mitochondrial subfractions. Typically these measures are best accompanied by immunofluorescence measures in single fibres in which autophagosomes can be visualized. Co-staining of GFP-tagged LC3 with MitoTracker can reveal the abundance of mitochondria associated with autophagosomes. Because mitophagy is a dynamic process, flux measures are required for a full interpretation of the data. This is quantified in animal models using prior treatment with microtubule or lysosomal inhibitors, such as colchicine or chloroquine. An extensive review of the methodology associated with autophagy and mitophagy can be found elsewhere [42].

In summary, mitochondrial content and turnover in muscle can be assessed using a variety of well-established, as well as more recently developed techniques. To have the greatest confidence in these methods, attempts should be made to use them in combination. Although they need not all be employed, the complexity of the organelle, including its derivation from two distinct genomes, as well as its unique phospholipid and protein composition, suggests that multiple approaches will provide the most easily interpretable results.

PGC-1 FAMILY OF TRANSCRIPTIONAL CO-ACTIVATORS

Transcriptional co-activators respond to cellular signals to enhance the production of target transcripts through binding with transcription factors or nuclear receptors, but not to DNA directly. The PGC-1 family is made up of three members, PGC-1α, PGC-1β and PGC-related co-activator (PRC). The mechanism through which PGC-1 family members up-regulate gene transcription is through docking with transcription factors through the known LXXLL motif, followed by the subsequent recruitment of additional proteins that can modify the DNA, such as p300, steroid receptor co-activator-1 (SRC-1) and the initiator complex TRAP/Mediator to promote transcription [43,44]. Interestingly, although each family member is capable of inducing a unique transcriptional profile, they are all involved in the co-activation of nuclear genes encoding mitochondrial proteins (NuGEMPs) to regulate mitochondrial content and function [45,46].

Of all the family members, PGC-1α has been studied most intensively and has often been regarded as the 'master regulator' of mitochondrial biogenesis. It was initially cloned from a two-hybrid screen of brown fat cells as a co-activator for PPARγ [47]. PGC-1α levels were found to robustly increase in response to cold exposure in both brown fat and skeletal muscle, concomitant with a rise in numerous mitochondrial markers. Both PGC-1α and PGC-1β are expressed in tissues with high mitochondrial activity, including skeletal muscle, and are particularly enriched in oxidative fibres. Muscle-specific overexpression of PGC-1α increases the transcription of a wide number of oxidative phosphorylation genes, and substantially augments muscle mitochondrial content [48]. In contrast, whole-body deletion or *in vitro* silencing of either PGC-1α or PGC-1β reduces skeletal muscle mitochondrial content and function as well as NuGEMP expression [16,49–51]. Investigations using muscle-specific knockout (KO) models have also revealed that the ablation of PGC-1α led to reductions in mitochondrial content and a fibre type shift from slow type I/IIa myosin isoforms to fast IIx/IIb [52]. Intriguingly, adult-inducible PGC-1β muscle KO animals exhibited no change in overall muscle mitochondrial content, but they did display abnormal structure and function of the organelles, along with reduced exercise capacity [53]. It has been suggested that with the deletion of only one co-activator, there may be compensation by the remaining family member. However, no changes in PGC-1β were noted in PGC-1α KO animals [54], or after PGC-1α knockdown in muscle cells in culture [16], suggesting that no compensation takes place with PGC-1β when PGC-1α is absent. Further investigation using dual KO strategies have been employed for a deeper understanding of the molecular mechanisms through which these co-activators combine to regulate mitochondrial content and function in skeletal muscle. One model used a PGC-1α whole-body KO crossed with a muscle-specific deletion of PGC-1β [55], whereas the other model used mice floxed for both PGC-1α and PGC-1β which were ablated in muscle with Cre-recombinase driven by a Myogenin-MEF2 promoter [56]. As expected, these genetic models produced animals with lower mitochondrial markers and impaired function, along with reduced exercise performance, implicating PGC-1 (mainly PGC-1α) as a logical target for pharmaceutical manipulations to modify organelle content/function in muscle.

It is well established that exercise can stimulate the expression of PGC-1α, as muscle contractile activity *in vivo* or *in vitro* increases the transcription and expression of PGC-1α [57,58], an effect which is believed to occur through a number of signalling pathways (Figure 1) that are responsive to changes in the intracellular environment [59]. Numerous studies have replicated this finding at the mRNA and protein level. The basis for this increase may be transcriptional, since a single bout of exercise in humans [60] or rodents [61,62] can result in increased PGC-1α transcription. Aerobic exercise also promotes PGC-1α nuclear translocation [63,64] to up-regulate the transcription of its own mRNA transcription through a positive feedback loop [65], as well as the synthesis of critical NuGEMPs to elicit mitochondrial biogenesis.

Given the impact of exercise on the expression of PGC-1α, determining the necessity of this factor in regulating the increase in mitochondrial content with exercise has been a subject of great interest. *In vitro* silencing of PGC-1α attenuates, but does not abolish the increase in mitochondrial content following contractile activity [16]. *In vivo*, PGC-1α KO animals display decrements in muscle mitochondrial content and function, which can be restored with chronic endurance training, despite the absence of PGC-1α [7,66]. Moreover, muscle-specific PGC-1α KO animals do not experience any noticeable impairments in exercise-induced mitochondrial biogenesis [67]. Thus, muscle contractile activity and exercise training undoubtedly activate pathways that are independent of PGC-1α to improve the mitochondrial phenotype.

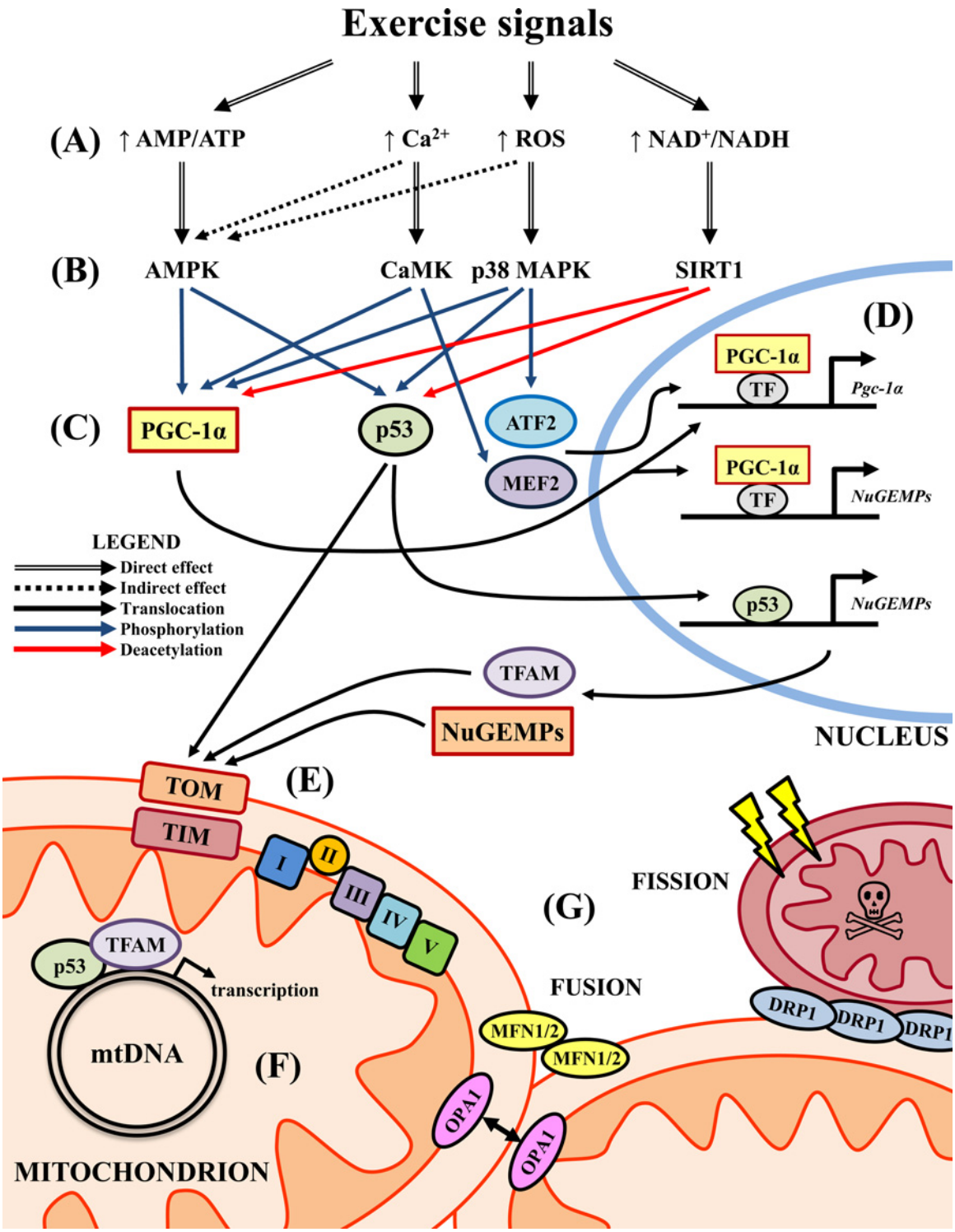

Figure 1 Exercise-induced mitochondrial biogenesis

(**A**) Muscle contractile activity alters the concentration of several metabolites and molecules involved in initiating mitochondrial biogenesis, including increasing the ratios of AMP/ATP and NAD + /NADH, as well as cytosolic calcium and ROS. These changes enhance the activity of a number of kinases (**B**), including AMPK, CaMK, p38 MAPK and the deacetylase SIRT1. These proteins can then modify factors involved in the transcriptional response to exercise by either phosphorylation or deacetylation, including the transcriptional co-activator PGC-1α (**C**) and transcription factors p53, ATF2 and MEF2. PGC-1α can then enter the nucleus, and interact with transcription factors to induce the transcription of its own gene, or of NuGEMPs. p53 also enters the nucleus in response to contractile activity, and can induce the expression of NuGEMPs. Once NuGEMPs have been transcribed (**D**) and the mRNAs have been translated in the cytosol, they are delivered to the mitochondrion through the PIM (**E**), consisting of the TOM and the TIM complexes. They are then sorted to different mitochondrial compartments. Expression of the mitochondrial genome is accomplished by the actions of TFAM and p53, which enter the matrix and interact with mtDNA to promote transcription following contractile activity (**F**). With the increase in expression of both nuclear- and mitochondria-encoded proteins, the mitochondrial reticulum can expand (**G**). Elongation of the reticulum involves the fusion of pre-existing organelles, which is accomplished by fusion proteins MFN1/2 and Opa1. Transient increases in ROS during exercise may prompt the fission of dysfunctional components of the reticulum, a process leading to mitophagy (see Figure 2) and driven by proteins Drp1, Fis1 and others (see text for details).

A larger portrait of complexity surrounding PGC-1α has begun to emerge through the identification of an additional upstream promoter, as well as alternative splicing of mRNA transcripts which generate distinct, functional protein products. Initial studies from independent laboratories describe two additional variants for PGC-1α in murine skeletal muscle which are synthesized from an alternative promoter and the use of an alternative first exon [68–71]. The original PGC-1α has been labelled as PGC-1α-a or PGC-1α1, depending on the scientific group, whereas these new splice variants of PGC-1α, PGC-1α-b and PGC-1α-c, have been found to code for a functional protein that transcriptionally co-activates gene expression *in vitro* and *in vivo* [69,70]. These variants of PGC-1α are also found abundantly in skeletal muscle and brown adipose tissue [69]. The alternative promoter that gives rise to these variants is located ∼14 kb upstream from the canonical (or proximal) PGC-1α promoter, and examination of the regulatory factors that control the alternative promoter has revealed similarity to the proximal promoter [71]. Gene expression from this upstream promoter appears to be coupled to the alternative exon1, known as exon1b, whereas the rest of the transcript is identical with the original PGC-1α-a. This alternative exon1b shortened the transcript and the translated protein at the N-terminal, which gave rise to PGC-1α-b [72,73]. Alternative transcript splicing from the upstream promoter also occurs to produce PGC-1α-c, which is further shortened at the N-terminus. These initially described variants were found to positively respond to aerobic exercise in skeletal muscle [69]. Furthermore, PGC-1α-b and PGC-1α-c exhibited a greater response to exercise than the traditional PGC-1α-a, and also accounted for the majority of the increase when total transcript change was calculated [69]. However, the functions of the translation products of these transcripts remain to be identified. In addition to the full-length variants of PGC-1α, there also exist a group of protein products approximately 30–37 kDa in size which include PGC-1α4 [57,73–75]. In contrast with the other isoforms, PGC-1α4 has been described to confer the benefits of resistance exercise in skeletal muscle [75], but this remains a controversial finding [76–78]. Foreseeably, through the use of more specific KO and/or transgenic experiments, and further exercise experiments, the importance of each PGC-1α splice variant will be determined.

SIGNALLING PATHWAYS TOWARDS MITOCHONDRIAL BIOGENESIS

A number of signalling pathways activated by contractile activity are now recognized to be involved in initiating mitochondrial biogenesis (Figure 1). The most commonly considered pathways based on extended experimental evidence include the contractile activity-associated rise of intracellular calcium, mainly derived from sarcoplasmic reticulum sources, and the turnover of ATP, leading to an increase in AMP and the activation of AMP-activated protein kinase (AMPK). Early studies, which applied calcium ionophores to myotubes in culture, revealed (1) elevated mitochondrial enzyme activities [79], (2) an increase in the expression of selected genes associated with mitochondrial biogenesis [80,81] and (3) a dependence on both calcium-calmodulin kinase [82,83] and protein kinase C [83]. The importance of calcium in instigating mitochondrial biogenesis has been further fortified by results using parvalbumin KO and overexpressing animals. By modulating the expression of parvalbumin, a protein which sequesters calcium, it appears that mitochondrial volume is positively correlated with the availability of cytosolic calcium in muscle [84,85]. However, *in vitro* experiments with cultured muscle cells have revealed that calcium is not solely responsible for triggering contractile activity-induced organelle biogenesis. When muscle cells were treated with various intracellular calcium chelators, the transcription of cytochrome *c* was reduced, but its transcriptional activity in response to contractile activity was unaffected [80,83]. These findings laid the groundwork for research seeking alternative, complementary pathways involved in exercise-induced biogenesis.

Contractile activity occurs as a result of myosin ATPase-induced hydrolysis of ATP to ADP, and the energy released is used for cross-bridge formation and force generation. Some of the resulting ADP is further dephosphorylated by myokinase to AMP. AMP binds to the γ subunit of the heterotrimeric AMPK molecule and enhances its activation [86], allowing AMPK to phosphorylate downstream targets which are intimately involved in the control of cellular metabolism [87]. These include acetyl-coA carboxylase and AS160, which promote increases in lipid oxidation and glucose uptake in muscle respectively. AMPK also plays an important role in the regulation of mitochondrial content in skeletal muscle. This is highlighted by the consequences of muscle-specific loss-of-function, which include attenuated expression of mitochondrial genes and reductions in mitochondrial volume [88]. On the other hand, the activation of AMPK increases the expression of a number of genes associated with mitochondrial content, including cytochrome *c*, SDH and CS [89–91]. This is probably due to several reasons, including the ability of AMPK to phosphorylate a protein target such as GATA4, and enhance its DNA binding within a GATA/E-box region of the PGC-1α promoter [92], thereby increasing the expression of PGC-1α [58,93,94]. Recent research has also identified protein kinase A anchoring protein 1 (AKAP1) as an additional substrate of AMPK in skeletal muscle, an interaction which provides a direct link between the activation of AMPK and the regulation of mitochondrial respiration [95]. AMPK also directly phosphorylates PGC-1α on threonine-177 and serine-538 residues [90], a modification which appears to be critical for PGC-1α feedback activity on its own promoter, and the subsequent induction of PGC-1α-regulated genes. Indeed, in PGC-1α KO animals, the effects of AMPK are minimized, suggesting that the presence of PGC-1α is necessary for many of the metabolic consequences of AMPK activation in muscle.

Research has indicated that these two signalling systems also operate together to induce PGC-1α. Chemical uncoupling of cells leads to increases in cytosolic calcium levels, which activates AMPK. This leads to an increase in the expression of PGC-1α along with oxidative phosphorylation genes, an effect which is abolished when the increase in calcium is blocked [96]. Under the more physiological conditions of myotube contractile activity, calcium and AMPK activation are critical for an increase in the transcriptional activity of PGC-1α, as well as PGC-1α promoter activity [59].

In addition to these two kinases, p38 MAPK also has pleiotropic roles in the regulation of PGC-1α. Similar to AMPK, p38 can also directly phosphorylate the co-activator [97]. p38 is also highly responsive to elevations in ROS produced during acute exercise in muscle [59], and it is sufficient to up-regulate PGC-1α mRNA, particularly through the γ isoform [98]. p38 also regulates PGC-1α in a transcriptional manner by the phosphorylation of transcription factors such as MEF2 and ATF2, known regulators of the promoter [99], and pharmacological inhibition of p38 in cultured muscle cells attenuates the transcription of PGC-1α [59]. In summary, elevations in cytosolic calcium, along with the activation of AMPK and p38 (possibly via ROS), are vital contractile activity-induced events which share the responsibility of activating PGC-1α during exercise.

REGULATION OF MITOCHONDRIAL MORPHOLOGY

In skeletal muscle, the pioneering work of Brooks and co-workers [24,100], Kayar and Banchero [101] and Ogata and Yamasaki [25,102] has led to an appreciation that skeletal muscle mitochondrial morphology is complex, and regionally distinct in various compartments of the muscle fibre. This has been further verified more recently [27] with implications for the effective subcellular distribution of ATP within muscle. As noted above, mitochondria in both heart and skeletal muscle can be divided into morphologically-distinct subfractions, differentiated by their location, function and biochemical composition [26,40,103,104]. SS mitochondria tend towards greater circularity and are less involved in reticular networks than IMF mitochondria, however some continuity exists between these mitochondrial fractions [26,101]. In cultured cells, mitochondria exhibit very dynamic movement behaviours which are undoubtedly constrained within the context of mature muscle cells *in vivo*. Further, there is evidence that dynamic changes in mitochondrial shape can take place in response to a single bout of exercise [105], which likely has implications for metabolism [106].

Small fragmented mitochondria are a product of fission events (Figure 1), regulated in part by dynamin-related protein 1 (Drp1), and its interaction with the mitochondrial receptors mitochondrial fission protein 1 (Fis1) and mitochondrial fission factor (Mff) [107]. In contrast, fusion of mitochondria into more reticular configurations is promoted by optic atrophy protein (Opa1) and mitofusin isoforms, Mfn1 and Mfn2 [108], all of which are regulated by PGC-1α [109,110]. Over-expression and selected deletion of these proteins in mouse models has clearly revealed their functionality in determining mitochondrial morphology [111–113]. Thus, the ratio of fission to fusion regulatory proteins seems to be a reliable index that determines the connectivity of the mitochondrial network within muscle, and these proteins are inducible under conditions of exercise, disuse and aging. For example, after a period of chronic contractile activity, the fission to fusion protein ratio diminishes [114], leading to a more intact mitochondrial reticulum, a conformation that favours less ROS production and more efficient lipid metabolism. In contrast, in aged muscle, or in muscle subject to chronic disuse, the ratio of these proteins is reversed [114], leading to more fragmented organelles, elevated ROS production and increased rates of mitophagy.

Interestingly, recent findings seem to indicate that the control of mitochondrial fission, mitophagy and biogenesis are exerted by similar signalling events. It has recently been shown that AMPK activation in response to cellular stress leads to the phosphorylation of Mff which promotes organelle fission [115]. Thus, it appears that AMPK is at the nexus of the control of mitochondrial content, as it is an important regulator of mitochondrial clearance, while simultaneously acting to promote biogenesis via its activation and induction of PGC-1α, as discussed above.

MITOCHONDRIAL PROTEIN IMPORT

The mitochondrial proteome consists of a vast number of proteins required for proper organelle function. When compared with other organelles, the mitochondrial proteome is unique as it is encoded by two sets of genomes. The MitoCarta2.0 database has revealed that the nuclear genome codes for over 1100 gene products [116], whereas only 13 proteins are encoded by mtDNA. Due to the vast number of NuGEMPs that must be transcribed from nuclear DNA, translated and subsequently imported into the mitochondrion, coordinated expression of these two genomes is vital for proper mitochondrial biogenesis and function. The value of a functional mitochondrial import system is highlighted by the number of diseases or disorders which arise when protein import machinery (PIM) components are dysfunctional or absent [117]. Further, it has been shown that the protein import system is adaptable in response to physiological perturbations in muscle such as chronic exercise or muscle disuse, as mentioned below.

Over the last several decades, techniques to study mitochondrial protein import have been developed and refined from the pioneering methods of the Schatz and Neupert laboratories [118–120]. Briefly, nuclear-encoded precursor proteins are transcribed from cDNA, then translated *in vitro* in the presence of ^{35}S-methionine. They are subsequently incubated with isolated mitochondria, and the import is monitored over time using autoradiography. This technique is a sensitive indicator of mitochondrial function, since import into the matrix requires an intact membrane potential, ATP synthesis, and adequate expression of the PIM.

The mitochondrial import system (Figure 1) encompasses several large machinery complexes found across the inner and outer mitochondrial membranes (IMM and OMM respectively) that facilitate the transfer of newly synthesized proteins destined for mitochondria. These proteins are synthesized in the cytosol and chaperoned to the mitochondrial outer membrane. However, due to the double membrane structure of mitochondria, a gateway for mitochondrial proteins is required. Mitochondria-destined cytosolic "precursor" proteins contain mitochondrial targeting sequences (MTS) that assist in the sorting of these proteins [121]. For example, a positively charged N-terminus presequence serves to target a protein to the matrix compartment [122]. The precursor protein is recognized by molecular chaperones such as cytosolic Hsp70, and unravelled in an ATP-dependent manner to make the protein import-competent, and then directed to the mitochondrion. This conformational change allows entry of the precursor into the translocase of the outer mitochondrial membrane (TOM) complex, which consists of several preprotein receptors (TOM20, TOM22, TOM70) responsible for the recognition of the MTS [123–125]. Once through the TOM complex, precursor proteins proceed to the translocase of the inner mitochondrial membrane (TIM) and are sorted via the TIM23 complex [126,127]. Thereafter, proteins are actively pulled into the matrix with the assistance of mitochondrial Hsp70 and the precursor sequence is cleaved by mitochondrial processing peptidase (MPP). Lastly, matrix proteins are refolded into their native conformation with the assistance of molecular chaperones, such as Hsp60 and CPN10. This results in the complete import and maturation of matrix-destined proteins [128]. Precursor proteins destined for other mitochondrial subcompartments use a variety of other proteins and routes to their final destination within the organelle, as reviewed elsewhere [129].

The arrival and incorporation of proteins into the organelle produces an expansion of the mitochondrial reticulum. As such, mitochondrial protein import is a tightly regulated system that is intimately linked to cellular energetic status, and is impacted in response to stimuli which can induce or suppress mitochondrial biogenesis. This likely operates as a mechanism through which mitochondrial reticulum size and continuity can be modified in response to the changing metabolic demands of the muscle. Indeed, protein import of matrix-destined proteins, such as mitochondrial transcription factor A (TFAM) and outer membrane proteins such as TOM40, into skeletal muscle mitochondria is accelerated as an adaptation to chronic contractile activity [130–132]. To assist in this increase in mitochondrial protein import, a concomitant elevation in the rate of the TOM complex assembly has also been observed during increases in muscle activity [132].

In contrast, muscle inactivity impairs import of mitochondrial matrix-destined proteins quite rapidly following the onset of the stimulus [133,134], coinciding with a loss of mitochondrial content. This is accompanied by depleted ATP levels and reduced mitochondrial membrane potential. Collectively, these data underscore the adaptive plasticity of protein import and assembly in response to changes in skeletal muscle contractile activity.

Interestingly, in cases where mitochondrial protein import is defective, endurance exercise may show promise as a therapeutic modality. In a recent study, a novel role for the apoptosis-associated outer membrane proteins Bax and Bak in the regulation of mitochondrial protein import was identified [135]. A double KO model of these two proteins reduced mitochondrial protein import and diminished the expression of components of the PIM. However, this impairment was reversed following an endurance training programme, accompanied by an increase in the expression of PIM components. Alternatively, pharmacological activation can also be utilized to augment components of mitochondrial PIM in muscle. For example, thyroid hormone is known to induce mitochondrial biogenesis [136], as well as enhance protein import in cardiac muscle [137].

With the renewed interest in mitochondrial protein import in recent years, it has become apparent that this system is a highly adaptable rheostat, which appropriately matches changes in mitochondrial content with the rate and capacity to import proteins into the organelle. Thus, targeting this system with exercise or pharmaceuticals appears to be an attractive means to modulate mitochondrial mass, and may serve as a useful therapy for mitochondrial protein import defects.

MITOCHONDRIAL TRANSCRIPTION FACTOR A

The incorporation of proteins derived from both the nuclear and mitochondrial genomes is crucial for the construction of the multi-subunit complexes which make up the ETC. mtDNA gene expression is controlled by a non-coding portion of the DNA, referred to as the D-loop regulatory region. This segment of DNA modulates the expression of 13 mRNAs encoding components of the ETC, in addition to 2 tRNAs and 22 rRNAs which assist in the translation of the mRNAs. Interestingly, the transcription of mtDNA is dependent on three factors which are completely nuclear-encoded, including mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor B2 (TFB2M) and TFAM [138]. Although controversy exists as to the exact nature through which these components are organized and interact to modulate transcription *in vivo*, their role in governing mtDNA transcription is widely accepted.

Within the mitochondrion, the circular mtDNA is condensed and packaged in multi-protein complexes called nucleoids. TFAM is one of the major components of the nucleoid [139–141], and is a core component of the mtDNA transcription machinery (Figure 1) [142], with an additional role in mtDNA replication and packaging [139,143,144]. This protein contains two high-motility group (HMG) domains, which permit it to bend, wrap and unwind DNA. Accordingly, TFAM is capable of distorting mtDNA promoters into a "U-turn"-like conformation [145–147], and provides the other core proteins with access to the promoter [148] to stimulate mitochondrial transcription. The indispensability of TFAM is highlighted by loss-of-function studies in which complete ablation of this protein is embryonic lethal [149], whereas tissue-specific or partial deletions result in severe respiratory chain defects [149–154]. TFAM-mtDNA binding and mtDNA transcription can be modulated through post-translational modification of TFAM, specifically by phosphorylation by cellular energy responsive kinases such as protein kinase A (PKA) and extracellular signal-regulated protein kinases (ERK1/2) [155,156]. This represents a mechanism by which mtDNA transcription can be modified in response to intracellular conditions.

The role of TFAM in skeletal muscle has been highlighted by muscle-specific loss-of-function studies, which have identified this protein as a required factor for maintaining normal mitochondrial respiratory chain function and muscle strength [151,154]. In skeletal muscle cells, the protein expression of TFAM and its localization to the mitochondrial matrix correlates well with the expression of mtDNA-encoded genes [157], a relationship which holds true following both chronic muscle activity and inactivity [131,134]. Increased Tfam gene expression in muscle has been reported following a single acute session of endurance exercise in both rodent and humans [158–160], which is not surprising since TFAM expression is regulated by PGC-1α [161], which is robustly responsive to exercise. Further, a rise in total cellular TFAM protein content has been reported in multiple exercise training paradigms [9,16,131,162–165]. The changes in TFAM protein content during mitochondrial biogenesis occur in conjunction with a general increase in organelle protein import during this process [130], resulting in an increase in the amount of TFAM within the mitochondrial matrix, as well as the quantity of TFAM bound to the D-loop region of mtDNA [131]. These changes undoubtedly contribute to the increased expression of mtDNA-encoded genes required for the expansion of the mitochondrial network in muscle for exercise-induced mitochondrial biogenesis.

p53 – NUCLEAR AND MITOCHONDRIAL GENOME EFFECTS

p53 is a transcription factor which has been canonically regarded as a tumour suppressor [166], as it promotes the expression of genes involved in antioxidant defence, autophagy, apoptosis and DNA damage [167–169]. Recently, p53 has been further categorized as a critical regulator of mitochondria [170–172], as work in various cell types has verified a requirement for p53 in maintaining basal mitochondrial content [8,173–177]. One of the unique traits of p53 is its apparent ability to regulate transcription in both the nuclear and mitochondrial genomes [178–180]. Since mitochondrial function relies on the synchronized expression of these genomes, it is not surprising that a protein with the capacity to modulate gene expression in both the nucleus and the mitochondrion should play a vital role in mitochondrial biogenesis.

Studies have confirmed that p53 is capable of regulating genes involved in oxidative metabolism at the transcriptional level, such as the nuclear-encoded transcription factors TFAM [174,181] and NRF-1 [174], in addition to synthesis of cytochrome *c* oxidase 2 (SCO2), a protein which assists in the assembly of the ETC [176]. Further, PGC-1α contains a putative binding site for p53 in its promoter [92], and its protein level appears to be reduced in p53 KO mice [8]. With respect to mtDNA-encoded genes, p53 can transcriptionally regulate the expression of cytochrome *c* oxidase subunit I (COX I) of the COX holoenzyme [160] and 16S RNA [177]. The ability of p53 to influence the transcription of mtDNA is presumably aided by the physical interaction between p53 and TFAM [160,182].

In skeletal muscle, whole-body p53 KO mice display a reduction in basal mitochondrial content, in conjunction with deficiencies in the assembly of the COX holoenzyme and disorganized and disrupted mitochondrial cristae [8,175]. These structural changes are correlated with a lower respiratory capacity,

and an increased mitochondrial ROS emission [8]. The excess ROS can damage mitochondrial proteins and serve as a signal for their ubiquitination and subsequent degradation by mitochondrial-specific autophagy [183]. Indeed, skeletal muscle mitochondria from p53 KO mice are ubiquitinated to a greater extent, and have higher levels of localized LC3-II, suggesting a greater basal rate of organelle degradation via mitophagy [174].

Although chronic endurance exercise training can increase total cellular p53 protein content in skeletal muscle [184], a role for p53 in the regulation of signalling towards mitochondrial biogenesis following a single session of endurance exercise has also been uncovered. p53 can be post-translationally modified by phosphorylation on serine-15 residue (serine-18 in mice), which increases its stability and activity. This site is a *bona fide* target of p38 MAPK [185] and AMPK [186], two kinases which are sensitive to intra- and extra-cellular changes which occur during exercise [58,61,187]. It is not surprising then that several models of acute endurance exercise have been shown to increase the phosphorylation of p53 at this site [8,188,189]. Interestingly, genetic deletion of p53 can hinder the responsiveness of p38 MAPK and AMPK to acute exercise [174], and in turn, can impair the rate of PGC-1α translocation to the nucleus, as well as the transcription of the *Pgc-1α* gene, as well as other NuGEMPs, following acute exercise [174].

Research has also provided evidence for the translocation of p53 to the mitochondria following exercise, stimulating the formation of complexes between p53 and Tfam, as well as between p53 and mtDNA D-loop region (Figure 1). This appears to be critical for the expression of mtDNA-encoded genes, since p53 KO animals have a reduced expression of mtDNA encoded transcripts following exercise [160]. Additionally, p53 is a known substrate of SIRT1, an NAD-dependent deacetylase [190]. Deacetylation of p53 also occurs following exercise, prompting its translocation to the nucleus [191]. Irrespective of where p53 is destined, its activation and stabilization in response to exercise looks to be a means through which exercise-induced mitochondrial biogenesis is mediated. Interestingly however, while p53 KO mice have a reduced skeletal muscle mitochondrial content, exercise training appears to be capable of restoring mitochondrial content even when p53 is absent [8]. This indicates that p53 alone is not necessarily required for the mitochondrial adaptations to long-term endurance exercise training, despite being vital for the acute signalling and transcriptional responses to a single bout of exercise [174]. Further delineation of the necessity of p53 for long-term mitochondrial adaptations to endurance training is merited.

MITOCHONDRIAL TURNOVER MECHANISMS/MITOPHAGY AND TFEB

Skeletal muscle cells are capable of maintaining their functional and structural quality through the breakdown of damaged and dysregulated cellular organelles by macroautophagy (hereafter referred to as autophagy). This cellular degradation system sequesters targeted substrates within autophagosomes which, in turn, fuse with lysosomes where the encapsulated substrates are degraded. Autophagy is initiated by the activation of unc-51-like kinase 1 (ULK1) [192] and Beclin1 complex [193], which assists in the formation of double-membrane structures called phagophores around organelles and substrates, isolating the target to be degraded (Figure 2). The phagophore membrane is then extended through the recruitment of additional LC3-II, which is created by the lipidation of LC3-I by the action of several select autophagy-related genes (ATGs) [194]. A more complete review of the pathways and molecules involved in initiating and executing autophagy can be found elsewhere [195,196].

The more selective autophagy of mitochondria is termed mitophagy, and in this pathway, additional regulatory steps are required. When mitochondria are functional, they maintain a specific mitochondrial membrane potential, permitting normal levels of mitochondrial protein import and oxidative phosphorylation. However, when mitochondria become dysfunctional, as indicated by a dissipation of the membrane potential, a series of steps leading to their degradation is initiated. The reduction in membrane potential arrests the import of PTEN-induced putative kinase 1 (PINK1) into the organelle, where it would normally be degraded [197]. Instead, PINK1 accumulates on the outer membrane where it recruits the E3 ubiquitin ligase Parkin [197,198]. Parkin ubiquitinates outer membrane proteins such as voltage-dependent anion channel 1 (VDAC1) [199,200] and mitofusin isoforms [201–203]. The ubiquitin chains formed on mitochondrial proteins then act as an anchor for p62, a protein which provides a physical link between ubiquitinated substrates and LC3, allowing for the dysfunctional component of the mitochondrion to be encapsulated in an autophagosome [200,204]. The autophagosome is then trafficked to the lysosome, the two structures fuse, and the contents of the autophagosome are degraded by a multitude of lysosomal proteolytic enzymes, such as Cathepsin D (Figure 2) [205].

Exercise induces changes in skeletal muscle which requires the remodelling of the tissue and its organelles, such as mitochondria. Although lysosomes have been relatively ignored in the adaptations to exercise, it has been speculated that remodelling through the autophagy system, including lysosomes, may be required for skeletal muscle adaptations to exercise [64,206]. Early work had described a role for autophagic clearance in maintaining protein homoeostasis in skeletal muscle following exercise [207,208]. Since then, a number of studies have highlighted the importance of autophagy in skeletal muscle during exercise, and it is now recognized that acute exercise is a reliable stimulus to induce autophagy [64,174,209–217]. Activation of ULK1 in skeletal muscle, a marker of autophagic induction, has been demonstrated following several acute exercise paradigms [213–215], suggesting an increase in the signalling towards autophagy. Further, Beclin1, another critical protein involved in the initial steps of the generation of the autophagosomal structure, has also been shown to be activated with an acute bout of exercise [209]. Acute exercise also increases total cellular Parkin protein expression, the localization of mitophagic markers to mitochondria and mitochondrial LC3-II flux in skeletal muscle [64,174]. PGC-1α appears to be involved in this process, as PGC-1α KO animals exhibit diminished rates of Parkin recruitment to the mitochondrion, and an attenuation in the rate at which mitophagy proceeds following a single bout of endurance exercise [64]. Alternatively, overexpression of PGC-1α augmented the expression of autophagy markers in skeletal muscle following endurance exercise and reduced basal p62 levels [218]. Together, these data suggest a dual role for this transcriptional co-activator, in both promoting exercise-induced mitochondrial biogenesis, while simultaneously modulating exercise-induced mitochondrial degradation via mitophagy [64].

A growing number of studies have attempted to identify a role of TFEB, a master regulator for lysosomal biogenesis, in autophagic regulation [219–221]. Lysosomal and autophagic genes possess specific promoter regions called Coordinated Lysosomal Expression and Regulation (CLEAR) sites which are targets for TFEB binding [220]. During non-stressful conditions, TFEB is located in the cytosol and is stabilized by mTORC1-mediated phosphorylation [222,223]. However,

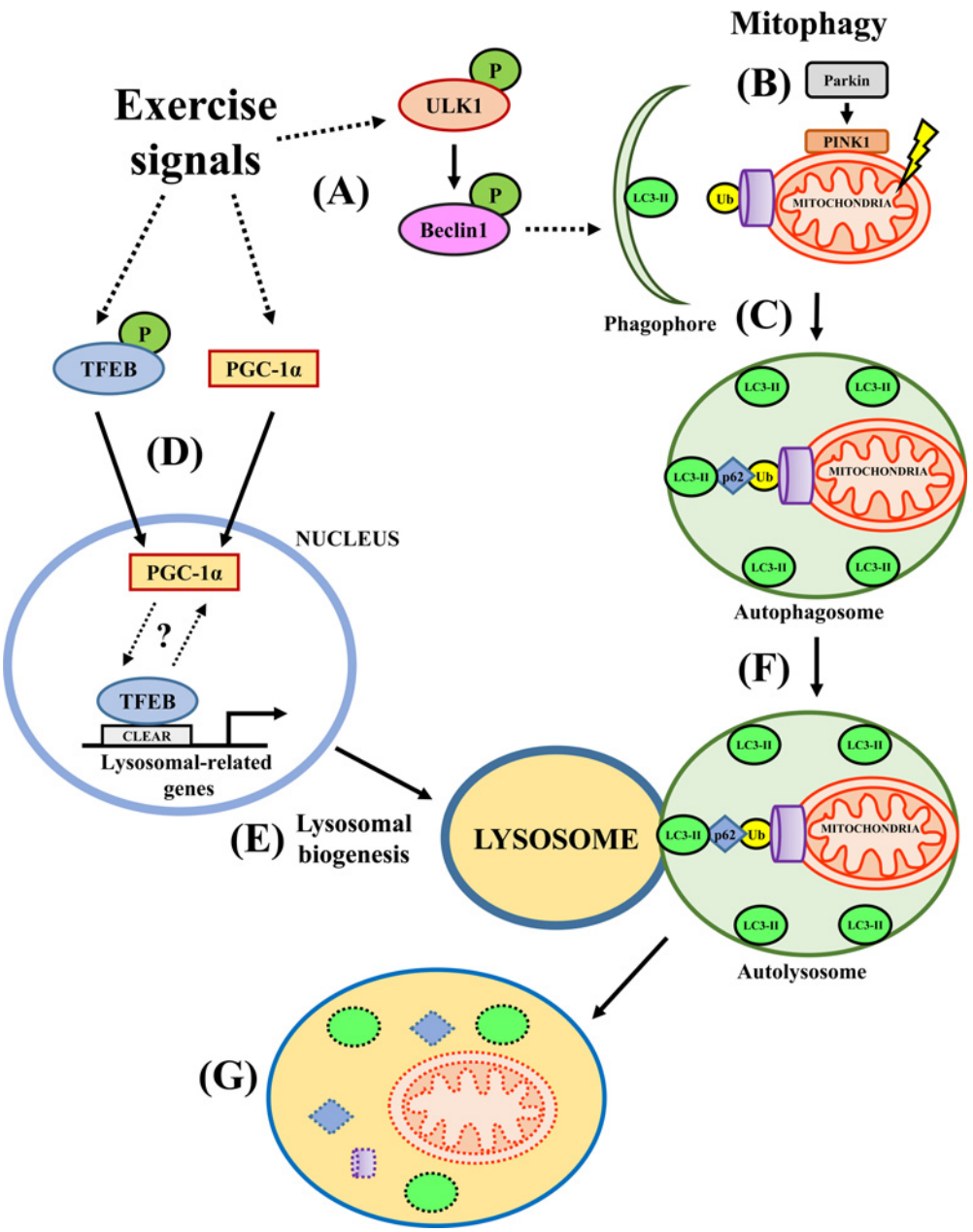

Figure 2 Mitophagy in skeletal muscle

Although low levels of basal mitochondrial turnover occur continuously, changes in the cellular environment, such as exercise, can increase the degree to which this process occurs. (**A**) Exercise can begin the initial steps required for the autophagosome formation, including the activation of the ULK1 and Beclin1 complexes. This permits the formation of the phagophore, which is composed of lipidated LC3-II molecules. In order to optimize the function of the mitochondrial network, dysfunctional portions of the mitochondrial reticulum must be removed. (**B**) Under basal conditions, the mitochondrial kinase PINK1 is imported into the mitochondrion and degraded. However, when mitochondria become dysfunctional, they lose their membrane potential, resulting in a stabilization of PINK1 on the outer mitochondrial membrane. (**C**) Once stabilized, it can serve as a receptor for the E3 ubiquitin ligase Parkin, which translocates to the mitochondrion, and can ubiquitinate mitochondrial proteins, such as VDAC and the mitofusins. These proteins that have been targeted for degradation are linked to the phagophore by p62, which serves as a bridge between ubiquitin-tagged proteins and LC3-II. The phagophore continues to expand and surround the targeted portion of the organelle (**C**), until completely encapsulated. (**D**) The changes in the cellular environment can also activate certain proteins involved in the regulation of autophagy, mitophagy and lysosomal biogenesis, namely TFEB and PGC-1α. TFEB is the master regulator of lysosomal biogenesis, which when activated by dephosphorylation, can enter the nucleus to initiate a transcriptional programme to increase lysosomal content in cells (**E**). PGC-1α is the master regulator of mitochondrial biogenesis, which has also been shown to be involved in the transcriptional regulation of autophagy and mitophagy. Interactions between PGC-1α and TFEB have been identified in other cell types, however the exact nature of this relationship is still under investigation in skeletal muscle. Nonetheless, autophagosomes containing cargo to be degraded can fuse with lysosomes (**F**), the cargo can be broken down into their constituent parts by lysosomal enzymes (**G**), and then released back into the cell.

TFEB is dephosphorylated and can translocate to the nucleus in response to various stimuli [219,221,224] including exercise [225], suggesting a potential role for this factor in exercise-associated lysosomal biogenesis and function. Although the activity of lysosomal enzymes cathepsin C and D in skeletal muscle are increased following acute exercise [208], the latter of which is a transcriptional target of TFEB [220], the exact role of TFEB in regulating this process remains unclear.

Recently, Vainshtein et al. [226] demonstrated that denervation, which promotes mitochondrial degradation and mitophagy flux,

markedly increases TFEB protein expression. Interestingly, deletion of PGC-1α attenuated this increase and impaired TFEB nuclear translocation, whereas over-expression of PGC-1α augmented TFEB expression. These data implicate a role for PGC-1α as a major regulator of TFEB activity and expression, as well as mitophagy flux, in skeletal muscle. The data also suggest the presence of a tight-knit relationship between PGC-1α and TFEB in skeletal muscle, which has been well documented in other cell types [221,227]. Future work further describing the relationship between these two transcriptional regulators in skeletal muscle metabolism will be important for a more complete understanding of mitochondrial turnover in muscle.

IMPACT OF MUSCLE DISUSE ON MITOCHONDRIAL TURNOVER

Chronic muscle inactivity is a powerful stimulus to induce muscle wasting. To study muscle disuse, several invasive and non-invasive experimental models have been traditionally employed, which provide insight into the molecular mechanisms through which this occurs. Rodent models of reduced muscle activity, such as hindlimb casting and tail suspension, have been utilized to investigate the impact of inactivity on muscle phenotype. These models have been developed to simulate selective human circumstances, such as microgravity during spaceflight or prolonged bed rest [228,229]. In contrast with these non-invasive techniques, denervation provides a surgical method to induce muscle disuse, whereby the neural innervation of the muscle is ablated by neurectomy. This completely removes nerve-muscle communication, and can rapidly induce atrophy [133,134,230].

Models of muscle inactivity are also useful as experimental paradigms to reduce mitochondrial content, and to study mitochondrial turnover. Studies using EM have noted that muscle disuse induces a loss of mitochondria [114,231,232], and the mitochondria which remain are smaller, more fragmented, and have reductions in cristae density [231–236]. These alterations are echoed by a reduction in the activity and expression of membrane-embedded and mitochondrial matrix enzymes such as COX, SDH and CS [33,133,236–238], alongside impairments in ATP synthesis and elevations in ROS emission [23,34,133,230,239]. These decrements in organelle function negatively impact mitochondrial protein import, which is also suppressed during muscle disuse [133]. Taken together, research in this area strongly indicates that prolonged muscle disuse causes reductions in both mitochondrial content and function, suggesting that the underlying molecular basis for this decay is worthy of study from a therapeutic perspective.

The loss in mitochondrial content with muscle disuse is, in part, due to a reduction in the drive for mitochondrial biogenesis. A dramatic drop in PGC-1α mRNA content occurs within the first day following denervation [240,241], which remains depressed for weeks [236,240–242]. PGC-1α protein content also is reduced [230,238], along with the expression of other factors which positively regulate mitochondrial biogenesis, including ERRα and NRF-1/2 [236,243]. This culminates in a loss of total cellular TFAM protein content [230,236] and intra-mitochondrial TFAM, which lessens mtDNA copy number and impairs mtDNA-encoded gene expression [134,243]. Further, the expression of fission and fusion proteins involved in modifying mitochondrial connectivity shift to favour mitochondrial fission, causing fragmentation of the mitochondrial network [114].

The overexpression of PGC-1α is sufficient to mitigate the loss of mitochondrial mass during muscle disuse, and this is accompanied by a reduction in muscle atrophy, likely via the direct or indirect inhibition of several intracellular degradation pathways [226,238,241,242,244–247]. It has also been shown that overexpression of mitochondrial fusion protein Opa1 preserves mitochondrial function, and also protects against loss of muscle mass during denervation [112]. Conversely, the over-activation of mitochondrial fission in muscle is sufficient to fragment the mitochondrial network and drastically impair organelle function, an effect which directly results in muscle atrophy [111,248]. Taken together, these studies highlight the intimate link between an intact mitochondrial network and optimal mitochondrial function, with the maintenance of muscle mass. Thus, directly targeting mitochondrial function, as well as preserving mitochondrial reticulum integrity, may be viable means to combating muscle atrophy during muscle inactivity.

Muscle disuse also increases the expression of a number of autophagy proteins, as well as the localization of LC3-II on mitochondria [23,249], resulting in an increase in autophagy and mitophagy flux [226]. Despite this apparent enhancement of the removal of dysfunctional organelles during disuse, the continued presence of poorly functioning mitochondria within disused muscle suggests that the elevated mitophagy is insufficient to adequately restore organelle homoeostasis. Thus, therapeutic methods to enhance mitochondrial biogenesis, or to stimulate further mitophagy, possibly via agents which signal through PGC-1α [64,226], would be beneficial in this respect.

IMPACT OF AGING AND EXERCISE ON MUSCLE MITOCHONDRIA

A wealth of scientific literature shows that aging and physical inactivity lead to reduced mitochondrial content and function in muscle (cf. [5,250,251] for reviews of current controversies in the field). As mentioned above, mitochondrial function is implicated in the maintenance of muscle mass. A decline in mitochondrial function is believed to be one of the major underlying causes of the sarcopenia (loss of muscle mass and strength) evident with old age. In addition, mitochondrial dysfunction is known to accompany the age-related diseases of obesity and type 2 diabetes [252,253]. Thus, the decrements in mitochondrial content and function with age play a vital role in the declining metabolic health landscape evident in modern society. Therefore a comprehensive understanding of these organelles and how they may be remedied with aging will ultimately contribute to greater health outcomes for the aging population.

A decline in organelle content with age is supported by many studies which report reduced protein markers and mtDNA content, along with Krebs' cycle and ETC enzyme activities [5,254–260]. Additionally, electron micrograph evidence of diminished IMF mitochondrial size and a reduced thickness of the SS mitochondrial layer [114,261–263], supports the biochemical findings of reduced mitochondrial content with aging. Mitochondrial fragmentation is also prominent in aging muscle, and is probably a result of an imbalance in the ratios of proteins that regulate morphology of the organelles through fission and fusion [114]. These alterations in mitochondrial structure are accompanied by defects in mitochondrial respiration [264,265], which may be a consequence of reduced MitoPS [35] or increased uncoupling of oxygen consumption to ATP synthesis [264]. Furthermore, accumulation of dysfunctional organelles may occur through impaired mitophagy/autophagy processes that are unable to clear the damaged organelle debris from the cellular milieu. Indeed, in aged muscle, evidence has shown an accumulation of undigested material, termed lipofuscin [266]. This phenomenon may also contribute to the cellular damage, exacerbating apoptosis and skeletal muscle loss.

The underlying cellular basis for the decline in mitochondrial content and function in skeletal muscle with age remains controversial. Maintenance of organelle content and function encompasses numerous molecular events and alteration in any aspect (i.e. decreased synthesis and/or increased degradation) could impact organelle homoeostasis. Important steps that could be altered include transcriptional regulation of NuGEMPs or of mtDNA, post-transcriptional trafficking of nuclear-encoded proteins into the organelle or alterations in clearance of the organelles. Mitochondrial protein import and holoenzyme assembly pathways have been found to be unaffected with age [132,261]. Further, mtDNA deletions and point mutations occur with increasing incidence with age, but appear to occur in later life, after the onset of the decline in mitochondrial function [256,267,268], suggesting that neither impairments in protein import, ETC enzyme assembly nor mtDNA integrity are primary causal factors in the decline of organelle function.

However, it is known that PGC-1α mRNA and protein content are reduced in both slow- and fast-twitch muscles with age [269] along with decrements in their downstream targets [262]. This suggests that reductions in mitochondrial function or content could be attributable to the loss of this important co-activator. In support of this, overexpression of PGC-1α specifically in skeletal muscle of aged animals retains mitochondrial content and function, and protects from sarcopenia [270]. Interestingly, endurance training is also capable of rescuing the deficiency in mitochondrial content and function in aged skeletal muscle. However, this beneficial effect of endurance training with age requires PGC-1α, as training is incapable of rescuing the mitochondrial decline in aged PGC-1α KO mice [271]. Thus, the decline in PGC-1α (and/or one of its isoforms) expression accompanied by a reduction in the transcription of NuGEMPs may be one of the most compelling reasons for the decrease in organelle content with age.

Lastly in regards to PGC-1α, it has recently been identified to also have a role in autophagy/mitophagy [272]. Therefore, a decline of this co-activator may impact quality control mechanisms in aged muscle, through decreased biogenesis and decreased clearance. If old organelles are not being purged from the cellular environment, and they are becoming increasing dysfunctional, this would lead to increased ROS production, mtDNA damage, nuclear DNA fragmentation and the induction of muscle atrophy. Further research in this area is warranted to gain a comprehensive picture of the dual role for PGC-1α in mitochondrial maintenance with aging.

An important issue to resolve in the aging literature is whether the decrements in mitochondria observed with age are a result of aging-related declines in the processes which determine mitochondrial maintenance in muscle (i.e. biogenesis and mitophagy), or whether these can be attributed, partially or entirely, to reductions in physical activity which accompany aging. It is known, for example, that very active older individuals retain higher levels of mitochondrial content and respiration [273–275], and that ROS production appears to be equal in older and younger subjects when matched for physical activity levels [276,277]. However, several studies have indicated that a variety of organelle functions remain depressed even when physical activity levels between young and old subjects are carefully matched [35,278], suggesting true age-related deficits in mitochondrial function. For example, older human subjects have exhibited mild organelle uncoupling, greater mitochondrial permeability transition pore (mtPTP) sensitization, and a 3-fold greater fraction of endonuclease G positive myonuclei [276], suggesting a greater propensity to undergo apoptosis. Nonetheless, this conclusion remains controversial [250,279,280], and the level of physical activity of the individual is certainly one of the most important determinants of organelle function in aging muscle. However, cross-sectional studies do not allow for the strongest conclusions because of the inherently divergent genetic and behavioural characteristics of the subjects. As discussed in Figure 3, appropriately-dosed exercise can be used to facilitate the interpretation of whether decrements in mitochondria (content or function) are due to physical inactivity, or aging *per se*. For example, if the largely "normal" decrease in mitochondrial content observed in aging individuals is completely reversed by a comparable training programme at the same relative intensity and duration as in young individuals, then one could interpret the decrement prior to training as a consequence of physical inactivity [281]. On the other hand, if a deficit in the adaptation of the muscle persists after the training regimen, then this would imply that aged muscle has an inherent deficit in the pathways that maintain mitochondria that is independent of physical activity levels.

Our understanding of the molecular regulation of mitochondrial biogenesis in aging muscle has benefited from the use of rodent models of exercise. These afford the possibility of strict control over the absolute training workload and in depth examination of the molecular mechanisms. Using genetically homogeneous Fischer Brown Norway/F1 hybrid rats, we and others have previously demonstrated that the initial signalling response to acute contractile activity [282], as well as the subsequent adaptation to a standardized chronic contractile activity regimen is attenuated [262], or delayed [283], in comparison with younger animals. This blunted adaptive mitochondrial response was attributable to reduced elevations of PGC-1α and TFAM, in addition to lack of exercise-induced alterations in PIM components in aged muscle. These data illustrate the potential corrective nature of exercise in ameliorating organelle dysfunction, but also suggest that the kinetics of mitochondrial adaptations in old muscle are delayed in response to an exercise regimen. These data also suggest that an inherent deficit exists in muscle which is 'age-related' and that can only be partially rescued by exercise. It should nonetheless be clear that exercise remains an important therapeutic intervention to ameliorate this mitochondrial decline and restore organelle function, at least in part, with age.

PHARMACOLOGICAL ACTIVATION OF MITOCHONDRIAL BIOGENESIS

Although it is abundantly clear that exercise promotes positive adaptations within muscle for tissue function and energy metabolism, there are portions of the population that are unable or disinclined to participate in regular physical activity. As a result, the search for pharmacological agents which activate molecular pathways similar to those stimulated by exercise is an active area of research [284,285]. Physical activity induces metabolic alterations in multiple tissues and improvements in multi-organ systems that are difficult to fully recapitulate pharmacologically. Nonetheless, molecular compounds can be exploited to encourage mitochondrial biogenesis and improve mitochondrial health, similar to that which is achieved following an endurance exercise training regimen. The utility of these drugs extends beyond improving athletic performance, as improving mitochondrial quality is a viable strategy to enhance systemic health and pathologies associated with numerous diseases.

AICAR

5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) is an analogue of AMP, and an intermediate in the synthesis pathway of

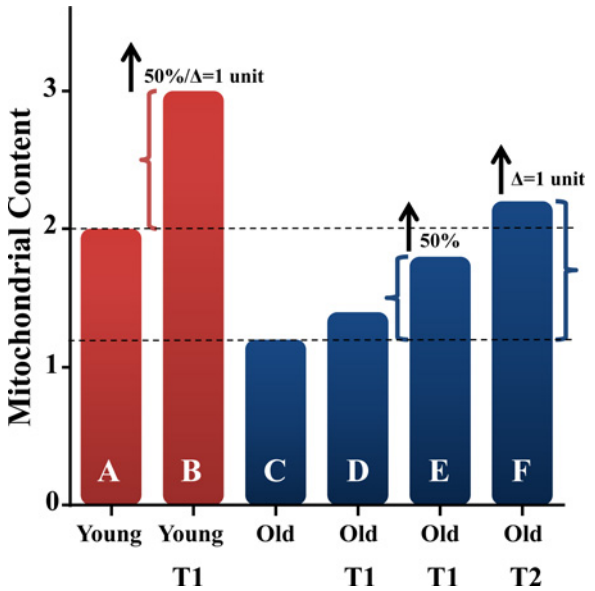

Figure 3 Is the reduction in mitochondrial content in aged muscle due to 'aging' *per se* or due to the inactivity which accompanies aging?

One way to determine the answer to this controversial question is to compare the mitochondrial adaptation of young and old subjects to training. In this hypothetical graph based on literature data, the mitochondrial content in young, healthy, untrained skeletal muscle is represented at a value of 2 (**A**). With an imposed training regimen (e.g. 75 % VO_2max for 6 weeks in humans, or 1 week of chronic contractile activity in rodent models), a typical strong adaptation in mitochondrial content can be represented by a 50 % increase, up to 3 units, representing a change (Δ) of 1 absolute unit (**B**). This degree of adaptation manifests because all mechanisms governing mitochondrial content are intact and responsive to the training protocol. In advanced age, and in the absence of other disease, skeletal muscle may lose 40 % of its mitochondrial content, to a level approximately 60 % of what is observed in young, untrained muscle (**C**). When a similar training regimen of the same *relative* workload (T1, e.g. 75 % VO_2max) and duration is applied to aged skeletal muscle, two scenarios may result. In the first (**D**), an adaptation significantly less than a 50 % increase reveals that the exercise level is incapable of restoring mitochondrial content in aged muscle. This suggests that the impairment in mitochondrial content with age is not simply due to inactivity, but also a result of decrements in the fundamental mechanisms of mitochondrial biogenesis (e.g. upstream signalling mechanisms, transcription of genes etc.) that cannot be reversed by exercise alone. In the second scenario (**E**), if the same relative workload paradigm elicits a 50 % increase relative to the starting point (D), this can be viewed as the same adaptive response in the young and aged muscle, indicating that the decrement in mitochondrial content in the aged muscle is simply due to the inactivity accompanying aging, and can be fully reversed by an appropriate physical activity programme. It also suggests that the molecular signalling mechanisms remain responsive to training in aged individuals. This adaptation is the same percentage increase as in young muscle, but the overall mitochondrial content of the muscle still falls below that seen in young, untrained subjects because of the lower starting level prior to training (C). Further interpretations are possible if the aged muscle is trained at the same *absolute* workload as the young muscle (T2). This may not be possible to achieve in human subjects, but can be accomplished using a chronic contractile activity paradigm in rodents (**F**). This workload would represent a greater relative workload for the aged muscle because of its lower initial mitochondrial content, and thus is a greater stimulus for adaptation. The expectation, in the absence of an impaired mitochondrial biogenesis activation pathway, is an increase in mitochondria of the same absolute magnitude, or more, as in young muscle (i.e. $\Delta = 1$ unit) if the signalling pathway towards biogenesis is fully intact. The absence of this response would suggest that the biogenesis pathway is impaired in aged muscle.

inosine monophosphate [286,287]. As an AMP analogue, it can allosterically activate and promote the phosphorylation of AMPK, a kinase which is intimately involved in metabolic sensing and the control of muscle adaptation to exercise [288]. When activated by AICAR, AMPK phosphorylates PGC-1α, increasing its activity, as well as its cooperation with other transcription factors to activate its own promoter [90,92]. This causes an increase in both PGC-1α mRNA and protein content in muscle cells *in vitro* and in muscle *in vivo* [58,92,94,289–291]. Consequently, this augments the expression of many genes related to fatty acid oxidation and oxidative phosphorylation in skeletal muscle [91,289–293], and induces a shift in muscle fibre composition towards that of a "slower" muscle [289]. Systemically, this contributes to an increase in basal oxygen consumption and a notable improvement in endurance exercise performance [289,292]. These data suggest that AICAR treatment brings about mitochondrial adaptations that are similar to that of endurance exercise. Despite the large number of positive adaptations that AICAR can induce in skeletal muscle, the true utility of AICAR alone as a pharamacotherapeutic agent or as an 'exercise pill' has been brought into question for a number of reasons, including off-target cellular effects, compound half-life *in vivo* and oral bioavailability when administered alone [294,295]. Developing techniques to improve the bioavailability of AICAR, or compounds to more specifically target AMPK in skeletal muscle without experiencing undesired cellular effects, are certainly areas of interest.

GW501516

This compound was developed several decades ago as a selective agonist for the nuclear receptor PPARδ/β [296]. In skeletal muscle, PPARδ/β has been identified as a positive transcriptional regulator of fatty acid oxidation, mitochondrial metabolism and a slower muscle fibre phenotype [297–299], and is thus an attractive agent for therapy. Activation of PPARδ/β by GW510516 increases the expression of genes involved in energy uncoupling, fatty acid oxidation and transport [292,300–303], and is reliant on PGC-1α for this effect [302]. However, its ability to induce mitochondrial biogenesis in skeletal muscle under basal conditions has been questioned [292,301,302,304]. Instead, GW501516 appears to produce large improvements in endurance performance, mitochondrial content and a shift towards a slower fibre type composition when combined with endurance exercise training [292]. Further, when administered alongside AICAR, an increase in the expression of a number of genes involved in oxidative metabolism is observed [292]. Due to some of the aforementioned drawbacks of AICAR treatment by itself, the use of GW501 516 in conjunction with a more specific AMPK agonist may be a preferred route to increasing mitochondrial volume.

SIRT1 activators

Resveratrol

Resveratrol is a natural occurring polyphenol, which has garnered attention in recent years for its proposed role in disease prevention and antioxidant capabilities [305,306]. This compound has also been recognized as an upstream activator of SIRT1 [10,307–309], a deacetylase which has a well-established role in regulating mitochondrial function and mass [10,310] through the deacetylation and activation of PGC-1α (Figure 1) [311,312]. In rodents, treatment with resveratrol has revealed increases in kinase signalling towards mitochondrial biogenesis, enriched expression of mitochondrial genes and proteins, as well as elevations in mitochondrial volume in skeletal muscle [10,308,309,313], an effect which is enhanced when resveratrol is administered concomitant with an exercise programme [10]. In turn, this contributes to an improvement in muscle function and whole-body endurance performance [10,314]. Unfortunately, recent results from human studies utilizing resveratrol supplementation have shown mixed success [315–319], perhaps owing to differences in study design, outcome measures, target population, treatment length and dose, in addition to other confounding variables. Thus, continued attention to optimizing resveratrol dose and duration

for the purpose of improving mitochondrial content and function in human skeletal muscle is still of value.

NAD + precursors

NAD + is a cofactor for SIRT1, and may be another means by which this deacetylase may be activated. Increasing intracellular concentrations of precursors to NAD +, such as nicotinamide riboside (NR), has been considered a worthwhile means to enhance mitochondrial biogenesis [320]. Indeed, oral intake of NR elevates the NAD + content in multiple tissues, increases mitochondrial content and cristae density in skeletal muscle, and enhances endurance exercise performance [321,322]. Further, treatment with acipimox, a niacin derivative and another NAD + precursor, improves mitochondrial biogenesis and function both *in vitro* and in human skeletal muscle *in vivo* [323]. The efficacy of NAD + precursors is promising for the treatment of various myopathies, which are characterized by compromised mitochondria, as these compounds can boost mitochondrial gene expression and rescue mitochondrial function [322,324].

CONCLUSIONS

Mitochondrial research in muscle has progressed at an astonishing rate in recent years thanks to substantial improvements in molecular and biochemical techniques, along with advances in animal and cell culture models used to study the organelle. It has become abundantly clear that there are a number of intersecting and overlapping molecular pathways that control mitochondrial reticulum connectivity, volume and function. At the same time, research is only beginning to scratch the surface in terms of uncovering novel pathways and factors controlling mitochondrial degradation and turnover. Developing a better understanding of the interplay between the pathways controlling organelle biogenesis and degradation is critical to the development of physiological or pharmaceutical therapies to optimize organelle health within muscle. This knowledge will be crucial to improving the state of the mitochondrial networks in instances where it is compromised, such as during muscle inactivity, aging or disease.

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