Topical Review

The role of AMP-activated protein kinase in mitochondrial biogenesis

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While it has been known for more than 75 years that physical activity is associated with increased mitochondrial content in muscle, the molecular mechanism for this adaptive process has only recently been elucidated. This brief review examines existing studies that have identified AMPK-activated protein kinase (AMPK) and several other key regulators of mitochondrial biogenesis, including peroxisome proliferator-activated receptor-γ coactivator-1α and -1β, calcium/calmodulin-dependent protein kinase IV, and nitric oxide. In addition, the potential role of mitochondrial dysfunction in the pathogenesis of insulin resistance associated with ageing and type 2 diabetes mellitus is also discussed.

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Introduction

The association between physical activity and mitochondrial content has been known for more than 75 years (Needham, 1926). Some of the first observations to establish this association were done by comparing the mitochondrial content in the breast muscle of chickens, which fly infrequently, to the breast muscle of pigeons, which regularly fly for extended periods of time. These studies found that that pigeon breast muscle has more mitochondrial activity and content than the chicken breast muscle (Paul & Sperling, 1952). Other studies also demonstrated that continuously functioning muscles, like cardiac muscle, have more mitochondrial activity and content than sporadically functioning muscles, such as back muscle (Paul & Sperling, 1952). These early studies in animals suggested that muscles responsible for long-lasting and regular physical activity are capable of increasing their mitochondrial activity and content to fulfill their roles.

Endurance exercise and mitochondrial biogenesis

Endurance exercise studies during the late 1960s and early 1970s provided further evidence that repeated bouts of physical activity increase mitochondrial activity and content. Mitochondrial biogenesis was observed in 6-week-old rats subjected to treadmill exercise 5 days per week for 3 months. At the end of their training, the exercising rats' skeletal muscle contained a higher concentration of cytochrome *c* and increased activities of key mitochondrial enzymes (Holloszy, 1967). Further studies established the same trend in humans (Morgan *et al.* 1971; Gollnick *et al.* 1972; Hoppeler *et al.* 1973). Fink and colleagues compared a subset of exercise-trained individuals, elite distance runners, and sedentary individuals and found that the runners have a much greater percentage of oxidative, slow-twitch skeletal muscle fibres and more succinate dehydrogenase activity than sedentary controls (Fink *et al.* 1977). Yet despite the long-recognized link between mitochondrial biogenesis and stimuli such as endurance exercise, the critical factors regulating mitochondrial biogenesis have remained elusive until recently.

Newly discovered factors regulating mitochondrial biogenesis

The first major regulator of mitochondrial biogenesis discovered was peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Puigserver *et al.* 1998; Wu *et al.* 1999). Puigserver and colleagues induced PGC-1α mRNA expression in mice by exposing them to cold (4°C) for either 3 or 12 h at a time. Doing so also increased the mRNA expression levels of ATP synthetase (β subunit), cytochrome *c*-oxidase II, and cytochrome *c*-oxidase IV. Irrcher *et al.* (2003) used electrical stimulation in rats to provide further *in vivo* evidence of a link between PGC-1α and mitochondrial

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biogenesis. They detected a rise in PGC-1α protein expression as the electrical stimulation induced an increase in cytochrome *c*-oxidase activity. Lastly, Wu *et al.* (1999) further investigated the link between PGC-1α and mitochondrial biogenesis by examining mitochondrial DNA content and mitochondrial content in mouse myotube cells expressing PGC-1α. They found increases in both, thus clearly illustrating a strong connection between PGC-1α and mitochondrial biogenesis.

PGC-1α is a cotranscriptional regulation factor that induces mitochondrial biogenesis by activating a group of transcription factors, including nuclear respiratory factor 1 (NRF1) and nuclear respiratory factor 2 (NRF2), which activate mitochondrial transcription factor A (mtTFA). Previous studies demonstrated that NRF1 and NRF2 are important contributors to the sequence of events culminating in the increase in transcription of key mitochondrial enzymes, and they have been shown to interact with mtTFA, which initiates the replication and transcription of mitochondrial DNA (Clayton, 1992; Scarpulla, 1997; Virbasius & Scarpulla, 1994). Wu *et al.* (1999) further explored the relationship between PGC-1α and the NRFs in a series of experiments that provided evidence that PGC-1α modulates the NRFs via physical interaction and PGC-1α-induced mitochondrial biogenesis requires NRFs. Exercise studies further linked PGC-1α and the NRFs to mitochondrial biogenesis as an acute bout of swimming increased PGC-1α protein expression and NRF-1 binding to the δ-aminolevulinate synthase (δ-ALAS) promoter and NRF-2 binding to the cytochrome *c*-oxidase IV promoter (Baar *et al.* 2002). Taken together, these studies illustrate that PGC-1α plays an indispensable role in mediating a pathway that connects stimuli such a cold or exercise to an internal metabolic response like mitochondrial biogenesis via the NRF transcription factors (Fig. 1).

A homologue of PGC-1α, termed peroxisome proliferator-activated receptor-γ coactivator-1β (PGC-1β), has also been found recently, and studies have demonstrated that it also regulates mitochondrial biogenesis. In L6 myoblasts expressing PGC-1β, mitochondrial biogenesis was observed (Meirhaeghe *et al.* 2003). Lin *et al.* (2002) also found that PGC-1β interacts with NRF-1 to mediate its transcriptional activity (Lin *et al.* 2002). Stimuli like cold and exercise, however, did not increase PGC-1β mRNA expression, thus suggesting that PGC-1α and PGC-1β are stimulated independently. Once stimulated, though, both PGC-1α and PGC-1β clearly regulate mitochondrial biogenesis through NRF-1 to enable the mitochondria to meet the energetic requirements of the cell.

Calcium/calmodulin-dependent protein kinase IV (CaMKIV) has also been identified as a major regulator of mitochondrial biogenesis. Previous studies have shown that CaMKIV influences gene expression in oxidative fibres of myocytes (Wu *et al.* 2000). To examine the potential role of CaMKIV in mitochondrial biogenesis, Wu and colleagues created a transgenic mouse with a skeletal muscle-specific constitutively active form of CaMKIV. They showed that the skeletal muscle of mice with constitutively active CaMKIV contain more copies of mitochondrial DNA and possess more mitochondria

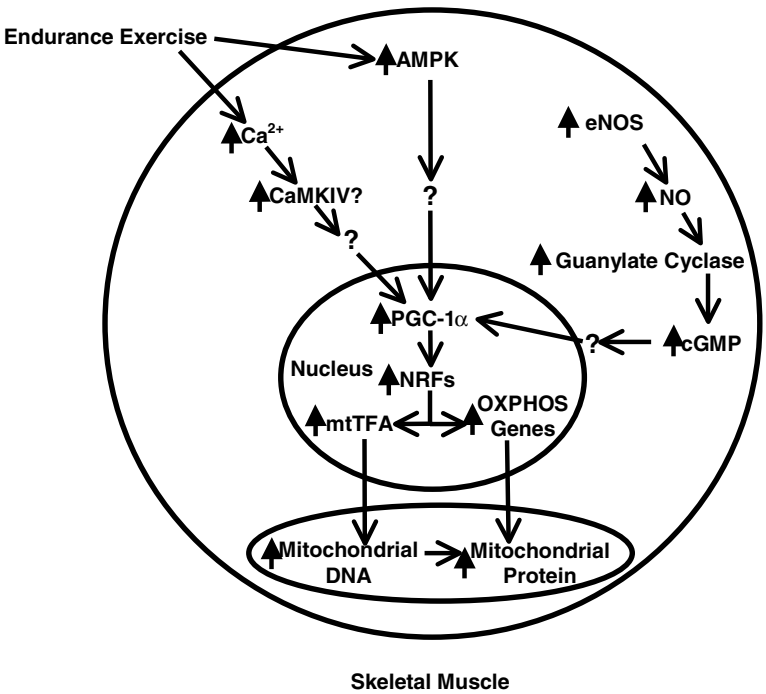

Figure 1. Critical factors involved in mitochondrial biogenesis
CaMKIV, AMPK, and NO cause an increase in PGC-1α gene transcription, which results in gene expression of the NRFs, to which PGC-1α also binds and activates, and finally, mtTFA expression and initiation of mitochondrial DNA replication. The NRFs also induce OXPHOS gene transcription, and the resulting nuclear-encoded proteins then travel to the mitochondria. Lastly, mtTFA is able to transcribe mitochondrial DNA, which leads to the production of mitochondrial-encoded proteins.

as a percentage of the myocytes' volume. In addition, mRNA expression of cytochrome *b* and carnitine palmitoyltranferase-1 was increased in the transgenic line (Wu *et al.* 2002). More recent studies, however, have created doubt about the role of CaMKIV in mitochondrial biogenesis. Akimoto *et al.* (2004) generated CaMKIV null mice, which had similar protein expression levels of PGC-1α and cytochrome *c*-oxidase IV to wild-type mice. Moreover, wild-type and CaMKIV null mice both increased their PGC-1α and cytochrome *c*-oxidase IV protein expression in response to voluntary running, thus indicating that CaMKIV is not required for mitochondrial biogenesis. Finally, Akimoto and colleagues did not detect CaMKIV protein expression in murine skeletal muscle. Thus, it remains unclear what physiological role CaMKIV plays in the regulation of muscle mitochondrial biogenesis in response to physical training.

Recently, nitric oxide (NO) has also been shown to have an effect upon mitochondrial biogenesis. HeLa cells expressing endothelial nitric oxide synthase eNOS (eNOS) displayed increases in mitochondrial DNA content, cytochrome *c* and cytochrome *c*-oxidase IV protein expression levels, and PGC-1α, NRF-1, and mtTFA mRNA expression (Nisoli *et al.* 2003). Nisoli and colleagues also found that the NO produced by eNOS activates guanylate cyclase to increase the amount of cyclic GMP (cGMP) present, which, through an unknown mechanism, transmits a signal to the nucleus that causes the induction of PGC-1α gene transcription and, consequently, mitochondrial biogenesis.

Given the critical role for the AMP-activated protein kinase (AMPK) in regulating intracellular energy metabolism in response to acute energy crises, it is perhaps not surprising that AMPK has also been

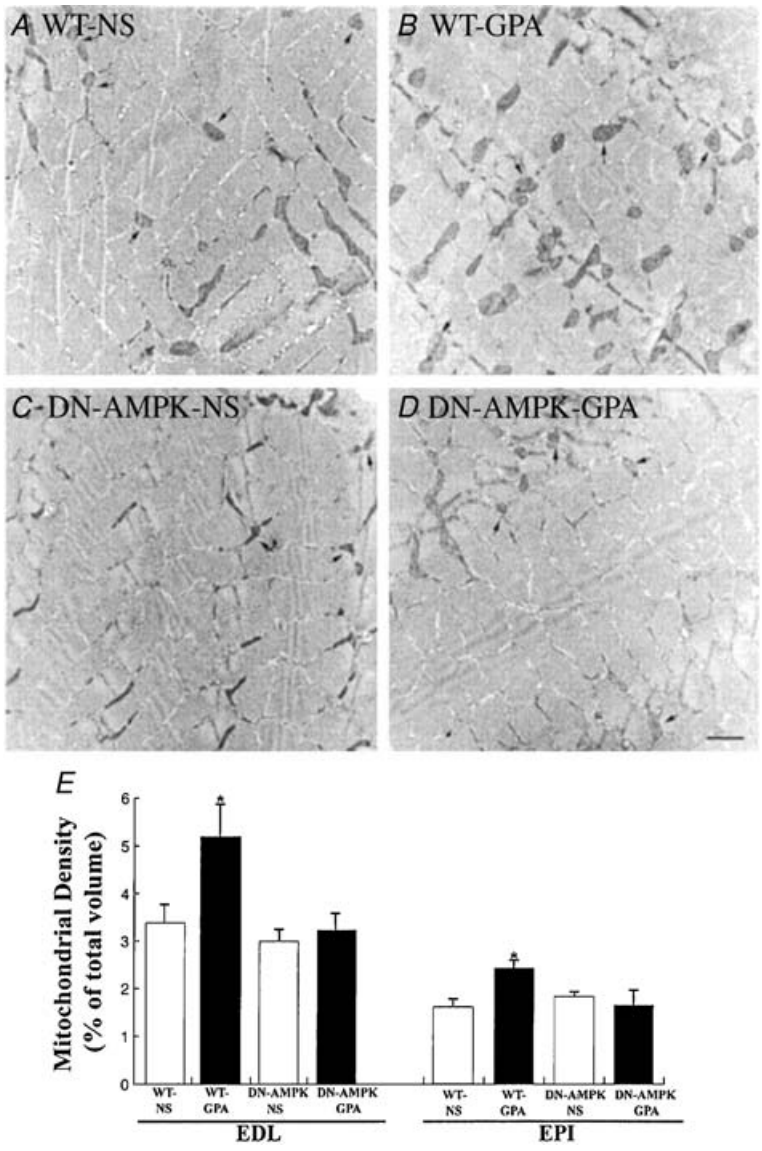

Figure 2. Effect of β-GPA treatment on mitochondrial content as assessed by electron microscopy
Transgenic mice expressing a dominant-negative mutant form of AMPK in skeletal muscle and wild-type mice were fed β-GPA for 8 weeks. Mitochondrial content was then assessed by electron microscopy of the epitrochlearis (EPI) muscle of wildtype (WT) mouse (saline treated) (*A*), WT mouse (GPA treated) (*B*), dominant-negative AMPK transgenic mouse (saline treated) (*C*), and dominant-negative AMPK transgenic mouse (GPA treated) (*D*). Arrows point to mitochondria. (Scale bar, 1 μm.) *E*, mitochondria density (% of total volume) in the extensor digitorum longus (EDL) ($n = 10$ in each group) and EPI muscles of each group of mice ($n = 5$ in each group). $^*P < 0.05$ compared with other groups. From Zhong *et al.* (2002).

identified as a major regulator of mitochondrial biogenesis in response to chronic energy depletion (Hardie, 2004; Hardie & Sakamoto, 2006; Kahn *et al.* 2005). In order to examine the link between chronic energy deprivation and mitochondrial biogenesis, our laboratory fed β-guanadinopropionic acid (β-GPA), a chronic pharmacological activator of AMPK that works by chronically depleting muscle phosphocreatine stores, to rats for 8 weeks. This resulted in chronic AMPK activation in skeletal muscle and increases in NRF-1 binding activity, δ-ALAS mRNA expression, cytochrome *c* protein expression, and mitochondrial content, thus clearly demonstrating that AMPK activation promotes mitochondrial biogenesis through PGC-1α and the NRFs (Bergeron *et al.* 2001). Other pharmacological studies have also established a link between chronic AMPK activation and the up-regulation of key mitochondrial enzymes in skeletal muscle. Winder *et al.* (2000) administered 5′-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) to rats for 4 weeks and observed increased protein expression of cytochrome *c* and δ-ALAS and increased activities of citrate synthase, malate dehydrogenase and succinate dehydrogenase in skeletal muscle. AICAR-induced AMPK activation has also been shown to increase uncoupling protein-3 mRNA and protein expression (Zhou *et al.* 2000; Putnam *et al.* 2003).

To determine if AMPK is required for mitochondrial biogenesis, transgenic mice expressing a dominant-negative mutant form of AMPK in skeletal muscle and wild-type mice were fed β-GPA for 8 weeks. While AMPK was activated in the skeletal muscle of the wild-type mice in response to β-GPA feeding, the transgenic mice fed β-GPA showed no similar increase in AMPK activation. Moreover, PGC-1α mRNA expression, cytochrome *c* protein expression levels, mitochondrial DNA content and mitochondrial density were all increased in the wild-type mice, and none of these parameters were increased in the β-GPA transgenic mice lacking a functional form of AMPK (Fig. 2) (Zong *et al.* 2002). Clearly, these data demonstrate that AMPK is necessary for mitochondrial biogenesis in response to chronic energy deprivation, and it appears likely that pharmacologically activated AMPK conveys its signal to induce mitochondrial biogenesis via the PGC-1α–NRF pathway.

Exercise studies also suggest that AMPK works through PGC-1α to promote mitochondrial biogenesis. Six hours of low intensity swimming resulted in increases in AMPK activation and PGC-1α mRNA expression (Terada *et al.* 2002). Electrical stimulation designed to mimic endurance exercise also activated AMPK and increased PGC-1α protein expression (Atherton *et al.* 2005). These results demonstrate that the physiological activation of AMPK also leads to changes in PGC-1α mRNA and protein expression, although exercise has been shown to increase PGC-1α mRNA expression in the absence of a functional copy of AMPK-α_2 (Jorgensen *et al.* 2005). Exploring further how exercise affects AMPK and PGC-1α and whether this leads to an increase in mitochondrial biogenesis are important issues that need to be addressed.

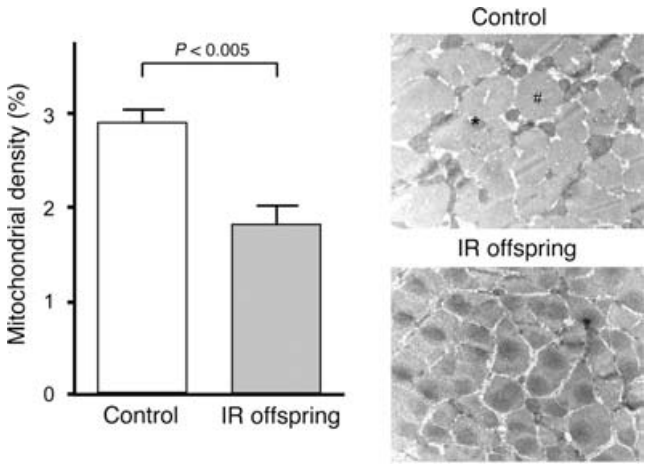

Figure 3. Mitochondrial density and gene expression data of young, lean and sedentary insulin-resistant offspring of parents with type 2 diabetes and control subjects
Mitochondrial density in control subjects ($n = 6$) and insulin-resistant offspring ($n = 8$), assessed by electron microscopy. #, muscle fibre; *, mitochondrion.

Clinical relevance of reduced mitochondrial function and content in the pathogenesis of insulin resistance and Type 2 diabetes mellitus

The link between mitochondria and diabetes was first observed in patients with a rare maternally transmitted form of diabetes-associated deafness, which was found to be due to a 10.4 kb mitochondrial DNA deletion encoding a mitochondrial tRNA (Ballinger *et al.* 1992). These patients have severe β-cell dysfunction, are insulin dependent, and resemble patients with type 1 diabetes. Recent studies by our group have found that a much more mild form of mitochondrial dysfunction may be responsible for causing insulin resistance associated with type 2 diabetes mellitus (T2DM). Using ^{31}P magnetic resonance spectroscopy to non-invasively assess muscle mitochondrial rates of ATP synthesis, our group found a 30% reduction in rates of ATP synthesis in the muscle of young, lean and sedentary insulin-resistant offspring of parents with T2DM (Petersen *et al.* 2004). These young, lean and sedentary insulin-resistant individuals also had increased intramyocellular lipid content, which has previously been shown to cause insulin resistance in muscle and was hypothesized to be secondary to lower mitochondrial activity (Shulman, 2000). In order to understand whether the reduced mitochondrial activity could be attributed to reduced mitochondrial content and/or mitochondrial dysfunction, our group performed

muscle biopsy studies to assess mitochondrial content by electron microscopy in a similar group of young, lean and sedentary insulin-resistant offspring (Morino *et al.* 2005). Using this approach, we found that these insulin-resistant subjects had a 38% reduction in mitochondrial content compared to the control subjects (Fig. 3). These data suggest that the reduced mitochondrial content may be the major factor responsible for the reduced mitochondrial activity and provide further evidence to support the hypothesis that hereditary mitochondrial dysfunction, due to reduced mitochondrial content, contributes to the development of insulin resistance and T2DM.

Alterations in nuclear gene expression essential for mitochondrial biogenesis have also been found in type 2 diabetics. Specifically, type 2 diabetics and first-degree relatives of patients with T2DM displayed reduced NRF-1-dependent gene expression and reduced PGC-1α and PGC-1β gene expression in muscle compared with non-type 2 diabetic subjects (Patti *et al.* 2003). In addition, genes involved in mitochondrial oxidative phosphorylation that are regulated by PGC-1α were expressed less in type 2 diabetics than in non-diabetics (Mootha *et al.* 2003). However, in contrast to these results, recent studies by our group did not detect any changes in the mRNA or protein expression levels of PGC-1α, PGC-1β, NRF1, NRF2, or mtTFA of the young, lean, sedentary insulin-resistant offspring of the type 2 diabetic parents, suggesting that the observed reductions in PGC-1 responsive genes in the previous studies were secondary to T2DM, obesity, or some other acquired factors (Yechoor *et al.* 2002). Furthermore, these data suggest that other as-of-yet unidentified nuclear-encoded factors regulating mitochondrial biogenesis may be responsible for the observed reduction in mitochondrial content. A key question that remains to be answered is whether the reduced mitochondrial content observed in the young, lean and sedentary insulin-resistant offspring is responsible for the increased intramyocellular lipid content or whether it is secondary in nature.

In addition to inherited forms of mitochondrial dysfunction and reduced mitochondrial content, evidence is accumulating that acquired mitochondrial dysfunction also occurs with ageing. Using ^{31}P and ^{13}C magnetic resonance spectroscopy to non-invasively assess rates of mitochondrial oxidative-phosphorylation activity, our group found reductions in mitochondrial oxidative and phosphorylation activity in muscle of healthy, lean and sedentary elderly individuals, who also had increased intramyocellular and intrahepatocellular lipid content and insulin resistance (Petersen *et al.* 2003). Why mitochondrial dysfunction occurs with ageing remains unknown, but age-associated accumulation of mutations in mitochondrial DNA may play a role. Mice with a defective mitochondrial DNA polymerase have been shown to age prematurely (Trifunovic *et al.* 2004) and recently discovered point mutations in the displacement loop (D-loop), where mitochondrial DNA replication is regulated by mtTFA, were shown to accumulate with ageing (Michikawa *et al.* 1999; Del Bo *et al.* 2002).

Intriguingly, young insulin-resistant and obese Zucker diabetic fatty (ZDF) rats showed defects in AMPK phosphorylation and in PGC-1α protein expression levels, and endurance exercise on a treadmill partially restored these abnormalities (Sriwijitkamol *et al.* 2005). These experiments suggest that obesity may impair AMPK signalling and that physical activity reverses obesity's effect. Another recent study further supports this idea as rats fed a high-fat diet for 5 months had reduced AMPK activity and AMPK-α mRNA and protein expression levels, and pharmacological activation of AMPK using metformin increased AMPK activity and AMPK-α mRNA and protein expression levels (Liu *et al.* 2006). While rodent studies have demonstrated a potential link between excess lipid accumulation and impaired AMPK activity, human studies have not found any similar associations (Hojlund *et al.* 2004; Steinberg *et al.* 2004). Nevertheless, given its ability to activate glucose transport through a PI-3 kinase-independent pathway, promote fat oxidation via inhibition of acetyl-CoA carboxylase, and direct mitochondrial biogenesis via promoting increased expression of PGC-1α in skeletal muscle, AMPK is a potentially attractive target to treat and prevent insulin resistance in muscle of patients with T2DM.

Summary

While it has been known for decades that physical activity is associated with increased mitochondrial content, it has been only recently that some of the critical factors involved in the regulation of mitochondrial biogenesis have been identified. Given the potentially important role of mitochondrial dysfunction and reduced mitochondrial content in the pathogenesis of insulin resistance, understanding the molecular mechanisms regulating mitochondrial biogenesis and function may provide potentially important novel therapeutic targets to prevent and treat T2DM.

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