

Role of calcium and AMP kinase in the regulation of mitochondrial biogenesis and GLUT4 levels in muscle

Edward O. Ojuka

Department of Human Biology, University of Cape Town, Cape Town 7700, South Africa

Contractile activity induces mitochondrial biogenesis and increases glucose transport capacity in muscle. There has been much research on the mechanisms responsible for these adaptations. The present paper reviews the evidence, which indicates that the decrease in the levels of high-energy phosphates, leading to activation of AMP kinase (AMPK), and the increase in cytosolic Ca^{2+} , which activates Ca^{2+} /calmodulin-dependent protein kinase (CAMK), are signals that initiate these adaptative responses. Although the events downstream of AMPK and CAMK have not been well characterized, these events lead to activation of various transcription factors, including: nuclear respiratory factors (NRF) 1 and 2, which cause increased expression of proteins of the respiratory chain; PPAR- α , which up regulates the levels of enzymes of β oxidation; mitochondrial transcription factor A, which activates expression of the mitochondrial genome; myocyte-enhancing factor 2A, the transcription factor that regulates GLUT4 expression. The well-orchestrated expression of the multitude of proteins involved in these adaptations is mediated by the rapid activation of PPAR γ co-activator (PGC) 1, a protein that binds to various transcription factors to maximize transcriptional activity. Activating AMPK using 5-aminoimidazole-4-carboxamide-1- β -D-ribose (AICAR) and increasing cytoplasmic Ca^{2+} using caffeine, W7 or ionomycin in L6 myotubes increases the concentration of mitochondrial enzymes and GLUT4 and enhances the binding of NRF-1 and NRF-2 to DNA. AICAR and Ca-releasing agents also increase the levels of PGC-1, mitochondrial transcription factor A and myocyte-enhancing factors 2A and 2D. These results are similar to the responses seen in muscle during the adaptation to endurance exercise and show that L6 myotubes are a suitable model for studying the mechanisms by which exercise causes the adaptive responses in muscle mitochondria and glucose transport.

Mitochondrial biogenesis: GLUT4: Ca^{2+} /calmodulin-dependent protein kinase: AMP kinase: Transcription factors

Regular physical exercise induces increases in the capacity of skeletal muscle to oxidize pyruvate and fatty acids (Holloszy & Booth, 1976) and take up glucose (Ren *et al.* 1994). These adaptations enhance endurance performance and increase the capacity to expend energy. They also prevent and even reverse type 2 diabetes. The increased capacity for respiration, oxidation and energy expenditure are mediated by an increase in muscle mitochondria (Holloszy & Booth, 1976), and the increase in glucose uptake capacity is, in part, the result of an increase in the content of GLUT4, the glucose transport protein (Ren *et al.* 1994). The present short review highlights the evidence, which indicates that the decrease in high-energy phosphates and the increase in intracellular Ca that occur

during contractile activity trigger the processes that cause the adaptive increase in GLUT4 and mitochondria in muscle.

Regulators of genes that express mitochondrial and GLUT4 proteins

Mitochondria comprise gene products derived from nuclear and mitochondrial genomes. Each mitochondrion houses multiple copies of a small circular DNA (mitochondrial DNA) that contributes thirteen mRNA, twenty-two tRNA and two rRNA molecules (Hood, 2001). The thirteen mRNA molecules encode proteins of the respiratory chain. Transcription factors and other proteins required for

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-1- β -D-ribose; AMPK, AMP kinase; CAMK, Ca^{2+} /calmodulin-dependent protein kinase; NRF, nuclear respiratory factor; PGC-1, PPAR γ co-activator-1.

Corresponding author: Dr Edward O. Ojuka, fax +27 21 6867530, email ejuka@sports.uct.ac.za

mitochondrial DNA transcription and mitochondrial assembly derive from nuclear genes. Mitochondrial biogenesis, therefore, requires an orchestrated expression of mitochondrial and nuclear genes. The regulation of this complex process is brought about by the interaction of various transcription factors and coactivators. The transcription factors that have been well characterized include nuclear respiratory factors (NRF) 1 and 2 and PPAR α . PPAR- α is a member of a family of nuclear receptors that stimulate transcription of genes following activation by a ligand. These transcription factors are activated to bind specific DNA sequences when they interact with a co-activator of nuclear receptors termed PPAR γ co-activator-1 (PGC-1; Puigserver *et al.* 1998; Wu *et al.* 1999; Scarpulla, 2002). Activation of NRF-1 by PGC-1 increases the expression of genes that encode mitochondrial respiratory chain proteins (Scarpulla, 2002) and 5-aminolevulinate synthase (Briadotti *et al.* 1993), as well as mitochondrial DNA transcription factor A, a regulator of mitochondrial DNA transcription and replication (Virbasius & Scarpulla, 1994). Kelly's group (Vega *et al.* 2000) showed that activation of PPAR α by PGC-1 stimulates the expression of enzymes of fatty acid oxidation. Overexpression of PGC-1 greatly stimulates mitochondrial biogenesis in cultured myocytes and adipocytes (Puigserver *et al.* 1998; Wu *et al.* 1999). A single bout of swimming induces an increase in PGC-1 mRNA (Terada *et al.* 2001) and protein (Baar *et al.* 2002), as well as NRF-1 and NRF-2 binding to DNA (Baar *et al.* 2002). These observations provide support for the view that PGC-1, NRF-1, NRF-2 and PPAR α are involved in the stimulation of mitochondrial biogenesis by exercise and that activation of NRF-1, NRF-2 and PPAR α by PGC-1 results in a well-coordinated expression of mitochondrial proteins.

GLUT4 expression in response to exercise is regulated at the transcriptional level. Support for transcriptional activation comes from nuclear run-on transcription assays of nuclei from exercised muscles, which show that 30 min after the exercise bout transcription was unaltered, but increased by 1.8-fold after 3 h, and returned to control values by 24 h (Neufer & Dohm, 1993). *GLUT4* gene transcription following exercise is regulated by a number of transcription factors, including myocyte-enhancing factor 2 and GLUT4-enhancing factor. It appears that full expression of GLUT4 after exercise occurs when both myocyte-enhancing factor 2 and GLUT4-enhancing factor are bound to their respective sites (Oshel *et al.* 2000).

Signals that trigger the adaptive processes

Some of the evidence that implicates changes in the concentrations of high-energy phosphates and Ca²⁺ as the initial events leading to the exercise-induced increase in mitochondrial biogenesis and GLUT4 up-regulation will be described.

Changes in high-energy phosphates

During muscle contraction ATP is hydrolysed to ADP by myosin ATPase to drive the cyclic association and dissociation of contractile proteins. Two reactions, driven

by creatine kinase and adenylate kinase, act to replenish ATP by hydrolysing creatine phosphate and ADP to produce creatine and AMP respectively. Thus, during contractile activity of sufficiently high intensity ATP and creatine phosphate levels fall while the level of AMP rises. These changes in the levels of high-energy phosphates stimulate increases in GLUT4 and mitochondrial proteins. Support for this explanation comes from studies in which muscle creatine phosphate is depleted by feeding rats the creatine analogue β -guanidopropionic acid. This treatment causes GLUT4 protein and mitochondrial enzymes (Bergeron *et al.* 2001) in muscle to increase.

A likely link between changes in high-energy phosphates and gene transcription is the enzyme AMP-activated protein kinase (AMPK). AMPK is activated during exercise and phosphorylates key target proteins that control flux through metabolic pathways (Winder & Hardie, 1999). Activation of α_2 -AMPK can be simulated by administration of the drug 5-aminoimidazole-4-carboxamide-1- β -D-ribose (AICAR) to animals. Injection of AICAR into rats activates AMPK and induces an increase in GLUT4 protein (Holmes *et al.* 1999). When epitroclearis muscles are incubated *in vitro* with AICAR for 18 h there is an increase in GLUT4 of approximately 50%, demonstrating that activation of AMPK by AICAR is independent of systemic factors and that the increase in expression of GLUT4 in muscle is rapid (Ojuka *et al.* 2000). A subsequent study using L6 myotubes has shown that chronic treatment with AICAR for 5 d results in increases in GLUT4 and insulin-stimulated glucose transport of approximately 60%. The increase is prevented when adenosine-9-D-arabino-furanoside, an inhibitor of AMPK, is included in the medium (Ojuka *et al.* 2002b). The results of these studies and others (Hayashi *et al.* 1998; Zheng *et al.* 2001) provide strong evidence that AMPK is a key signalling intermediate that improves glucose transport by up regulating GLUT4 expression following exercise. Recently, Fryer *et al.* (2002) have demonstrated more directly, by over-expressing a constitutively-active AMPK gene in skeletal muscle, that AMPK improves glucose uptake and induces GLUT4 biogenesis.

Activation of AMPK also stimulates, at least in part, increases in mitochondrial proteins. After injection of rats with AICAR for 4 weeks there are marked increases in some (e.g. citrate synthase, succinate dehydrogenase and cytochrome c), but not all (e.g. carnitine palmitoyltransferase, hydroxyacetyl-CoA), mitochondrial enzymes in muscle (Winder *et al.* 2000). Chronic incubation of L6 myotubes with AICAR for 5 d increases 5-aminolevulinate synthase and cytochrome c protein by 30–50% compared with controls (Ojuka *et al.* 2002a).

The mechanism by which activated AMPK induces gene expression is not yet clear. As the enzyme is localized in the nucleus of many cells, it has been proposed that it directly regulates gene expression (Salt *et al.* 1998). It is also possible that gene expression occurs secondarily to AMPK activation. Support for the latter view comes from Bergeron *et al.* (2001), who have shown that chronic activation of AMPK by β -guanidopropionic acid results in NRF-1 activation and mitochondrial biogenesis in muscle.

Increase in cytosolic calcium

There is mounting evidence that the increase in cytosolic Ca triggered by each wave of sarcolemmal depolarization during muscle contraction also triggers the events that lead to mitochondrial biogenesis and increased GLUT4 expression. Lawrence & Salsgiver (1983) have reported that exposure of primary cultures of fetal rat muscles to A-23187 (3 μM), a Ca ionophore, induces increases in a number of mitochondrial proteins. Similarly, Freyssenet *et al.* (1999) have shown that continuous exposure of L6E9 myoblasts to A-23187 induces an increase in cytochrome c mRNA. As changes in high-energy phosphate were not measured in these experiments, there is the possibility that the effect of A-23187 on mitochondrial biogenesis was mediated by a decrease in ATP and an increase in AMP, resulting in activation of AMPK, rather than by Ca *per se*. Continuous exposure of cells to A-23187 decreases cell viability and number (E Ojuka and J Holloszy, unpublished results). Thus, because enzyme activity varies markedly between individual myotubes in primary cultures of rat skeletal muscle, it is possible that the sustained increase in cytosolic Ca may have selectively killed myotubes with a low mitochondrial content rather than stimulated mitochondrial biogenesis. Furthermore, because A-23187 mediates Ca entry through the sarcolemma and not from the sarcoplasmic reticulum, it is not clear whether these experiments mimicked the effects of exercise.

In order to address these questions a series of experiments have been conducted in which L6 myotubes were used and various agents that increase cytosolic Ca²⁺ by releasing Ca²⁺ from the sarcoplasmic reticulum (caffeine and W7) were compared with an ionophore (ionomycin) that allows Ca²⁺ entry through the sarcolemma. As L6 myotubes do not contract in response to an increase in cytosolic Ca²⁺, the generation of other potential signals induced by the breakdown of high-energy phosphates (increases in glycolytic intermediates, changes in redox state and pH etc.) that occur in contracting muscle were avoided. To improve cell viability and address the issue of selective destruction of myotubes with low mitochondrial content, myotubes were incubated intermittently for 3–5 h/d, instead of continuously, with the Ca²⁺-raising agents.

The results provide strong evidence that increases in cytosolic Ca²⁺, mediated either by intermittent release of Ca²⁺ from the sarcoplasmic reticulum or Ca entry through the sarcolemma, stimulate mitochondrial biogenesis and GLUT4 expression and that these effects are not mediated by a decrease in high-energy phosphate (Ojuka *et al.* 2002a,b, 2003). Furthermore, in support of the role of Ca, dantrolene, in concentrations that inhibit most of the increase in cytosolic Ca²⁺ induced by caffeine or W7, also inhibits the increases in mitochondrial and GLUT4 proteins. Similarly, removal of Ca²⁺ from the medium with [ethylenebis(oxonitrilo)]tetra-acetate attenuates both the increase in cytosolic Ca²⁺ and the stimulation of mitochondrial biogenesis by ionomycin. Data showing increases in PGC-1, NRF-1 and NRF-2, and mitochondrial transcription factor A in response to raising cytosolic Ca²⁺ (Ojuka *et al.* 2003) provide evidence that the same mechanisms that mediate increases in GLUT4 and mitochondrial

proteins in response to exercise also increase GLUT4 and mitochondrial proteins in L6 myotubes. This evidence shows that L6 myotubes are a good model for studying the adaptations in response to exercise.

As a preliminary step in the investigation of the pathway by which an increase in cytosolic Ca²⁺ stimulates mitochondrial biogenesis and GLUT4 expression, KN93, an inhibitor of Ca²⁺/calmodulin-dependent protein kinase (CAMK; Corcoran & Mean, 2001), was used to evaluate the possibility that the pathway activated by Ca²⁺ that leads to increased mitochondrial biogenesis involves CAMK. Inclusion of 10 μM -KN93 completely blocks the Ca²⁺-induced increases in GLUT4, representative proteins of the mitochondria and NRF-1, NRF-2 and myocyte-enhancing factor 2 transcription factors (Ojuka *et al.* 2002a,b, 2003). These results provide strong evidence that these Ca²⁺-induced effects on GLUT4 and proteins of the mitochondria involve CAMK. Further evidence in support of the role of CAMK have come from Williams' group (Wu *et al.* 2002), who have reported increases in mitochondrial density, cytochrome c and PGC-1 mRNA in transgenic mice that overexpress a constitutively active CAMK IV compared with mice that overexpress WT CAMK. Up-regulation of 5-aminolevulinate synthase, cytochrome c, and GLUT4 in L6 myotubes infected with adenovirus expressing a constitutively-active construct of CAMK IV has also been observed (E Ojuka and J Holloszy, unpublished results).

Concluding remarks

Evidence has been provided that shows that the adaptive increases in muscle GLUT4 and mitochondria induced by exercise are mediated by both the increase in cytosolic Ca²⁺, resulting in activation of CAMK, and the decrease in high-energy phosphates, resulting in activation of AMPK. On the basis of this observation, it seems reasonable to propose that during prolonged steady-state exercise that results in modest perturbations in the high-energy phosphate concentration and minimal activation of AMPK the primary adaptive stimulus is the increase in cytosolic Ca²⁺. On the other hand, intense training that involves periods of very strenuous exercise such as that used in interval training would, by activating both CAMK and AMPK, result in a more powerful stimulus to mitochondrial biogenesis and GLUT4 expression. This outcome would explain the greater effectiveness of high-intensity interval training compared with steady-state moderate-intensity exercise in inducing the adaptive response (Terada *et al.* 2001).

As AMPK and CAMK are closely-related enzymes that belong to the same protein kinase subfamily and recognize the same amino acid consensus sequence (Hawley *et al.* 1995), it seems possible that either CAMK or AMPK may activate the next step in the enzymic pathway leading to the stimulation of mitochondrial biogenesis and GLUT4 expression. As these adaptations are necessary for survival under conditions requiring prolonged vigorous exercise, it is not surprising that redundant mechanisms evolved for stimulating mitochondrial biogenesis and GLUT4 expression.

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