Topical Review

Role of AMPK in skeletal muscle metabolic regulation and adaptation in relation to exercise

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The 5′-AMP-activated protein kinase (AMPK) is a potent regulator of skeletal muscle metabolism and gene expression. AMPK is activated both in response to *in vivo* exercise and *ex vivo* contraction. AMPK is therefore believed to be an important signalling molecule in regulating muscle metabolism during exercise as well as in adaptation of skeletal muscle to exercise training. The first part of this review is focused on different mechanisms regulating AMPK activity during muscle work such as alterations in nucleotide concentrations, availability of energy substrates and upstream AMPK kinases. We furthermore discuss the possible role of AMPK as a master switch in skeletal muscle metabolism with the main focus on AMPK in metabolic regulation *during* muscle work. Finally, AMPK has a well established role in regulating expression of genes encoding various enzymes in muscle, and this issue is discussed in relation to adaptation of skeletal muscle to exercise training.

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Skeletal muscle is characterized by a dramatic increase in energy turnover in response to contraction and exercise. In order to maintain the muscle energy charge, both oxidative and non-oxidative ATP syntheses are increased. To feed the ATP production of the working muscle, carbohydrate originating from plasma glucose and muscle glycogen stores as well as lipid originating from plasma lipids and intramuscular triacylglycerol stores are oxidized (Holloszy *et al.* 1998; Richter *et al.* 2001; Kiens, 2006).

The ability of the skeletal muscle to produce ATP is dependent on content and activities of organelles and proteins responsible for substrate processing, oxidation and ATP production. Acutely, during exercise, the alterations in cytosolic messengers (e.g. Ca^{2+}, free AMP, P_i, creatine, H^+, lipid intermediates) are associated with activation of several signalling cascades and a rapid increase in uptake and intracellular mobilization of glucose and fatty acids. Simultaneously, signalling pathways eliciting transcription of genes encoding metabolic proteins are initiated. Such alterations may change muscle metabolic capacity in the period after exercise, and will in particular be manifested if these pathways are repeatedly activated as during regular physical activity. Even though the acute and chronic regulations may be different in nature and temporally separated, common initiating factors activated during exercise may exist. The 5′-AMP-activated protein kinase seems to be a candidate for this role because it can both regulate cell metabolism acutely and induce transcription of genes encoding various proteins.

The 5′-AMP-activated protein kinase (AMPK) is a multisubstrate serine/threonine protein kinase which is ubiquitously expressed and functions as an intracellular fuel sensor activated by depletion of high energy phospho compounds (Corton *et al.* 1994; Hardie *et al.* 1998). The overall function of AMPK is in response to cellular energy deprivation to increase the potential for ATP production while concurrently decreasing cellular energy-consuming anabolic processes (Corton *et al.* 1994; Kahn *et al.* 2005). The heterotrimeric AMPK holoenzyme consists of one catalytic subunit (α) and two functionally and structurally different regulatory subunits (β, γ). Two isoforms have been identified of the α- and β-subunit (α1 and -2, and β1 and -2) and three isoforms of the γ-subunit (γ1–3) (Kemp *et al.* 2003).

Activation of AMPK in exercising skeletal muscle

It is well established that muscle contraction is associated with a vast increase in energy turnover (>100-fold) and

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introduces a major energetic challenge to the muscle fibre (Sahlin *et al.* 1998). Under conditions of high energy turnover, AMP concentration increases with only a small decrease in ATP concentration. Even though free cytosolic AMP content is heavily buffered by binding to proteins and by deamination to inosine monophosphate (IMP), estimates of the pool of free cytosolic AMP suggested that it increases in response to contraction and exercise and that the increase is exercise intensity dependent (Tullson & Terjung, 1990; Rundell *et al.* 1993). AMP activates AMPK by binding to the two CBS domains on the γ-subunit, which activates AMPK directly by an allosteric mechanism and indirectly by rendering AMPK a better substrate for upstream kinase(s) and a worse substrate for phosphatases thus enhancing a potent activating phosphorylation on Thr^{172} on the α-subunit (Adams *et al.* 2004; Scott *et al.* 2004; Kahn *et al.* 2005). The binding of AMP to the γ-subunit is antagonized by ATP and the $[ATP]/[AMP_{free}]$ ratio is therefore alleged to be the best predictor of AMPK activity (Corton *et al.* 1994), and reduction of this ratio is thought to be one of the major factors activating AMPK during muscle contraction and exercise (Park *et al.* 2002*b*; Chen *et al.* 2003).

A vast line of studies have shown that AMPK is activated in rodent muscle by electrical stimulation *ex vivo* and by motor nerve stimulation of both alive animals and perfused rat hindlimb *in situ* (Hutber *et al.* 1997; Vavvas *et al.* 1997; Hayashi *et al.* 1998; Derave *et al.* 2000). *In vivo*, exercise studies have furthermore shown that AMPK is activated in rat muscle during treadmill running and in human muscle during cycle exercise in a time and exercise-intensity-dependent manner (Winder & Hardie, 1996; Wojtaszewski *et al.* 2000; Fujii *et al.* 2000; Chen *et al.* 2003). In general, in rodent muscle both $\alpha 1$ and $\alpha 2$-associated AMPK complexes are activated during contraction/exercise whereas the majority of human studies display a more pronounced sensitivity and response of activation of $\alpha 2$-AMPK compared with $\alpha 1$-AMPK (Wojtaszewski *et al.* 2000; Musi *et al.* 2001; Park *et al.* 2002*b*; Chen *et al.* 2003). Although this may relate to both intensity and fibre type recruitment, it has become apparent that the expression pattern of AMPK isoforms varies between rodent and human muscle and between muscles types (Chen *et al.* 1999; Durante *et al.* 2002; Frosig *et al.* 2003). In mouse muscle for instance, $\alpha 2$-, $\gamma 1$- and both β-isoforms are fairly evenly distributed between red and white muscle types whereas the $\alpha 1$-, $\gamma 2$- and $\gamma 3$-isoform content is higher in white muscle (Yu *et al.* 2004; Jorgensen *et al.* 2004*b*; Mahlapuu *et al.* 2004; authors' unpublished observations). Furthermore, a recent study investigated the isoform compositions of AMPK complexes in mixed human vastus lateralis muscle and found that only 3 of the 12 theoretically possible AMPK complexes were present in detectable amounts ($\alpha 2\beta 2\gamma 1 \gg \alpha 2\beta 2\gamma 3 = \alpha 1\beta 2\gamma 1$) (Wojtaszewski *et al.* 2005). Even though AMPK in muscle has been shown to be sensitive to circulating factors such as leptin (Minokoshi *et al.* 2002) and adiponectin (Yamauchi *et al.* 2002), the activation of AMPK in exercising muscle seems to depend, at least in part, on local mechanisms. This is so, because AMPK activation is retained in contracting *ex vivo* incubated rodent muscle (Hayashi *et al.* 1998; Jorgensen *et al.* 2004*b*) and because AMPK activation during one-legged exercise in human is restricted to the exercising leg (authors' unpublished observations).

Interestingly, activation of $\alpha 2$-AMPK during *ex vivo* muscle contraction seems mainly to depend on covalent modification on α-Thr^{172} by the upstream LKB1 kinase, since knocking out LKB1 almost completely prevents both AICAR- and contraction-induced $\alpha 2$-AMPK signalling (Sakamoto *et al.* 2005). The increase in α-Thr^{172} AMPK-phosphorylation seems mainly to depend on conformational changes in AMPK which result in it becoming a better substrate for LKB1 as LKB1 activity is not increased during contraction of rodent muscle (Sakamoto *et al.* 2004; Hurst *et al.* 2005). However, LKB1 does not seem to be the only existing AMPK kinase as one study has recently shown increased activity of an unidentified AMPK kinase in exercising human muscle (Chen *et al.* 2003). Since some studies have shown that CaMK kinase phosphorylates AMPK *in vitro* (Hawley *et al.* 1995; Hurley *et al.* 2005), calcium signalling may also have a role in activating AMPK in muscle during exercise. Recent unpublished data from the authors' laboratory obtained in contracting mouse muscle supports this possibility.

Muscle glycogen seems to be an important factor in regulation of muscle AMPK activity. Several studies have shown that glycogen can be a powerful negative controller of AMPK because glycogen loading of muscle suppresses AMPK signalling in response to both exercise/contraction and AICAR (Derave *et al.* 2000; Wojtaszewski *et al.* 2002*a*, 2003). Interestingly, the AMPK β-subunit possesses a glycogen-binding domain binding AMPK to glycogen *in vitro*, but since incubation of AMPK with glycogen particles does not affect AMPK activity the mechanistic link does not seem to be via a direct interaction (Polekhina *et al.* 2003; Hudson *et al.* 2003). Furthermore, if the $\gamma 3$-subunit is knocked out in mouse muscle then the inverse relationship between glycogen content and AMPK activity is no longer seen (Barnes *et al.* 2005) suggesting that only AMPK activity associated with $\gamma 3$-complexes is affected by glycogen. Thus, the reduction in muscle glycogen during exercise could in part explain the steady increase in AMPK activity seen during prolonged bicycle exercise (Wojtaszewski *et al.* 2002*b*; Stephens *et al.* 2002; Rose *et al.* 2005) possibly due to a gradual relief from a glycogen related inhibition.

Interestingly, AMPK also seems to respond to availability of extra-cellular fuel sources by an as yet unknown mechanism(s). For instance, lowering of glucose

in the medium of *ex vivo* incubated muscles and cultured β-cells increases AMPK activity (Salt *et al.* 1998; Itani *et al.* 2003), and also the AMPK homologue in yeast, SNF1, is activated by low availability of external glucose (Hardie *et al.* 1998). Two studies have investigated the influence of oral glucose ingestion on AMPK activation during exercise in human muscle (De Bock *et al.* 2005; Akerstrom *et al.* 2006). Both studies showed that AMPK activity expressed as phosphorylation of AMPK or its downstream substrate ACCβ was not affected significantly by glucose ingestion. However, further analysis in the study by Akerstrom *et al.* revealed that when AMPK signalling was measured as α-isoform specific kinase activity, α2- but not α1-AMPK activity was attenuated during exercise when glucose was ingested simultaneously. Moreover, *in vitro* studies have shown that long-chain fatty acid esters reduce AMPK activation by making AMPK a poorer substrate for LKB1 suggesting that muscle AMPK may be regulated by fatty acid (FA) availability (Taylor *et al.* 2005*b*). However, prolonged exposure to palmitate of resting *ex vivo* incubated muscle does not influence AMPK activity (Olsen & Hansen, 2002), and lowering plasma free fatty acid by prior ingestion of nicotinic acid (acipimox) also does not influence human muscle AMPK at rest (Watt *et al.* 2004*a*). On the other hand, lowering plasma free fatty acids (FFAs) during exercise in humans with nicotinic acid accelerates the exercise-induced AMPK activation (Watt *et al.* 2004*a*), but whether this is due to a reduction in cellular FA or the disturbance of muscle energy balance in not clear. Finally, an earlier study indicated that the energy-rich phosphocreatine inhibits AMPK activity allosterically, and the dramatic decrease in phosphocreatine during the onset of intensive exercise could be speculated to contribute to activation of AMPK by a 'relief of inhibition' mechanism (Ponticos *et al.* 1998). However, recent evidence has shown that phosphocreatine does not affect AMPK activity directly, although it may still be an important factor in regulating AMPK activation during exercise by buffering muscle ATP content and in turn diminishing AMP accumulation (Taylor *et al.* 2005*a*).

AMPK in acute regulation of muscle metabolism at rest and during exercise

In skeletal muscle, AMPK has been shown to have regulatory effects on fatty acid oxidation, glycogen metabolism and presumably also protein synthesis, but the most well-documented action of AMPK in skeletal muscle today is its ability to activate glucose uptake.

Glucose uptake

It was initially shown by Merrill *et al.* (1997) that the adenosine analogue 5-amino-4-imidazolecarboxamide riboside (AICAR) increased glucose uptake and AMPK activity in perfused rat muscle. This association between AMPK activity and glucose transport has been verified as causal by studies in *ex vivo* incubated muscles from transgenic mice where ablation or reduction of AMPK signalling by either over-expressing a kinase-dead α2-AMPK construct or knocking out the catalytic α2-isoform or the regulatory γ3-isoform completely abolished AICAR-stimulated glucose uptake (Mu *et al.* 2001; Jorgensen *et al.* 2004*b*; Barnes *et al.* 2004; Fujii *et al.* 2005). Activation of AMPK by AICAR or hypoxia increases the plasma membrane content of the transmembrane glucose transporter GLUT4, and in this way AMPK seems to control muscle glucose uptake, at least in part (Kurth-Kraczek *et al.* 1999; Mu *et al.* 2001; Koistinen *et al.* 2003) (Fig. 1).

Based on these observations, and because AMPK is activated during muscle contractions, AMPK has been proposed to be a key player in initiating signalling to contraction-stimulated glucose uptake (Merrill *et al.* 1997; Hayashi *et al.* 1998)(Fig. 1). Although obvious, such a connection has been surprisingly difficult to verify even with the use of genetic approaches. Several different genetic strategies have been used to evaluate the relationship between contraction-induced AMPK activation, GLUT4 translocation and glucose transport. Two transgenic mouse models with a muscle specific over-expression of a kinase-dead α2-AMPK construct displayed partially reduced contraction-stimulated glucose uptake in the EDL muscle (Mu *et al.* 2001; Fujii *et al.* 2005). Furthermore, in both mouse models force production was compromised during the *ex vivo* experimental conditions applied which could play some role in the observed reduction in contraction-stimulated glucose uptake (Mu *et al.* 2001; Fujii *et al.* 2005). In contrast, the soleus muscle from the AMPK kinase-dead mouse has normal force production *ex vivo* yet glucose transport is substantially reduced, although not totally eliminated (Mu *et al.* 2001) (T. E. Jensen, A. J. Rose & the authors, unpublished observations). However, the α1-isoform is only partially displaced by the kinase-dead α2 over-expression and the muscle still expresses some α1-AMPK activity which seems to be regulated during contractions (T. E. Jensen, A. J. Rose & the authors, unpublished observations). Similar to the data obtained in the α1- or α2-AMPK knockout models, in which *ex vivo* contraction-stimulated glucose uptake and force production is normal (Jorgensen *et al.* 2004*b*), the interpretation of the data from the two transgenic models may be biased by this residual AMPK activity. Taken together, these findings might be interpreted to mean that if both α1- and α2-AMPK activity are markedly decreased then contraction-induced glucose uptake is diminished, but as long as one of the two α-isoforms is expressed normally then contraction-induced glucose uptake is not decreased, at least during the *ex vivo* conditions studied. Recently, data obtained in mice with

a muscle-specific knockout of LKB1 was reported. In this model, covalent activation of $\alpha 2$-AMPK is largely prevented during contraction and glucose uptake is severely blunted (Sakamoto *et al.* 2005). Although this model depicts LKB1 as a major upstream regulator of AMPK during contraction it does not necessarily link AMPK and glucose transport directly, as LKB1 regulates several other kinases the role of which is not fully elucidated (Sakamoto *et al.* 2004). Finally, it should be committed to memory when evaluating results obtained in transgenic animals that the risk of compensatory alterations of alternative signalling pathways always exists which may affect the interpretations of data.

The signalling pathway(s) by which AMPK regulates the sarcolemma GLUT4 content is far from clear but recent evidence suggests that the Rab binding protein AS160 may be involved in AMPK regulated glucose uptake in muscle (Bruss *et al.* 2005). Studies in 3T3-L1 adipocytes/fibroblasts have shown that AS160 is a negative regulator of Rab-dependent GLUT4 vesicle translocation and is phosphorylated and inactivated by Akt in addition to AMPK (Kane *et al.* 2002; Zeigerer *et al.* 2004). This view is reinforced by the observation that mutating several potential Akt sites on AS160 decreases insulin-induced membrane translocation of GLUT4 in adipocytes (Sano *et al.* 2003; Zeigerer *et al.* 2004). Interestingly, studies in rat muscle have shown that AS160 is phosphorylated both by AICAR and *ex vivo* contraction, suggesting that AS160 is involved in AMPK-dependent GLUT4 translocation (Bruss *et al.* 2005). This view is supported by recent observations showing that lowering AMPK signalling in white muscle by either $\alpha 2$- or a $\gamma 3$-knockout (KO) or over-expressing the kinase-dead AMPK construct severely blunts AICAR-induced increases in AS160 phosphorylation indicating that AMPK complexes containing $\alpha 2$- and $\gamma 3$-complexes are necessary for AICAR-induced AS160 phosphorylation in muscle (Treebak *et al.* 2006). During contraction, the increase in AS160 phosphorylation is still completely prevented in $\alpha 2$-AMPK KO and AMPK kinase-dead muscles but not in $\gamma 3$-AMPK KO muscles indicating that contraction-induced AS160 phosphorylation is independent of AMPK $\gamma 3$-complexes (Treebak *et al.* 2006). It has previously been shown that AICAR-induced glucose uptake is completely prevented in muscle from the $\alpha 2$- and $\gamma 3$-AMPK KO mice and the AMPK kinase-dead mouse, while contraction-induced glucose uptake *ex vivo* is normal in $\alpha 2$- and $\gamma 3$-AMPK KO muscles and only partially reduced in AMPK kinase dead muscles (Mu *et al.* 2001; Barnes *et al.* 2004; Jorgensen *et al.* 2004*b*; Fujii *et al.* 2005). Thus, the relationship between AMPK activity, AS160 phosphorylation and contraction-induced glucose uptake is not clear at this point.

Taken together, these investigations clearly show that AMPK can regulate glucose uptake in resting mouse muscle and that AMPK likely plays a partial role in contraction-stimulated glucose uptake in mouse muscle. However, all studies above employed high-intensity electrically induced isometric contraction which could have led to supra-physiological activation of AMPK, and extrapolation of these results to *in vivo* exercise is not straightforward. Even though several exercise studies in general have reported good agreements between the average increase in glucose uptake and AMPK activity in muscle biopsies from bicycling human subjects (Wojtaszewski *et al.* 2003; Nielsen *et al.* 2002), correlations do not necessarily imply causality. Hence, it seems evident that it is still too early to define the role of AMPK in regulating muscle glucose uptake during exercise, and that more *in vivo* approaches using transgenic mice or specific inhibitor strategies seem warranted to elucidate this aspect.

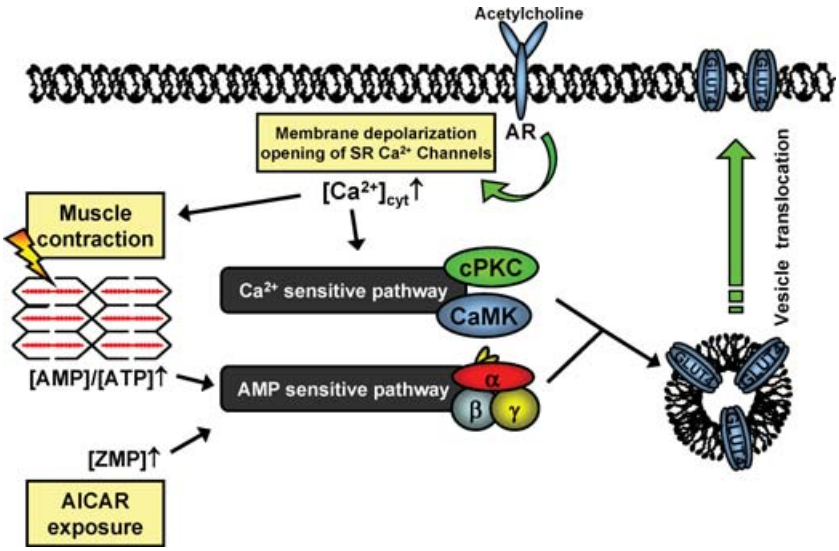

Figure 1. Two pathways thought to regulate muscle glucose uptake during contraction
The increase in cytosolic Ca^{2+} initiates contraction and is also suggested to be a feed-forward stimulus to glucose transport. Lowering of the muscle energy charge is in addition suggested to regulate muscle glucose in a feed-back manner. AR: acetylcholine receptor; SR: sarcoplasmatic reticulum.

Furthermore, alternative signalling pathways to AMPK such as Ca^{2+}-dependent signalling molecules (e.g. CaMK and PKC) may be involved (Rose & Richter, 2005; Jessen & Goodyear, 2005) (Fig. 1).

Glycogen metabolism

Glycogen is an important energy source for the working muscle, especially at moderate to high intensities, and it is broken down by glycogen phosphorylase (GP) and glycogen disbranching enzyme to provide glycosyl units for glycolysis and oxidative phosphorylation. After a bout of exercise, or in response to insulin, glycogen is under the consumption of ATP (re)synthesized from cellular glucose by a chain of enzymes, and the incorporation of UDP-glucose to glycogen by glycogen synthase (GS) is believed to be the rate limiting step (Roach, 2002)(Fig. 2). Regulation of GP and GS is complex and depends on both allosteric and covalent mechanisms. Originally GP-kinase was found to be a target for AMPK, potentially enhancing GP activity and glycogenolysis (Carling & Hardie, 1989; Young *et al.* 1996). However, more recent *in vitro* evidence has shown that GP-kinase is unlikely to be an AMPK substrate suggesting that AMPK does not regulate glycogenolysis (Beyer *et al.* 2000). The finding that glycogen breakdown during treadmill running in muscle from either the AMPK kinase-dead mouse, or the α2- or γ3-AMPK KO mouse is not compromised (Mu *et al.* 2003; Barnes *et al.* 2004; Jorgensen *et al.* 2005) could support such a view.

In contrast to GP, several lines of *in vivo* and *in vitro* evidence have shown that AMPK phosphorylates GS on Site2 (Ser^7) with a concomitant reduction in GS activity (Carling & Hardie, 1989; Jorgensen *et al.* 2004*a*; Halse *et al.* 2003) which is in agreement with the role of AMPK as a protector of cellular energy homeostasis (Fig. 2). In mouse muscle, the α2-isoform seems to be the most important α-AMPK isoform in phosphorylating GS because knocking out the α2-subunit completely prevented AICAR-induced deactivation of GS (Jorgensen *et al.* 2004*a*). A logical assumption from this finding could be that the α1-isoform is of minor importance in regulation of GS activity in response to AICAR. Even though it seems obvious that AMPK covalently modulates GS activity, it has been difficult to establish that activation of AMPK in fact lowers the rate of glycogenesis in resting muscle. A somewhat surprising finding was that chronic activation of AMPK with AICAR in rat muscle actually results in glycogen accumulation rather than lowering glycogen content (Winder *et al.* 2000). This is probably because activation of AMPK with AICAR in addition to directly inhibiting GS activity also increases glucose uptake and subsequently the cellular content of the allosteric GS activator, glucose-6-P. An AMPK-dependent increase in glucose-6-P would therefore be expected to induce an allosteric activation of GS potentially overruling the deactivating Site2 phosphorylation. This idea is indirectly supported by the finding that if basal AMPK signalling is *increased* by, for example, natural mutations or transgenic approaches, glycogen content is increased (Milan *et al.* 2000; Barnes *et al.* 2004) and if AMPK signalling is *lowered* then muscle glycogen is lowered (Mu *et al.* 2001; Jorgensen *et al.* 2004*b*) in spite of reduced kinase activity towards Site2 on GS (Jorgensen *et al.* 2004*a*). Despite these observations in resting muscle, it could be argued that one function of the activation of AMPK during exercise/contraction is to counteract activation of GS and thus the ATP and glucose consuming glycogenesis.

In working muscle, GS activity is in general found to be increased except during very intense exercise where unchanged or even decreased GS activity has been reported (Nielsen & Wojtaszewski, 2004). Although likely to be complex in nature, one plausible explanation for the increase in GS activity is that the breakdown of muscle glycogen during exercise is associated with dephosphorylation of GS at Site3a and -3b which leads to an elevated GS activity (Nielsen & Wojtaszewski, 2004). However, concurrent phosphorylation of GS on Site2 would act to counter-regulate the effects mediated by dephosphorylation on Site3a and -3b. Studies in the α2-KO mouse suggest that α2-AMPK does not fulfil such a role as the increase in GS Site2 phosphorylation is normal both during treadmill exercised and in *ex vivo* contracted white muscle lacking the α2-isoform (authors' unpublished

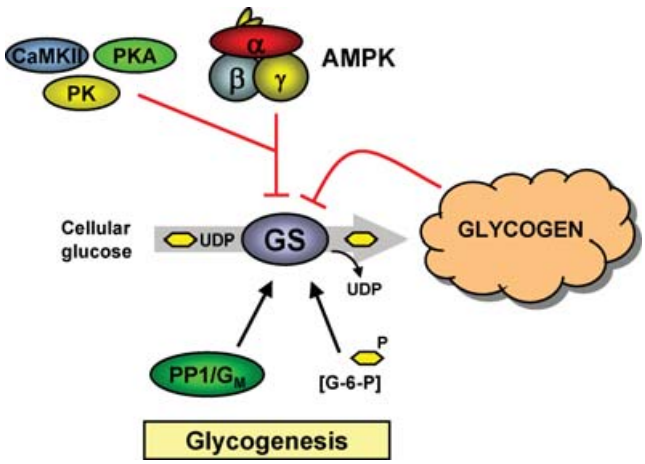

Figure 2. Glycogen synthesis
Glycogen is synthesized by incorporation of cellular UDP-glucose into the glycogen particle. Glycogen synthase (GS) is believed to be the rate limiting step in this process. GS activity is increased by glucose-6-phophate (G-6-P) and phosphatase activity (PP1/G). Conversely, GS activity is reduced by increasing levels of glycogen and by phosphorylation on at least nine serine residues (e.g. Site2/Ser^7) induced by several protein kinases where AMPK, calmodulin-activated protein kinase II (CaMKII), protein kinase A (PKA) and glycogen phosphorylase kinase (PK) are believed to be important Site2 kinases. Black arrow: activation; red arrow: inhibition/deactivation

observations). However, it cannot entirely be ruled out that activation of the remaining α1-isoform in α2-KO muscle had normalized AMPK signalling towards GS during exercise/contraction. Also, it should be remembered that during exercise, several additional GS Site2 kinases (PKA, PKC, CaMK and GPK) are likely to be activated making it difficult to tease out the specific role of each single kinase (Fig. 2).

Fatty acid metabolism

Both resting and exercising muscle oxidize long-chain fatty acids (LCFAs) to generate ATP. These are either transported from the plasma into the muscle or are derived from hydrolysis of intracellular triacylglycerol (IMTG) stores. The transsarcolemmal uptake of LCFAs in muscle is believed to depend on both facilitated diffusion aided by fatty acid translocase (FAT/CD36) and the membrane associated fatty acid-binding protein (FABPpm) as well as simple diffusion (Kiens, 2006). Studies in giant sarcolemmal vesicles obtained from rat muscles have shown that with contractions, FAT/CD36 translocates to the plasma membrane with a parallel increase in fatty acid uptake and this mechanism has therefore been suggested as a regulatory step in contraction-induced LCFA uptake (Bonen *et al.* 2000). This view is in addition supported by the observation that activation of AMPK with AICAR increases FFA uptake in perfused rat muscle (Raney *et al.* 2005). It is also noteworthy that the lowering of AMPK activity by resistin in L6 skeletal muscle cells decreases cell surface FAT/CD36 content (Palanivel & Sweeney, 2005) and that activation of AMPK with AICAR in cardiac myocytes increases FAT/CD36 plasma membrane translocation (Luiken *et al.* 2003). A recent study has, however, shown that inhibition of ERK 1/2 without inhibition of AMPK completely prevented the contraction-induced increase in CD36 translocation to plasma membrane and LCFA uptake (Turcotte *et al.* 2005). Thus, the role of AMPK in contraction-induced uptake of LCFA of muscle is not clear.

Several studies have in addition shown that AMPK can enhance muscle LCFA oxidation. For instance activation of AMPK with AICAR, leptin or adiponectin increases palmitate oxidation in both perfused rat muscle and *in vivo* in mouse muscle and in cultured muscle cells (Merrill *et al.* 1998; Winder & Holmes, 2000; Minokoshi *et al.* 2002; Yamauchi *et al.* 2002). Studies in transgenic animals support these observations since expression of the activating γ3 R225Q mutation in muscle increased oleate oxidation and prevented accumulation of triglycerides in mice fed a high-fat diet (Barnes *et al.* 2004). Interestingly, recent data have shown that resistin lowers AMPK signalling in muscle-like cells and that this reduction is associated with a suppressed fatty acid oxidation (Palanivel & Sweeney, 2005).

The mechanism by which AMPK controls LCFA oxidation in muscle is believed to be at the level of mitochondrial entry of LCFA via phosphorylation of the muscle isoform of acetyl-CoA carboxylase (ACCβ) at Ser^{218}. This interaction does not *per se* alter ACCβ carboxylase activity but desensitizes ACCβ to allosteric activation by cytosolic citrate (Vavvas *et al.* 1997) and also sensitizes ACCβ to inhibition by palmitoyl-CoA (Rubink & Winder, 2005). A decrease in activity of ACCβ will decrease formation of malonyl-CoA (Winder & Hardie, 1996) (Fig. 3). Some studies (Saha *et al.* 2000; Park *et al.* 2002*a*), but not all (Habinowski *et al.* 2001), suggests that AMPK also lowers cellular malonyl-CoA

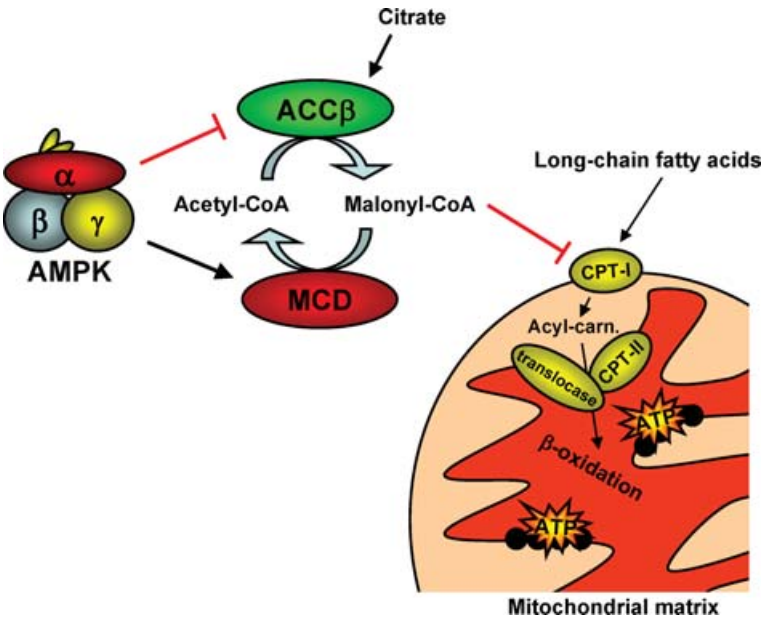

Figure 3. The mechanism by which AMPK is believed to stimulate fatty acid oxidation in the mitochondrial matrix
Production of malonyl-CoA is reduced by inhibiting acetyl-CoA carboxylase-β activity (ACCβ) and possibly by activating malonyl-CoA decarboxylase (MCD) activity. Since malonyl-CoA inhibits carnitine palmitoyl transferase-I (CPT-I) activity, the reduction in malonyl-CoA content will relieve CPT-I from its inhibition leading to increased mitochondrial fatty acid uptake and oxidation. Red oval arrow: inhibition/deactivation. Acyl-carn: acyl-carnitine.

content by phosphorylating and activating malonyl-CoA decarboxylase (MCD), the enzyme responsible for decarboxylating malonyl-CoA to acetyl-CoA (Fig. 3).

Because malonyl-CoA is an potent inhibitor of carnitine palmitoyl transferase-I (CPT-I), the rate limiting enzyme in mitochondrial FA uptake, a reduced level of malonyl-CoA relieves CPT-I from its inhibition in turn allowing carnitinization of LCFA-CoA and subsequent entry to the mitochondrial matrix for β-oxidation (McGarry & Brown, 1997) (Fig. 3). Since AMPK phosphorylates ACCβ (and MCD) and thereby lowers endogenous ACCβ activity during exercise/contraction, AMPK has been hypothesized to be a key player in enhancing muscle LCFA oxidation during exercise.

If the AMPK $\rightarrow$ ACCβ/(MCD) $\rightarrow$ malonyl-CoA axis is a central mechanism in up-regulating LCFA oxidation during exercise, then some degree of correlation between malonyl-CoA and lipid oxidation might be expected. While studies in treadmill exercised rats have shown a decrease in muscle malonyl-CoA at the same time as lipid oxidation presumably is increased (Winder & Hardie, 1996), studies of humans have shown conflicting results. This might to some extent be explained by the fact that the malonyl-CoA level is $\sim$100-fold lower in human than rat muscle (Winder *et al.* 1990; Dean *et al.* 2000; Roepstorff *et al.* 2005; Kraegen *et al.* 2006), perhaps suggesting that malonyl-CoA has a less important role in regulating FA oxidation in human muscle and/or that different mechanisms regulate the malonyl-CoA level in human muscle compared with rat muscle. Thus, in human muscle older studies failed to detect a decrease in malonyl-CoA during both prolonged submaximal and short-term bicycle exercise at various intensities (Odland *et al.* 1996, 1998) while others have reported a decrease in muscle malonyl-CoA levels during submaximal bicycle exercise and one-legged exercise (Dean *et al.* 2000; Roepstorff *et al.* 2004*b*). However, in the study by Roepstorff *et al.* muscle lipid oxidation was manipulated by varying pre-exercise muscle glycogen levels but the decrease in malonyl-CoA concentration was identical in the two conditions despite differences in AMPK activity. In the study by Dean *et al.* it was found that malonyl-CoA concentrations only decreased at higher exercise intensities when lipid oxidation in fact decreased. Finally, a recent study showed that the higher lipid oxidation in females than males during exercise cannot be explained by a higher muscle AMPK activity in females. On the contrary, due to better maintenance of energy status in female muscle during exercise, AMPK activation was in fact lower in females than in males (Roepstorff *et al.* 2006). Taken together, these data may be interpreted to mean that while activation of AMPK and decrease in malonyl-CoA could be responsible for the increase in fat oxidation that occurs from rest to exercise, further fine tuning of fat oxidation during exercise is not carried out by the AMPK $\rightarrow$ ACCβ/(MCD) $\rightarrow$ malonyl-CoA axis. This fine tuning may be related to the muscle availability of free carnitine because carnitinization of LCFA-CoA by CPT-I is necessary for entry into the mitochondrial matrix (Roepstorff *et al.* 2005). However, a recent study has shown that electrically induced contraction of perfused rat muscle *in situ* by a low-intensity protocol increases FA uptake and oxidation without increasing AMPK activity or decreasing ACCβ activity (Raney *et al.* 2005). These findings imply that if AMPK is involved in regulating muscle FA metabolism during exercise this action seems to occur during more intensive exercise and that AMPK-independent mechanisms predominate during low-intensity muscle contraction (Raney *et al.* 2005).

Hormone sensitive lipase

AMPK has also been suggested to be involved in regulation of breakdown of IMTG during exercise via phosphorylation of the enzyme hormone sensitive lipase (HSL) thought to be important if not the rate-limiting enzyme in IMTG breakdown (Kiens, 2006). HSL activity can be modulated by phosphorylation of specific sites and five phosphorylation sites on HSL have so far been identified as regulatory sites. *In vitro* studies have demonstrated that Ser^{563}, Ser^{659} and Ser^{660} are cAMP-dependent protein kinase A (PKA) targets on HSL (Holm, 2003) whereas Ser^{565} (Garton *et al.* 1989) and Ser^{600} (Shen *et al.* 2001) are targets for AMPK and MAPK, respectively. In adipocytes, activation of AMPK with AICAR has been found to inhibit subsequent HSL activation and lipolysis by β-adrenergic stimulation (Sullivan *et al.* 1994) suggesting an antilipolytic role of AMPK (Corton *et al.* 1995).

In skeletal muscle the role of AMPK in regulation of HSL activity and degradation of IMTG during exercise is controversial. No conclusive studies using genetic manipulation of AMPK expression in intact animals have been published. Available evidence stems mainly from human studies in which AMPK activity was manipulated by altering pre-exercise muscle glycogen levels. Thus, Roepstorff *et al.* (2004*a*) found that low pre-exercise muscle glycogen values as expected increased α2-AMPK activity in the vastus lateralis muscle and was associated with increased phosphorylation of HSL on Ser565 during exercise. However, there was no effect on HSL activity measured *in vitro* or on IMTG breakdown during exercise, leading the authors to conclude that AMPK activation and HSL phosphorylation on Ser^{565} do not play a major role in regulation of HSL activity during exercise. In another human study with a similar design, the different conclusion was that activation of AMPK can inhibit HSL activation by β-adrenergic stimulation (Watt *et al.* 2004*b*). This was further supported by studies in L6-myotubes in

which constitutively active AMPK decreased HSL activity (Watt *et al.* 2006). However, in isolated contracting rat soleus muscle, HSL activity varied in the face of constant AMPK activity (Donsmark *et al.* 2004). Taken together, the available data do not allow any firm conclusions about the role of AMPK in regulation of HSL activity in skeletal muscle during exercise.

Protein synthesis

It is well established that global ribosomal synthesis of proteins is a potent consumer of cellular energy, a process among others requiring energy-rich aminoacyl-tRNA, mRNA and ATP/GTP. Protein synthesis can be regulated at several levels such as content of mRNA and ribosomes as well as the specific rate of translating mRNA to the nascent peptide where translation conventionally is subdivided into three phases, initiation, elongation and termination (Proud & Denton, 1997). Furthermore, short-term regulation of protein synthesis is believed to be predominately at the translation level (Bolster *et al.* 2004*a*). The acute regulation of protein synthesis is controlled by several classes of regulatory proteins modulated by the mTOR pathway or the eEF-2 kinase pathway. The mTOR pathway signals to initiation of mRNA translation by activating eukaryotic initiation factor 2 (eIF2) and S6 kinase (S6K1) and assembly of the eukaryotic initiator 4F complex where the latter is achieved by phosphorylating and inactivating eukaryotic initiation factor 4E binding proteins (4E-BP) (Proud, 2004). The calcium sensitive eukaryotic elongation factor 2 kinase (eEF2K or CaMKIII) phosphorylates and deactivates eukaryotic eEF2 thereby inhibiting its interacting with ribosomes and thus impairing the rate of peptide elongation (Carlberg *et al.* 1990).

Some evidence suggests that AMPK can reduce cellular energy expenditure by lowering protein synthesis. Interestingly, subcutaneous injection of AICAR in rat reduces incorporation of phenylalanine to proteins by ∼50% suggesting that the concomitant activation of α2-AMPK leads to an acute reduction of protein synthesis in resting rat muscle (Bolster *et al.* 2002). Since transfection of HEK293 kidney cells with a kinase-dead AMPK construct abolishes AICAR stimulated protein synthesis (Horman *et al.* 2002), the above effect of AICAR *in vivo* seems to depend on AMPK signalling rather than side-effects of AICAR induced by, for example, hypoglycaemia or other AMP-mediated effects in the cell. The mechanism by which AMPK inhibits protein synthesis potentially depends on down- and up-regulation of mTOR and eEF2 kinase signalling to 4E-BPs and eEF2, respectively (Horman *et al.* 2002; Inoki *et al.* 2003; Chan *et al.* 2004). In accordance, AICAR treatment of rat skeletal muscle and cardiomyocytes reduces both 4E-BP-dependent assembly of the eIF4 F complex and eEF2 activity (Bolster *et al.* 2002; Horman *et al.* 2003).

Studies in rat and untrained human have shown that global protein synthesis is reduced during acute dynamic exercise in a time- and intensity-dependent manner (Rennie & Tipton, 2000; Rennie, 2005). For instance, in electrically stimulated perfused rat muscle, protein synthesis is reduced by almost 90% in white muscle types (Bylund-Fellenius *et al.* 1984). Whether the exercise-induced activation of AMPK is directly involved in lowering global protein synthesis in muscle seems convoluted to address, because the mTOR pathway is activated by growth factors/hormones and amino acids and the eEF-2 kinase pathway is activated by Ca^{2+}, and AMPK (Richter *et al.* 2004; Bolster *et al.* 2004*b*; Proud, 2004). However, studies in exercising humans have shown that the inhibitory Thr^{56} phosphorylation on eEF-2 is rapid and sustained during 90 min of exercise, contrasting with the more gradual increase in AMPK activity during the exercise bout (Rose *et al.* 2005). This could indicate a role of other regulatory pathways, e.g. Ca^{2+}-dependent signalling towards mRNA translation during exercise. Due to the complex matrix of intracellular signalling potentially involved in regulating global muscle protein synthesis during exercise, it seems too early to conclude on the role of AMPK in the acute regulation of protein synthesis during muscle exercise. More causal approaches than correlative comparisons are clearly warranted.

AMPK in metabolic adaptation in muscle

In addition to the acute metabolic functions of AMPK, AMPK also regulates expression of specific genes. Repetitive pharmacological activation of AMPK *in vivo* results in protein expression of muscle mimicking some of the effects of exercise training. For instance, activation of AMPK by daily injections with AICAR or chronic intake of the creatine analogue β-guanadinopropionic acid (β-GPA) in rodent increases muscle expression of GLUT4 and hexokinase II (HKII) (Holmes *et al.* 1999; Winder *et al.* 2000; Zheng *et al.* 2001) (Fig. 4). These findings are supported by studies of the AMPK kinase-dead or the α2-KO mouse which showed that muscle mRNA content of GLUT4 and HKII fails to increase in response AICAR (Holmes *et al.* 2004; Jorgensen *et al.* 2005). Furthermore, chronic activation of AMPK with AICAR or β-GPA increases mitochondrial content and expression of mitochondrial proteins (Winder *et al.* 2000; Bergeron *et al.* 2001; Zong *et al.* 2002) (Fig. 4). Again, transgenic approaches to lower or abrogate muscle AMPK signalling strongly suggest a causal role of AMPK in this scheme. In the AMPK kinase-dead mouse, β-GPA-induced increase in mitochondrial content is totally abolished (Zong *et al.* 2002) and an α2-knockout hinders AICAR in increasing protein/mRNA of several mitochondrial markers (citrate

synthaste, HAD, COX1, cytochrome c, CPT-I) (Jorgensen *et al.* 2005; authors' unpublished observations). In accordance with the decreased mitochondrial enzyme content, we have shown that the α2-KO mouse has a disturbed muscle energy balance during exercise as indicated by a reduced ATP content with a comparable increase in IMP content (Jorgensen *et al.* 2005). Thus, these data are in fact the first to demonstrate a crucial role for α2-AMPK in maintaining energy-status in muscle during *in vivo* exercise. Our findings are supported by recent observations showing that LKB1 knockout muscles have disturbed energy balance and α2-AMPK signalling during *ex vivo* contraction (Sakamoto *et al.* 2005).

The mechanism(s) by which AMPK exerts its actions on GLUT4 protein expression and on mitochondrial biogenesis seems to depend on activation of several cellular transcription factors and coactivators. The coordinated induction of mitochondrial biogenesis in muscle is believed to involve the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Scarpulla, 2002). This transcriptional coactivator increases expression of the transcription factors nuclear respiratory factor (NRF) 1 and 2, mitochondrial transcription factor A (mtTFA) and peroxisome proliferator-activated receptor-α (PPARα) (Wu *et al.* 1999; Scarpulla, 2002) (Fig. 4). Interestingly, PGC-1α mRNA and protein increases in response to activation of AMPK with AICAR, β-GPA or thyroid hormone in differentiated muscle and L_6E_9 muscle cells which could indicate that AMPK exerts its mitochondriogenic action, at least in part, by enhancing PGC-1α expression (Zong *et al.* 2002; Irrcher *et al.* 2003; Jorgensen *et al.* 2005). Furthermore, over-expression of both PGC-1α and NRF-1 in cultured muscle cell lines and mouse muscle increases GLUT4 protein and transcriptional activity of myocyte enhancer factor (MEF) isoforms (GLUT4 transcription factors) (Michael *et al.* 2001; Baar *et al.* 2003) which could indicate that AMPK increases GLUT4 expression by a PGC-1α-dependent pathway. Furthermore, activation of AMPK with AICAR increases GLUT4 promoter DNA binding activity of MEF isoforms as well as GLUT4 (Zheng *et al.* 2001; Ojuka *et al.* 2002). In contrast, signalling molecules downstream of AMPK connecting it to protein expression of HKII are far from understood and clearly requires more research.

Based on these findings, a key role of AMPK in inducing metabolic adaptations of skeletal muscle to exercise-training might be hypothesized but only a few studies have provided more conclusive results concerning this aspect. Holmes *et al.* (2004) investigated whether the acute exercise-induced increase in GLUT4 gene activation is compromised in muscle from the AMPK kinase-dead mice. They somewhat surprisingly reported that GLUT4 mRNA increased normally after treadmill running in muscle with impaired AMPK signalling. In concert with this observation, we have shown that PGC-1α, HKII and FOXO1 mRNA increase normally in muscle from the α2-KO mouse after treadmill running despite a ~60% reduction in AMPK signalling during both rest and exercise (Jorgensen *et al.* 2005). A complicating aspect of the study in the α2-KO mice is that the remaining α1-isoform in α2-AMPK KO muscle is still activated and it could be argued that the remaining α1-isoform in α2-KO muscle was sufficient in initiating metabolic adaptations in muscle to exercise. On the other hand, if AMPK is mandatory in initiating metabolic adaptations of muscle to exercise, then the reduction in AMPK signalling should be expected to be manifested at some point. Disregarding these speculations, alternative signalling pathways to AMPK such as CaMK, PKC, MAPK or calcineurin may be involved (Fig. 4).

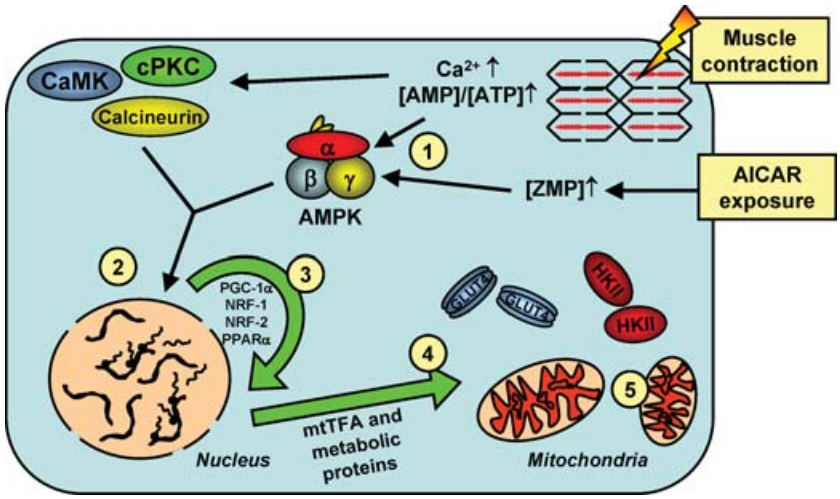

Figure 4. Cellular events proposed to be involved in inducing mitochondrial biogenesis and up-regulation of some metabolic proteins in muscle at rest and during exercise
1, increases in the [AMP]/[ATP] ratio activates AMPK in exercising muscle and ZMP activates AMPK in resting muscle. 2, AMPK and several other signalling molecules increase transcription of nuclear genes encoding metabolic proteins and transcriptional modulators. 3, transcription modulators subsequently activate nuclear genes encoding various mitochondrial and metabolic enzymes as well as mitochondrial genes. 4, synthesis of mitochondrial and metabolic enzymes as well as transcription factor(s) important for activation of mitochondrial genes in the mitochondrial matrix. 5, formation of new mitochondria.

Concluding remarks

A large body of experimental evidence has clearly shown that pharmacological activation of AMPK in resting muscle has profound actions on metabolism of glucose, lipids and proteins as well as on the expression of a large number of genes. Because AMPK is activated in muscle by exercise/contraction it is tempting to assign an important role for AMPK in regulation of muscle metabolism and gene expression during exercise/contraction. However, it has been surprisingly difficult to establish such a role for AMPK unequivocally. One reason for this may be the many additional signalling pathways that are activated during exercise such as MAP kinases, PKCs, CaMKs and calcineurin (Figs 1, 2 and 4) which may have overlapping actions to AMPK. Another reason is the lack of potent and specific pharmacological tools to inhibit AMPK signalling in differentiated muscle. So far, the most conclusive evidence has come from studies using genetic manipulation of AMPK expression/function but even so the available mouse models have sometimes offered less than conclusive findings. While it is somewhat surprising that the role of AMPK in exercise-induced gene activation has not been demonstrated so far, the $\alpha 2$-AMPK KO mouse in fact has decreased expression of mitochondrial proteins and markedly disturbed balance of ATP and AMP in muscle during exercise. The reason for the disturbed energy balance could be related to decreased mitochondrial protein expression or disturbance in mobilization or uptake of substrates. In terms of substrate availability, a role for AMPK in contraction-induced glucose uptake is emerging whereas the jury is still out on the role of AMPK in lipid and protein metabolism during muscle contraction.

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