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Symposium 1: Exercise signalling pathways controlling fuel oxidation during and after exercise

Regulation of glucose transport by the AMP-activated protein kinase

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The AMP-activated protein kinase (AMPK) is an energy-sensing enzyme that is activated during exercise and muscle contraction as a result of acute decreases in ATP:AMP and phosphocreatine:creatine. Physical exercise increases muscle glucose uptake, enhances insulin sensitivity and leads to fatty acid oxidation in muscle. An important issue in muscle biology is to understand whether AMPK plays a role in mediating these metabolic processes. AMPK has also been implicated in regulating gene transcription and, therefore, may function in some of the cellular adaptations to training exercise. Recent studies have shown that the magnitude of AMPK activation and associated metabolic responses are affected by factors such as glycogen content, exercise training and fibre type. There have also been conflicting reports as to whether AMPK activity is necessary for contraction-stimulated glucose transport. Thus, during the next several years considerably more research will be necessary in order to fully understand the role of AMPK in regulating glucose transport in skeletal muscle.

Exercise: Glucose uptake: AMP-activated protein kinase

Exercise increases glucose uptake into contracting skeletal muscles, enhances post-exercise insulin sensitivity and promotes the oxidation of circulating fatty acids in muscle. In individuals with type 2 diabetes, these changes play an important role in the beneficial long-term effects of exercise, such as lowering blood glucose concentrations and improving lipid profiles. Regular physical exercise can also prevent or delay the development of type 2 diabetes. Thus, exercise is an important metabolic stimulus that can regulate glucose and lipid homeostasis in man.

Despite many years of research, the molecular mechanisms by which exercise promotes these beneficial effects are not fully understood. In recent years the AMP-activated protein kinase (AMPK) has been implicated in the regulation of many of the acute effects of exercise on skeletal muscle metabolism. There is also evidence that this enzyme may be involved in the chronic adaptations to exercise training in skeletal muscle through the modification of gene expression of several proteins. In the present paper background on AMPK is provided and the literature suggesting that AMPK is involved in the regulation of glucose transport in skeletal muscle is discussed.

AMP-activated protein kinase

AMPK is the mammalian homologue of the sucrose non-fermenting 1 protein kinase in yeast, which plays a key role in the adaptation of yeast to nutrient stress (Hardie *et al*. 1998). AMPK is a heterotrimer formed by an α subunit, which contains the catalytic domain, and by the β and γ subunits, which are important in maintaining the stability of the heterotrimer and for substrate specificity (Hardie *et al*. 1998; Kemp *et al*. 1999). AMPK is activated through Thr^{172} phosphorylation of the α subunit by one or

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; FDB, flexor digitorum brevis; NOS, NO synthase; PKC, protein kinase C.

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more upstream kinases (AMPK kinase) and allosterically by increases in AMP:ATP and creatine:phosphocreatine (Hardie *et al*. 1998; Kemp *et al*. 1999). The α subunit has two known isoforms (α1 and α2) and contains the catalytic domain that transfers a high-energy phosphate from ATP to serine and threonine residues on a number of different target proteins. The α subunit also contains the specific threonine residue (Thr^{172}) that functions as an activating phosphorylation site for one or more upstream AMPK kinase. Multiple isoforms of β (β1, β2) and γ (γ1, γ2, γ3) regulatory subunits have also been identified, which are essential for full enzymic activity and may have many other functions, including localization of the AMPK molecule within the cell and binding to glycogen. While AMPK is regarded as a ubiquitous enzyme, the expression pattern of individual α, β and γ subunits occurs in a tissue-specific manner. Thus, the composition of the AMPK heterotrimer can vary greatly from tissue to tissue.

Both the α1 and α2 catalytic isoforms are expressed in skeletal muscle. These isoforms share 90% amino acid sequence identity in the catalytic domain and 60% amino acid sequence identity outside this domain. Both isoforms have a molecular mass of approximately 63 kDa (Stapleton *et al*. 1996; Thornton *et al*. 1998). The α2 isoform represents approximately 66% of the total expression level of α subunit mRNA in human skeletal muscle (Fujii *et al*. 2000; Musi *et al*. 2001*a*). Although both α1 and α2 isoforms are distributed throughout the cytosol of skeletal muscle, there is some evidence that a substantial percentage of α2 AMPK is associated with nuclei (Salt *et al*. 1998; Ai *et al*. 2002), suggesting that this isoform may also play a role in regulating gene transcription. The α1 and α2 isoforms are present in muscles composed of both slow-twitch (type I) and fast-twitch (type IIa and IIb) fibres.

The β regulatory subunits of AMPK have an approximate molecular mass of 30–40 kDa and at least the β1 subunit is capable of being modified post-translationally by phosphorylation and myristoylation (Warden *et al*. 2001). These modifications may enable the β subunits not only to regulate AMPK catalytic activity, but also to target the AMPK heterotrimer to the nuclei, cytoplasm and membranes within cells. Fast-twitch muscle fibres (types IIa and IIb) appear to contain both β1 and β2 AMPK, whereas red slow-twitch fibres may contain only the β1 isoform (Chen *et al*. 1999*a*).

AMPK γ subunits may function in the regulation of AMPK catalytic activity and sensitivity to AMP (Cheung *et al*. 2000). Several γ subunit mutations have been identified and can have profound effects on skeletal muscle fuel reserves. Both mRNA (Cheung *et al*. 2000) and protein (Durante *et al*. 2002) of γ1, γ2 and γ3 isoforms have been detected in skeletal muscle, although the fibre-type expression pattern of individual isoforms is not entirely clear. The γ1 isoform has been shown to be similarly expressed in red and white muscle, γ2 is weakly expressed in red muscle and abundantly expressed in white muscle and γ3 has been detected only in red muscle (Durante *et al*. 2002). However, recent data (H Yu and LJ Goodyear, unpublished results) demonstrate that γ3 is the isoform primarily expressed in white fast-twitch muscle fibres.

Exercise increases AMP-activated protein kinase activity

AMPK is an energy-sensing enzyme, such that when the cell is under conditions associated with energy depletion, catalytic activity of the enzyme increases. This activation of kinase activity is thought to primarily function to switch off ATP-consuming pathways and switch on pathways for ATP regeneration. Given that physical exercise can result in marked decreases in muscle energy stores, it is not surprising that there can be marked increases in AMPK activity in contracting skeletal muscles. Regardless of the experimental systems used, it has consistently been shown that contraction increases AMPK activity. For example, treadmill-running exercise in rats *in vivo* (Rasmussen & Winder, 1997; Musi *et al*. 2001*b*), sciatic nerve-stimulated muscle contractions *in situ* (Hutber *et al*. 1997; Vavvas *et al*. 1997) and contraction of isolated muscles *in vitro* (Hayashi *et al*. 1998, 2000; Ihlemann *et al*. 1999*b*; Musi *et al*. 2001*b*) all markedly increase AMPK activity in muscle. Cycle exercise also increases AMPK α2 activity in human subjects in an intensity- and time-dependent manner (Fujii *et al*. 2000; Wojtaszewski *et al*. 2000; Stephens *et al*. 2002). AMPK α2 is activated during moderate-intensity exercise, while, in general, AMPK α1 is more resistant and requires a stronger stimulus to be activated, such as electrically-stimulated isolated rat muscle contractions (Musi *et al*. 2001*b*) and, in human subjects, supramaximal exercise (Chen *et al*. 2000). Moderate-intensity exercise does not increase AMPK α1 activity (Fujii *et al*. 2000; Wojtaszewski *et al*. 2000; Stephens *et al*. 2002). In addition to exercise and contractile activity, stimuli such as hypoxia, hyperosmolarity and uncouplers of oxidative phosphorylation increase activity of the enzyme in skeletal muscle (Hayashi *et al*. 2000; Hardie & Hawley, 2001).

AMP-activated protein kinase and muscle glucose uptake

Initial evidence in support of a role for AMPK in contraction-stimulated glucose transport came from studies using 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR). AICAR is a compound that is taken up into skeletal muscle and metabolized by adenosine kinase to form 5-amino-4-imidazolecarboxamide riboside 5′-monophosphate, the monophosphorylated derivative that mimics the effects of AMP on AMPK (Merrill *et al*. 1997; Hayashi *et al*. 1998). AICAR infusion enhances insulin-stimulated glucose transport in perfused rat hindlimb skeletal muscles (Merrill *et al*. 1997). In the isolated intact rat epitrochlearis muscle AICAR can stimulate glucose transport in the absence of insulin, similar to the effects of contraction (Hayashi *et al*. 1998; Bergeron *et al*. 1999). Insulin, muscle contraction and AICAR all robustly increase glucose transport in isolated rat epitrochlearis muscles incubated *in vitro* and, similar to contraction-stimulated transport, AICAR-stimulated transport is not inhibited by wortmannin. Furthermore, the increase in glucose transport with the combination of maximal AICAR plus maximal insulin treatments is partially additive, while there is no additive

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effect on glucose transport with the combination of AICAR plus contraction (Hayashi *et al*. 1998). Short-term infusion of rats with AICAR (and glucose to maintain euglycaemia) also increases glucose transport in multiple muscle types.

Similar to contraction, AICAR-stimulated muscle glucose uptake involves GLUT4 glucose transporter translocation to the plasma membrane (Kurth-Kraczek *et al*. 1999). In H-2K^{b} skeletal muscle cells overexpression of constitutively-active AMPK stimulates glucose uptake accompanied by GLUT1 and GLUT4 translocation, indicating that AMPK is sufficient to stimulate glucose uptake and, furthermore, that AMPK-mediated glucose uptake involves glucose transporter translocation (Fryer *et al*. 2002).

Although experiments using AICAR as an AMPK activator have generated important information for the function of AMPK, there are limitations to this approach, as AICAR does not have strict specificity to AMPK (Vincent *et al*. 1991; Young *et al*. 1996). Specific activation and inhibition of AMPK by pharmacological agents would be a valuable approach for more clearly defining the role of AMPK in glucose transport and other metabolic effects but, unfortunately, such compounds are not currently available. Two putative AMPK inhibitors, i.e. iodotubercidin and adenosine-9-D-arabino-furanoside, are not specific to AMPK, and although these compounds inhibit AICAR-induced activation of AMPK, they fail to inhibit contraction-induced AMPK activation in skeletal muscle (Musi *et al*. 2001*b*). A very valuable approach for the elucidation of the functions of skeletal muscle AMPK *in vivo* has been the generation of transgenic mice overexpressing a kinase-inactive AMPK protein (Mu *et al*. 2001). Interestingly, glucose transport in response to electrically-stimulated contractions of hindlimb muscles *in situ* is reduced by only 30% in these mice (Mu *et al*. 2001). This finding suggests that there may be other mechanisms in addition to AMPK that regulate contraction-stimulated glucose transport.

Using adenoviral-mediated gene transfer into rat soleus muscles, it has recently been reported that overexpression of an α2 inactive subunit completely abolished AICAR-induced glucose transport in isolated soleus muscles (Sakoda *et al*. 2002). These findings are very interesting, but results must be interpreted cautiously, since the general consensus and published reports suggest that AICAR does not increase glucose transport in isolated rat soleus muscles. In the adenovirus study (Sakoda *et al*. 2002) overexpression of the mutant α2 isoform was found to decrease total endogenous α subunit expression by 50%, but it was not reported whether this outcome was a result of substitution of the endogenous α1 or α2 isoforms. Given that the mutant transgene was the α2 isoform, it is likely that endogenous α2 was reduced, suggesting that the α2 isoform has the predominant role in glucose transport. In contrast to these findings, it has recently been reported that α2-specific AMPK null mice have normal contraction-stimulated glucose transport in skeletal muscle (Jorgensen *et al*. 2003). These animals have a 5-fold increase in α1 isoform expression in skeletal muscle, although they show no change in contraction-stimulated AMPK activity. Since AMPK is a heterotrimeric complex, the possibility that alterations in complex formation may be independent of specific subunit function cannot be ruled out.

Putative molecules 'downstream' of AMP-activated protein kinase

Several molecules have been implicated in mediating AMPK-stimulated glucose uptake. For example, NO has been proposed to function in the regulation of muscle glucose transport during exercise (Balon, 1999). Furthermore, Kemp and colleagues (Chen *et al*. 1999*b*) have shown that AMPK can phosphorylate endothelial NO synthase (NOS) on Ser^{1177} *in vitro*. In human subjects a high-intensity exercise protocol that increases AMPK activity is associated with increases in the phosphorylation of neuronal NOSμ on Ser^{1451}, a site that is comparable with endothelial NOS Ser^{1177} (Chen *et al*. 2000). These findings, together with studies using cultured muscle cells (Fryer *et al*. 2000), suggest that the effects of AMPK on muscle glucose transport could be mediated through NOS (Fryer *et al*. 2000). However, inhibition of NOS does not decrease contraction-stimulated glucose uptake in rat muscles (Etgen *et al*. 1997; Higaki *et al*. 2001); therefore, the role that NOS plays in AMPK-mediated muscle glucose uptake is unclear.

The p38 mitogen-activated protein kinase has also been proposed as a mediator of contraction-stimulated glucose uptake (Somwar *et al*. 2000). In clone 9 cells AICAR phosphorylates both p38 mitogen-activated protein kinase and AMPK, and AICAR-stimulated glucose uptake into these cells is p38 mitogen-activated protein kinase-dependent (Xi *et al*. 2001). Based on these findings, the authors propose that AMPK stimulates glucose uptake through activation of p38 mitogen-activated protein kinase (Xi *et al*. 2001). It remains to be determined whether the regulation of p38 mitogen-activated protein kinase by AMPK occurs in animal and human skeletal muscle *in vivo*.

As discussed earlier, it is likely that there are multiple signalling mechanisms involved in the regulation of exercise-stimulated glucose transport. One family of proteins that may function in both an AMPK-dependent or -independent mechanism is protein kinase C (PKC). There is longstanding evidence for PKC involvement in contraction regulation of glucose transport (Richter *et al*. 1987; Henriksen *et al*. 1989*a*; Ihlemann *et al*. 1999*a*), although identifying a definitive role for this family of proteins has remained elusive. Inhibition of PKC using polymyxin B has been associated with decreases in contraction-stimulated glucose transport (Henriksen *et al*. 1989*b*; Young *et al*. 1991), although these findings are limited because polymyxin B can decrease contractile force. Calphostin C, an inhibitor of diacylglycerol-sensitive PKC isoforms, has also been shown to inhibit contraction-stimulated glucose transport in rat epitrochlearis muscles, even at concentrations that do not affect contractility (Ihlemann *et al*. 1999*a*).

Other intriguing evidence that PKC may be involved in contraction-stimulated glucose transport comes from a study that suggests that the atypical PKC ζ and λ are

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downstream of AMPK (Chen *et al*. 2002). This study, performed in L6 myotubes and isolated rat muscles, suggests that the effects of AMPK on glucose transport are mediated through the sequential activation of extracellular signal-regulated kinase, proline-rich tyrosine kinase-2, phospholipase D and atypical PKC. However, this hypothesis must be interpreted with caution since inhibition of contraction-induced phosphorylation of extracellular signal-regulated kinase by a mitogen-activated protein kinase kinase inhibitor does not affect contraction-stimulated glucose transport in rat muscles (Hayashi *et al*. 1999; Wojtaszewski *et al*. 1999). Clearly, further research is needed to clarify the role of these molecules in regulating AMPK-mediated metabolic responses.

Regulators of AMP-activated protein kinase activity in skeletal muscle

Several factors can modify the magnitude of AMPK activation in response to various stimuli. Glycogen supercompensation in rat skeletal muscle markedly blunts the acute increases in AICAR- and contraction-stimulated AMPK activity and glucose uptake (Kawanaka *et al*. 2000; Wojtaszewski *et al*. 2002). In contrast, subjects with McArdle's disease who have a high muscle glycogen content, a result of the lack of functional glycogen phosphorylase, have exaggerated muscle AMPK $\alpha2$ activation and glucose disposal during exercise (Nielsen *et al*. 2002). This finding suggests that the inhibitory effect of glycogen on stimulated-AMPK activity and glucose disposal is offset when glycolysis is impaired.

The nutritional state also modifies the activation of AMPK. In rat epitrochlearis and flexor digitorum brevis (FDB) muscles there is a tendency for contraction-stimulated AMPK activity to be higher in the fasted state compared with the fed state, while AICAR increases AMPK activity in these muscles at a similar extent regardless of the nutritional state (Ai *et al*. 2002). In epitrochlearis muscle AICAR- and contraction-simulated glucose uptake is also higher in the fasted state, but in FDB and soleus muscle the increases in glucose uptake by contraction are not affected by the nutritional state.

The activation of AMPK by different stimuli also varies depending on fibre type (Ai *et al*. 2002). In rat muscles contraction produces a strong activation of AMPK in epitrochlearis (type IIb fibres), FDB (type IIa fibres) and soleus (type I fibres), while AICAR increases AMPK activity in both epitrochlearis and FDB but has no effect in soleus. Similarly, AICAR stimulates glucose uptake in epitrochlearis and FDB muscles but has no effect in soleus muscle (Ai *et al*. 2002).

Endurance training also modifies the activation of AMPK by exercise (Durante *et al*. 2002). In red quadriceps muscle from rats exercise strongly activates AMPK $\alpha2$ but not AMPK$\alpha1$, while 7 weeks of exercise training blunts exercise-stimulated AMPK $\alpha2$ activity. In white gastrocnemius muscle exercise does not increase AMPK activity but, interestingly, training leads to increased basal AMPK $\alpha2$ activity and Thr^{172} phosphorylation of AMPK. Exercise causes only a small increase in AMPK $\alpha1$ and $\alpha2$ activities in soleus and training does not alter this response.

Diabetes and obesity effects on AMP-activated protein kinase

By virtue of the fact that AMPK activation is associated with increased glucose uptake in skeletal muscle, this enzyme has become an interesting pharmacological target (Winder & Hardie, 1999; Musi & Goodyear, 2002). Furthermore, the expression profile and activation of AMPK have been investigated in animal models of type 2 diabetes and in human subjects with this disease. In lean Zucker rats contraction of isolated epitrochlearis muscles increases both AMPK $\alpha1$ and $\alpha2$ activities, but in obese Zucker rats only AMPK $\alpha2$ activity increases (Barnes *et al*. 2002). This differential activation of the α subunit isoforms does not affect contraction-stimulated muscle glucose uptake (Barnes *et al*. 2002). The authors studied subjects with type 2 diabetes and found that moderate-intensity exercise increases AMPK $\alpha2$ activity to a similar extent in skeletal muscle from these subjects compared with non-diabetic controls, while the $\alpha1$ isoform does not change in either group (Musi *et al*. 2001*a*). The findings from these studies suggest that AMPK $\alpha1$ is not indispensable for contraction to increase glucose uptake in muscle (Barnes *et al*. 2002) and that AMPK $\alpha2$ is the predominant isoform involved in AMPK-regulated responses to exercise (Wojtaszewski *et al*. 2000; Musi *et al*. 2001*a*,*b*; Stephens *et al*. 2002). Moreover, subjects with type 2 diabetes have a normally-functioning AMPK amenable for pharmacological activation.

Summary

AMPK activity is increased with physical exercise and muscle contraction and this relationship is found in mice, rats and human subjects. There are considerable data in support of a role for AMPK in the regulation of multiple metabolic changes during exercise. The fact that activation of AMPK by pharmacological agents can increase glucose uptake has raised the possibility that this enzyme might be an attractive target for the treatment of insulin-resistant conditions, such as type 2 diabetes and perhaps obesity. Much more work is needed to understand the role of AMPK in the regulation of glucose uptake in contracting skeletal muscle.

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