

AMP-activated Protein Kinase and the Regulation of Glucose Transport

Nobuharu Fujii¹, Niels Jessen^{1,2}, and Laurie J. Goodyear¹

¹Research Division, Joslin Diabetes Center and Department Medicine, Brigham and
Women's Hospital and Harvard Medical School, Boston, Massachusetts

²Medical Research Laboratory and Department of Clinical Pharmacology, University of
Aarhus, Aarhus, Denmark

Correspondence:

Laurie J. Goodyear, Ph.D.

Research Division, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215

Phone: 617-732-2573, Fax: 617-732-2650

Email: Laurie.Goodyear@joslin.harvard.edu

ABSTRACT

The AMP-activated protein kinase (AMPK) is an energy-sensing enzyme that is activated by acute increases in the cellular [AMP]:[ATP] ratio. In skeletal and/or cardiac muscle, AMPK activity is increased by stimuli such as exercise, hypoxia, ischemia, and osmotic stress. There are many lines of evidence that increasing AMPK activity in skeletal muscle results in increased rates of glucose transport. Although similar to the effects of insulin to increase glucose transport in muscle, it is clear that the underlying mechanisms for AMPK-mediated glucose transport involve proximal signals that are distinct from that of insulin. Here, we discuss the evidence for AMPK regulation of glucose transport in skeletal and cardiac muscle, and describe research investigating putative signaling mechanisms mediating this effect. We also discuss evidence that AMPK may play a role in enhancing muscle and whole body insulin sensitivity for glucose transport under conditions such as exercise, as well as the use of the AMPK activator AICAR to reverse insulin resistant conditions. The identification of AMPK as a novel glucose transport mediator in skeletal muscle is providing important insights for the treatment and prevention of type 2 diabetes.

INTRODUCTION

In December of 1997, the *American Journal of Physiology* published a paper by Winder and colleagues showing that perfusion of hindlimb skeletal muscle with AICAR, an AMP-activated protein kinase (AMPK) activator, enhanced glucose transport in the presence of insulin (79). Several months later, our laboratory, in collaboration with Dr. Winder, reported that direct incubation of isolated rat muscles with AICAR increased glucose transport, an effect that was independent of insulin (47). One year later, Bergeron, Shulman and colleagues showed that infusion of AICAR in vivo was also effective in increasing glucose transport into multiple rat skeletal muscles (10). These papers provided the first evidence that AMPK can regulate glucose metabolism in skeletal muscle, and began an intense period of investigation aimed at studying the relationship between AMPK activity and insulin-independent mechanisms to increase glucose transport in this tissue and others. In fact, since those first studies, more than 400 papers have been published examining the relationship between AMPK and glucose metabolism in mammalian cells. In this review, we will focus on studies testing the hypothesis that AMPK regulates glucose transport in skeletal muscle, and touch briefly on the potential role of AMPK in regulating glucose transport in the heart.

AMPK: A CELLULAR FUEL SENSOR

AMPK was first identified as a kinase for hydroxymethylglutaryl-CoA reductase (HMG-CoA) and acetyl-CoA carboxylase (ACC), key regulatory enzymes of steroid and fatty acid synthesis, respectively (reviewed in (40)). AMPK is a serine/threonine kinase that is part of a multi-protein family of kinases that extends from plants to mammals. The mammalian homolog of AMPK in *S. cerevisiae* is the SNF-1 protein kinase, critical for the adaptation of yeast to nutrient stress (40; 82; 113). The AMPK heterotrimer consists of a catalytic α subunit and regulatory β and γ subunits (15; 40; 64). There are multiple isoforms for each subunit (α 1 and α 2, β 1 and β 2, γ 1, 2 and 3) with tissue-specific distribution. In skeletal muscle, α 2 (19; 84), β 2 (17; 116), and γ 1 (19) or γ 3 (78) are the major isoforms expressed and form the majority of AMPK heterotrimer complexes. In heart, all isoforms have been reported to be expressed (39). The α subunit of AMPK is a ~63 kDa protein that exhibits catalytic activity. The α 1

isoform is widely expressed, while the $\alpha 2$ isoform has its highest levels of expression in liver, heart, and skeletal muscle (114). The β and γ subunits appear to be important in substrate specificity and maintenance of heterotrimer stability (40; 64). The β subunit acts as a scaffold for the binding of the α and γ subunits (125), and by virtue of having a putative glycogen binding domain, may also function in the regulation of glycogen metabolism (52; 90). The γ subunit appears to be involved in AMP binding, and has also been implicated in regulating glycogen metabolism, since mutations in the CBS domains of γ subunit lead to altered glycogen metabolism in both skeletal muscle and heart (6; 19; 23; 81; 81).

AMPK has been called the “fuel gauge” of the mammalian cell, activated by a mechanism that involves allosteric modification and phosphorylation (40). When cells sense decreased ATP, AMPK acts to switch off ATP-consuming pathways and switch on alternative pathways for ATP regeneration. One hypothesis is that decreases in intracellular ATP and concomitant increases in AMP increase the AMP:ATP ratio (15; 40; 64), causing a conformational change in the AMPK complex and phosphorylation of the α subunit by one or more upstream kinases (15; 40; 64). Threonine 172 is the major regulatory phosphorylation site within the α subunit (42) and its phosphorylation is essential for catalytic activity (21; 115). Recently, LKB1 (41; 50; 107) and Ca^{2+} /calmodulin kinase kinase (CaMKK) (43; 53; 124) have been identified as enzymes that phosphorylate threonine 172 of the α subunit. In skeletal muscle, LKB1 appears to be the major kinase that regulates AMPK $\alpha 2$ activity in vivo, as our recent work with a muscle-specific LKB1 knockout (68), or in comparing a hypomorphic LKB1 knockout with a muscle LKB1 knockout (102), show ablation of AMPK $\alpha 2$ activity under basal and stimulated conditions. Interestingly, AMPK $\alpha 1$ activity was not significantly reduced (68; 102).

A major physiological stimuli that changes the ATP:AMP ratio is contraction of skeletal muscles during exercise. Thus, as would be expected from a change in cellular energy status, AMPK activity increases in rat skeletal muscle with exercise in vivo (93; 94), sciatic nerve-stimulated muscle contractions in situ (54; 119) and contraction of isolated muscles in vitro (46; 47; 56). The degree of force production generated by contraction (56), as well as the intensity of treadmill exercise (94), are directly related to

the level of AMPK activation. Human studies have shown that both moderate and high intensity exercise increase skeletal muscle AMPK activity and/or Thr¹⁷² phosphorylation (18). In addition to exercise and contractile activity, AMPK activity increases in skeletal muscle and cultured muscle cells during other forms of cellular stress including hypoxia (46; 83), hyperosmolarity (30; 46; 87), and by uncouplers of the mitochondrial respiration chain (46; 87).

Since AMPK was initially identified as an HMG-CoA and ACC kinase, in the early years of AMPK research, AMPK was thought to regulate cellular metabolism by virtue of affecting steroid and fatty acid metabolism. However, as will be discussed in this review, it is likely that the stimulation of glucose transport is also an important mechanism by which AMPK regulates cellular energy status. Since glucose transport is the rate-limiting step in glucose utilization and permissive for ATP generation under most physiological conditions, this hypothesis has greatly expanded the importance of AMPK in cellular energetic homeostasis. Given the dramatic worldwide increase in the prevalence of type 2 diabetes and associated muscle insulin resistance, understanding if and how AMPK functions in insulin-independent muscle glucose transport has great potential for translation into novel therapies.

AICAR INCREASES GLUCOSE TRANSPORT IN SKELETAL MUSCLE AND SEVERAL CELL LINES

As mentioned briefly above, early studies showing a relationship between AMPK and glucose transport in skeletal muscle and heart were done using AICAR, a compound taken up into skeletal muscle and metabolized by adenosine kinase to form 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranotide (ZMP), the monophosphorylated derivative that mimics the effects of AMP on AMPK (47; 79). In addition to studies from the Winder, Goodyear and Shulman laboratories, numerous other groups subsequently demonstrated that AICAR increases glucose transport in rat skeletal muscle, a phenomenon that is now universally accepted. The magnitude of AICAR-stimulated glucose transport is dependent on nutritional status (3) and muscle fiber type (3; 7; 63). Interestingly, in isolated rat soleus muscles, AICAR incubation does not increase glucose transport (3; 7; 63), but AICAR also does not increase AMPK activity ((3; 63) Fujii and Goodyear, unpublished observations). In mouse skeletal

muscle, AICAR incubation increases glucose transport in both EDL (7; 33; 61; 83; 99) and soleus (7; 61). AICAR also increases glucose transport and GLUT4 translocation in vastus lateralis muscle strips from healthy subjects, an effect that was partially impaired in patients with type 2 diabetes (69). AICAR is also effective in increasing glucose transport in a number of cell lines, including Clone 9 cells (1; 127), 3T3L1 pre-adipocytes (1), and several muscle cell lines such as L6 (16; 103), C2C12 (1), and H-2K^b (30; 31). Hindlimb perfusion studies in rats revealed that AICAR-induced increases in skeletal muscle glucose transport are mediated by the translocation of GLUT4 to the plasma membrane (74), and AICAR increased GLUT4 translocation in incubated ventricular papillary muscles and hearts perfused in vivo (96).

LIMITATIONS OF STUDIES USING AICAR

Although experiments using AICAR as an AMPK activator have generated a wealth of information on AMPK function, there are limitations to this approach, and conclusions based using this compound as a surrogate for other stimuli must be treated cautiously. AICAR does not have strict specificity to AMPK, activating all enzymes directly activated by AMP, and AICAR can also have systemic effects when administered in vivo (37; 131). Specific activation and inhibition of AMPK by pharmacologic agents would be a valuable approach to more clearly define the role of AMPK in glucose transport and other metabolic effects, but unfortunately, such compounds are not currently available. Two putative AMPK inhibitors, iodotubercidin and araA, 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 (85). Compound C, which can inhibit AMPK activity in hepatocytes (132), is ineffective in attenuating AMPK activity in skeletal muscle (Musi N, Hirshman MF and Goodyear LJ, unpublished observations). To overcome these limitations, non-pharmacologic approaches have been used to activate and inhibit AMPK activity in vivo, including studies using transgenic and knockout mice and expression of adenoviral-mediated constructs as discussed below.

DOES AMPK REGULATE CONTRACTION-STIMULATED GLUCOSE TRANSPORT?

Much of the initial interest in examining AMPK and glucose transport was focused on testing the hypothesis that AMPK is the signaling mechanism necessary for contraction-stimulated glucose transport. This has always been an important question, given the physiological relevance of exercise in the regulation of glucose transport and control of glucose homeostasis in healthy people and those with type 2 diabetes. Contraction and insulin are two major stimuli that activate glucose transport in skeletal muscle, and there are several lines of evidence that the underlying mechanisms leading to contraction- and insulin-induced glucose transport are distinct (36). AICAR-stimulated glucose transport was not affected by inhibition of PI3-kinase using wortmannin (10; 47), similar to numerous other insulin-independent stimuli that increase glucose transport. The combination of a maximal AICAR stimulus with a maximal insulin treatment had partially additive effects on glucose transport, suggesting that signaling to glucose transport by these two stimuli was via different mechanisms. In contrast, there was no additive effect on glucose transport with the combination of AICAR plus contraction (47), providing evidence that AMPK may be the long-elusive signaling mechanism regulating exercise-stimulated glucose transport. Bergeron *et al* also reported similar findings and further demonstrated that AICAR-stimulated glucose transport is not affected by an adenosine receptor inhibitor that diminishes contraction-induced glucose transport, suggesting that the AICAR effect is directly due to AMPK activation and not to adenosine receptor activation (10). In H-2K^b skeletal muscle cells, overexpression of constitutively active AMPK stimulates glucose transport accompanied with GLUT1 and GLUT4 translocation, also indicating that AMPK-mediated glucose transport involves glucose transporter translocation (30). Contraction of isolated muscle was shown to activate AMPK in a dose-dependent manner and at a similar rate compared to increases in glucose transport in skeletal muscle (85).

In spite of considerable enthusiasm for the idea that AMPK is part of the signaling pathway responsible for mediating contraction-stimulated glucose transport in skeletal muscle, not all findings have been consistent with this hypothesis. To evaluate the contribution of AMPK to glucose transport in skeletal muscle, Mu *et al.* generated transgenic mice that express a dominant negative form of AMPK α 2 subunit in skeletal muscle using the muscle specific-creatine kinase promoter (83). Consistent with the hypothesis that AMPK regulates glucose transport in rat skeletal muscle, they found that

AICAR- and hypoxia-stimulated increases in AMPK activity and glucose transport were completely inhibited. This result provides evidence that AMPK activity is necessary for AICAR-stimulated glucose transport. On the other hand, contraction-induced glucose transport was reduced by only 30-40% in the muscles from the transgenic mice. These results provided the first direct evidence that AMPK is involved in the regulation of contraction-induced glucose transport, but that AMPK is not the sole intermediary of the contraction effect.

While the data from Mu et al suggest that AMPK may be partially regulating glucose transport in contracting skeletal muscle, Jørgensen et al came to a different conclusion. Using either $\alpha 1$ or $\alpha 2$ whole body AMPK knockout mice, they found that contraction-induced glucose transport in isolated muscles was normal (61). Since the $\alpha 2$ knockout mice had a 2- to 3-fold increase in $\alpha 1$ expression and a 2-fold increase in contraction-stimulated $\alpha 1$ activity, as the authors state, it is possible that the upregulation of $\alpha 1$ compensated for the loss of $\alpha 2$ function with regard to glucose transport. We generated muscle-specific transgenic mice (TG) carrying cDNAs of inactive $\alpha 1$ and $\alpha 2$, and crosses of these animals (33). Surprisingly, despite 20-fold greater expression of the $\alpha 1$ transgene (compared to wild type), the $\alpha 1$ transgenic mice had only partially reduced AMPK $\alpha 1$ activity, suggesting there may be very little $\alpha 1$ expressed in skeletal muscle. AICAR- and contraction-stimulated AMPK $\alpha 2$ activity were fully inhibited in these mice, but only AICAR-stimulated glucose transport was fully ablated (33). For glucose transport measured in isolated muscle contracted *in vitro*, we found that similar to Mu et al, there was an approximately 20-30% decrease in the transgenic mice. However, with tedious characterization of muscle force generation with contraction, we determined that the entire decrease in glucose transport could be accounted for by a reduction in force production (33). Thus, our data are consistent with the hypothesis that AMPK is not essential for contraction-stimulated glucose transport in skeletal muscle.

Another approach to addressing the question of whether AMPK is necessary for contraction-stimulated glucose transport would be to disrupt the upstream kinase for AMPK. Recently, the serine/threonine kinase LKB1, in complex with the two accessory subunits, STRAD and MO25 has been identified as an upstream kinase for

AMPK (41; 50; 107), as well as a kinase for 11 of the 12 proteins in the AMPK family (76; 112). Phosphorylation of the Thr¹⁷² site on the $\alpha 1$ and $\alpha 2$ catalytic subunits is essential for AMPK-activity (21; 115). To study LKB1 function in skeletal muscle and its putative role in contraction-stimulated glucose transport, our group (68) has generated a muscle-specific LKB1 knockout mouse (MLKB1KO) where Sakamoto et al have studied a hypomorphic LKB1 mouse that has a 70-80% loss of LKB1 throughout all tissues in the body and complete ablation of LKB1 in skeletal muscle (102). In contrast to studies in AMPK whole body knockout mice and our muscle-specific AMPK $\alpha 2$ inactive transgenic mice, contraction-stimulated glucose transport was partially inhibited in these two LKB1 knockout models, a finding that could not be attributed to a reduced force production during contraction. Since AMPK $\alpha 2$ knockout mice and AMPK $\alpha 2$ transgenic mice have normal contraction-stimulated glucose transport (33; 61), the decrease in contraction-stimulated glucose transport in MLKB1KO mice may be a function of altered AMPK $\alpha 1$ activity, although the majority of data suggest that there may be little AMPK $\alpha 1$ activity in muscle. Alternatively, or in addition, another LKB1 substrate may be necessary for contraction-stimulated glucose transport. The fact that our MLKB1KO mice did not fully inhibit contraction-stimulated glucose transport demonstrates that LKB1 is necessary for a normal response, but also suggests there are additional signals involved in the regulation of this process.

If AMPK $\alpha 2$ activity is not essential for contraction-stimulated glucose transport, then how can one explain the many findings that led to the hypothesis that AMPK regulates glucose transport in skeletal muscle? For example, if increasing AMPK activity using AICAR increases muscle glucose transport, then why would increasing AMPK by muscle contraction not have the same physiological outcome? Furthermore, the combined effects of a maximal AICAR treatment and contraction on glucose transport are not additive, strongly suggesting that these two stimulus work through the same mechanism. The most likely explanation is that there are multiple contraction-stimulated signals that simultaneously contribute to an increase in glucose transport, and that there is redundancy in the system (33). In recent years there has been accumulating evidence that calcium mediated signaling, including regulation of the calcium-calmodulin kinases, are involved in the contraction effect (20; 59). Therefore, it may be that contraction-stimulated glucose transport is regulated by multiple signals

that originate from reductions in energy status of the muscle fibers, redox changes, and calcium release from the sarcoplasmic reticulum. It is also possible that signals emanating from mechanical stress to the muscle fibers could also contribute to changes in glucose transport, although such signals have yet to be linked to the transport process.

AMPK AS A MEDIATOR OF MULTIPLE INSULIN-INDEPENDENT STIMULI OF GLUCOSE TRANSPORT

Exercise and contractions have drawn a great deal of attention as stimulators of an insulin independent stimulus of glucose transport because of the obvious clinical benefits of exercise in metabolic disorders like type 2 diabetes. However, numerous metabolic stressors like hypoxia, hyperosmolarity, the chemical uncoupler 2,4-dinitrophenol (DNP), and the electron transport inhibitor rotenone can all increase glucose transport independently of insulin (46). The discovery of the differences in the involvement of AMPK in AICAR- and contraction-induced glucose transport has made it clear that several pathways may exist leading to an insulin-independent stimulus of glucose transport.

Hypoxia, Rotenone (a Complex I inhibitor), and DNP (a mitochondrial uncoupler), activate AMPK through energy depletion and result in an increase in glucose transport (46). Comparable results are seen when L6 cell and H-2k^b cells are incubated with DNP (31; 87). When muscles from mice expressing AMPK α 2 dominant negative isoforms are subjected to hypoxia or incubated with Rotenone the increase in glucose transport is completely abolished (33; 83). These results indicate there is no compensatory effect on α 2 function by α 1, and strongly support the idea that AMPK α 2 represents the predominant, if not the only, regulator of hypoxia-, and rotenone-stimulated glucose transport. Interestingly, AMPK does not seem to be involved in insulin- (33; 61), phorbol ester- (30), or passive stretch- (56; 57) stimulated glucose transport, which occurs without decreases in intracellular energy status. Interestingly, AMPK α 1 activity, though partly diminished by ~40%, was still significantly increased by rotenone (33). There is a discrepancy in whether osmotic stress activates AMPK by decreasing energy status in cells. While increased AMPK activity after osmotic shock induced by sorbitol, was associated with decreased intracellular ATP, phosphocreatine, and glycogen content in isolated rat epitrochlearis muscles (46), there was no effect of

sorbitol on the ATP/AMP ratio in H-2k^b cells (30). Unlike rotenone and hypoxia, but similar to contractions, glucose transport induced by osmotic shock was not affected in muscles from AMPK dominant-negative mice (33). This is unlike H-2K^b cells where osmotic shock stimulated glucose transport is completely abolished by adenoviral-mediated overexpression of the AMPK α dominant-negative mutant (30).

Hyperosmolarity-induced glucose transport in skeletal muscles may therefore be regulated by a mechanism in which AMPK α 2 is one of multiple signaling pathways. Nevertheless, it is clear that in skeletal muscle, AMPK α 2 activation is essential for some, but not all, insulin-independent stimuli of glucose transport.

SIGNALING INTERMEDIARIES FOR AMPK EFFECTS ON GLUCOSE TRANSPORT

Although it is now well documented that activation of AMPK using various stimuli can increase glucose transport in skeletal muscle and other tissues, the downstream signals that mediate glucose transport have remained elusive. Akt Substrate of 160 kDa (AS160) has recently been identified as a protein that modulates GLUT4 trafficking in insulin-sensitive 3T3-L1 adipocytes (105) and L6 myoblasts (62). In the non-phosphorylated state AS160 prevents the translocation of GLUT4 to the cell surface (26; 80), and when AS160 is phosphorylated by Akt on several phospho-motifs (12; 62) GLUT4 translocates. However, it is not known if AS160 phosphorylation is mandatory for GLUT4 translocation in skeletal muscle. The substrate-motifs of AMPK and Akt show similarities and both kinases can phosphorylate 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase on Ser⁴⁶⁶ (91). AS160 could possibly also be a shared substrate and thereby serve as a point of convergence and integration for multiple effectors of glucose transport. Bruss and co-workers reported that in rat epitrochlearis muscles contraction and AICAR treatment increase AS160 phosphorylation in muscle samples *in vitro* when probed with a phospho-Akt substrate (PAS) antibody (12). However, because AICAR is not a specific AMPK activator it is still not possible to conclude that AS160 is a substrate for AMPK. To determine if AS160 mediates AMPK signaling, our laboratory has assessed AS160 phosphorylation in AMPK α 2-inactive transgenic mice after various stimuli (70). We saw that AICAR-stimulated phosphorylation of AS160 phosphorylation was fully inhibited in AMPK α 2-

inactive transgenic mice, whereas contraction-stimulated AS160 phosphorylation was only partially reduced. Contraction activates Akt (101) and this activation can be inhibited by wortmannin (101). However, combined AMPK α 2 and Akt inhibition by wortmannin treatment of AMPK α 2 transgenic mice did not fully ablate contraction-stimulated AS160 phosphorylation. Similar to glucose transport AMPK α 2 activity is essential for AS160 phosphorylation by AICAR, but AMPK is not indispensable for the entire effects of contraction on AS160 phosphorylation. These results indicate that AMPK can be upstream of AS160 but if AS160 phosphorylation is obligatory for the AMPK signal to glucose transport is not known and several other proteins activated by AMPK have also been suggested as mediators.

Studies in Clone 9 cells identified the stress-activated protein kinase, p38 as a mediator of AMPK- induced glucose transport. Addition of a p38 inhibitor, SB203580, and overexpressing of a dominant negative p38 mutant inhibited AICAR-induced glucose transport in these cells (127). The p38 inhibitor, SB203580, was also shown to diminish contraction-stimulated p38 α and β activities and concomitantly reduced glucose transport in isolated rat EDL and soleus muscles (110). However, recent data show that SB203580 acts as a competitive inhibitor of glucose transport through direct interaction with the glucose transporter (95) and overexpression of dominant negative p38 mutants in skeletal muscle does not affect glucose transport (5). These results are supported by work from our laboratory where we find that another p38 inhibitor, SB202190 non-specifically diminishes both contraction- and insulin-induced glucose transport without inhibition of p38 activation (Boppart and Goodyear, unpublished findings and (34)), and that AICAR activates only AMPK but not p38 in L6 myotubes (48). Taken together a role of p38 as a mediator of the signal from AMPK to glucose transport in skeletal muscles therefore seems unlikely.

Based on studies using treadmill running of mice and L6 myotubes, Chen et al. proposed a new hypothesis that the effects of exercise on glucose transport are mediated by sequential activation of AMPK, Pyk2, Grb2, SOS, Ras, Raf, MEK1, ERK, PLD, and aPKCs (16). This signaling pathway has also been suggested to explain osmotic stress-stimulated glucose transport in L6 cells (100). However, Pyk2 does not seem to be activated by exercise in human skeletal muscle with contraction (120) and by

contraction in rat muscle in vitro (126). ERK inhibition using the MAP kinase kinase inhibitor PD98059 does not affect glucose transport either in slow-twitch fiber rich muscles, such as soleus (44; 45; 123), nor in fast-twitch fiber rich muscle, such as red gastrocnemius (123) and white gastrocnemius muscles in rats (123). Furthermore, the role of Grb2 in muscle contraction-induced ERK pathway activation has been ruled out (109). It is also suggested that genestein, an agent used to inhibit tyrosine kinase by Chen et al., may have a direct effect on GLUT4 (8) and GLUT1 (2). Roles for PLD (71; 72) and aPKCs (27) in glucose transport in skeletal muscle have been reported, although the association between AMPK activation and the function of these molecules has not been elucidated.

Collectively, it is clear that despite intense research no obvious mediators of the signal from AMPK to glucose transport have emerged. Future investigation is needed to determine the role of newly discovered regulators of GLUT4 trafficking, such as AS160, in glucose transport. The development of tissue specific transgenic animal models might help shed new light on the role of these regulators in AMPK mediated glucose transport.

AMPK AND INSULIN SENSITIVITY FOR GLUCOSE TRANSPORT

It is well established that the period after exercise is characterized by an increase in the sensitivity of the muscle to insulin. This phenomenon is not fully understood, but recently it has been hypothesized that AMPK may be part of the mechanism for enhanced insulin action in skeletal muscle following exercise. Fisher et al. found that prior incubation of isolated rat epitrochlearis muscles with AICAR for 1 hour enhanced insulin-stimulated glucose transport by twofold (29). This AICAR-induced increase in insulin sensitivity was not inhibited by addition of cyclohexamide, an inhibitor of protein synthesis, and was thus not dependent on increased expression of glucose transporters. More recently, this group has used Compound C, an AMPK inhibitor, to show that the insulin-sensitizing effects of both AICAR and hyperosmotic stress in C2C12 myotubes is dependent on AMPK. On the other hand, studies in primary human muscle cells have shown no effect of prior AICAR treatment on insulin-stimulated glucose transport (4), but incubation of these cells with AICAR also did not increase

glucose transport (4). Why these reports come to different conclusions is not clear, and further studies in this area are essential.

One mechanism for enhanced insulin action induced by AICAR may be through direct regulation of components of the insulin signaling pathway. Purified AMPK from rat liver phosphorylated IRS-1 on Ser⁷⁸⁹ in a cell-free assay (58). In C2C12 myotubes, activation of AMPK by AICAR caused Ser⁷⁸⁹ IRS-1 phosphorylation while preincubation of these cells with AICAR increased IRS-1-associated PI 3-kinase activation by insulin (58). This study raised the intriguing possibility that IRS-1 phosphorylation on Ser⁷⁸⁹ may be involved in the mechanism of increased insulin-sensitivity associated with AMPK activation. However, the effect of AMPK activation on insulin-stimulated PI3-kinase activity was independent of changes in IRS-1 tyrosine phosphorylation or binding of p85 to IRS-1 in the myotubes (58), and studies of adult intact skeletal muscles have shown that AICAR preincubation does not enhance insulin stimulated phosphotyrosine-associated PI3-kinase activity (29). Furthermore, it is unlikely that phosphorylation of IRS-1 by AMPK plays a role in the insulin-sensitizing effects of exercise, because prior contraction of rat hindlimb skeletal muscles actually causes a decrease in insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1, and in IRS-1-associated PI 3-kinase activity (35; 122). Moreover, it has been suggested that Ser⁷⁸⁹ may play a role in attenuating insulin signaling in insulin-resistant rodent models (106), and AMPK is not involved in serine kinases that phosphorylate the IRS-1 Ser⁷⁸⁹ site in liver (92). Evidently, further work is required to clarify the importance of IRS-1 Ser⁷⁸⁹ in insulin sensitivity, and the function of AMPK in the regulation of IRS-1.

AMPK AND INSULIN RESISTANCE IN SKELETAL MUSCLES

AMPK has also been suggested as a key component in the more prolonged insulin sensitizing effect of chronic exercise training where increased expression of proteins involved in glucose transport plays a key role. Winder and colleagues administrated 5 daily doses of AICAR to Sprague-Dawley rats (49). Twenty four hours after the last AICAR treatment they saw accumulation of glycogen in the muscles together with increases in the expression of GLUT4 and hexokinase (49), very similar to well-established effects of endurance exercise training. Using the same protocol

Buhl and colleagues showed increased insulin-stimulated GLUT4 translocation and glucose transport in isolated rat muscles (14). Besides the increase in GLUT4 and hexokinase expression, AICAR-treatment also increased the insulin stimulated activity of the insulin signaling cascade (60). These observations imply that the increase in insulin sensitivity following endurance training may be mediated by AMPK in skeletal muscle. However, when administrated *in vivo* the effects on muscles could also be secondary to AICAR-effects on fat and glucose metabolism in other tissues such as the liver.

Based on the numerous studies showing that a single AICAR treatment can increase muscle glucose transport, lower blood glucose concentrations and increase insulin sensitivity, there have been many investigations examining the effects of AMPK activation on specific insulin resistant conditions. In obese Zucker rats, insulin was ineffective in increasing glucose uptake, whereas a 90 min infusion of AICAR activated AMPK and increased glucose uptake in gastrocnemius muscle of obese and lean and to a similar degree (9). Furthermore, in insulin-resistant high-fat-fed rats, administration of a single injection of AICAR increased whole-body, liver, and muscle insulin action measured 24 h after AICAR was administrated (55). These studies showed that pharmacological activation of AMPK decreases blood glucose concentrations in an animal model of insulin resistance.

In addition to the short term studies in insulin resistant rats several long term studies have been conducted in both mouse and rat models of insulin resistance. In KKAY-CETP mice administration of AICAR intraperitoneally for seven days decreased the abnormally elevated glucose and insulin concentrations and improved glucose and insulin tolerance tests (28). However, insulin stimulated glucose transport into isolated muscles did not increase. In *ob/ob* mice, subcutaneous administration of AICAR for seven days corrected the hyperglycemia and improved glucose tolerance (111). Similar to the KKAY-CETP mice, in *ob/ob* animals AICAR had no effect on insulin stimulated glucose transport measured in muscles removed 24 h after the last *in vivo* dose, however glucose transport did increase normally during *in vitro* isolated muscle incubations with the compound. Similar to the effects of AICAR in *ob/ob* mice, this compound also lowered glucose concentrations acutely in *db/db* mice (38).

In these insulin resistant mice (28; 38; 111) while 7-8 days of AICAR treatment improved glucose and insulin concentrations, it also caused an increase in free fatty acid and triglyceride concentrations, an effect that could be detrimental. Buhl et al studied the effect of AICAR treatment for seven weeks in Zucker rats (13). AICAR caused a decrease in glucose and insulin levels and improved glucose tolerance. Moreover, after seven weeks of treatment, AICAR-treated animals had improvements in their lipid profile and systolic blood pressure. Similarly, eight weeks AICAR-treatment in the ZDF-rat, an animal model characterized by a progressive β -cell loss and development of diabetes, prevented or delayed the onset of diabetes without any increases in plasma lipids (89). Hyperinsulinemic-euglycemic clamp studies of these animals showed that the improved insulin sensitivity was mainly due to an increased glucose transport in skeletal muscle (89). AICAR treatment also preserved β -cell mass (89) but it is not known if this was due to a direct effect of AICAR on the β -cells or if it was a result of the improved insulin sensitivity in the peripheral tissues.

Whether the differences between the studies in insulin resistant mice vs. rats are due to species differences or duration of treatment needs to be investigated. Furthermore, given the non-specificity of AICAR and the inconsistent findings discussed here, in future studies it will be important to use AMPK knockout or transgenic mice, especially muscle specific, to determine if AMPK activation is necessary for post-exercise enhancement of insulin sensitivity for glucose transport.

AMPK AND GLUCOSE TRANSPORT IN THE HEART

Ischemia is a potent activator of AMPK in the heart (73) and it has been suggested that AMPK mediates the ischemia-induced increase in glucose transport (reviewed in (104)). During cardiac ischemia the glucose transporters GLUT1 and GLUT4 translocate to the sarcolemma and facilitate glucose transport into the heart (25; 98; 118). In vitro incubation of rat papillary muscles with AICAR increased GLUT4 translocation and glucose transport in a PI3-kinase independent manner (96). However, AICAR has been shown to have AMPK independent effects on glucose metabolism in the heart (77). To determine a causal relationship between AMPK and glucose transport in the heart Dr. Tian's group in collaboration with our laboratory generated

heart specific AMPK α 2 inactive transgenic mouse (128). During in vitro perfusion the ischemia-induced increase in glucose transport was partly reduced in the AMPK α 2 inactive hearts (128), providing evidence that AMPK plays a critical role glucose transport during ischemia. This observation was supported by the findings of Russell et al in a different AMPK transgenic mouse model (97). In these mice activation of both the α 1 and α 2 isoform of AMPK was inhibited and hypoxia completely failed to augment glucose transport in the hearts (97). Together these studies clearly demonstrate the importance of AMPK in ischemia-induced glucose transport in the heart.

The mechanisms by which AMPK stimulates GLUT4 translocation in the heart are unknown. Similar to skeletal muscle, nitric oxide synthase (NOS) and p38 MAPK have been suggested as possible mediators, but the findings have been inconsistent. Li et al found that incubation of rat papillary muscles with the two NOS inhibitors L-NAME and L-NMMA partly inhibited AICAR induced glucose transport, while the NO-donors sodium nitroprusside and S-nitroso-N-acetyl-DL-penicillamine increased glucose transport (75). However, the reduction of glucose transport was only modest and the finding is in contrast to other studies that show increased glucose transport in hearts perfused with L-NMMA (24). Further research is needed to determine the role of NOS in glucose transport by AMPK in cardiomyocytes.

p38 MAPK is activated in heart muscle during ischemia (11) and inhibition of AMPK with either araA or overexpression of a dominant negative form of AMPK abolishes p38 phosphorylation in isolated cardiomyocytes incubated with DNP (88). When the same cells are incubated with the p38 inhibitor PD169316, DNP-induced glucose transport is reduced by 70%, indicating a role for p38 in glucose transport in cardiomyocytes. This is an interesting hypothesis, but due to the possible non-specific effects of the inhibitor and the fundamental differences between cultured cells and intact cardiomyocytes, it will be important to further investigate the putative role of p38 in mediating the signal from AMPK to glucose transport in the heart in vivo.

AMPK AS A DRUG TARGET FOR DIABETES

The identification of AMPK as a regulator of glucose transport has made it a promising target for pharmaceutical development, supported by the discovery that metformin activates glucose transport via AMPK activation (121). Metformin is a

widely used drug for the treatment of type 2 diabetes, and its mechanism of action, however, had until recently not been defined. Zhou et al. showed that metformin activates AMPK in primary culture of rat hepatocytes, mediating inhibitory effects of the drug on hepatic glucose production, and in isolated rat skeletal muscle, stimulating glucose transport (132). The observation was recently confirmed by Shaw et al who provided strong experimental evidence for AMPK as the only therapeutic target of metformin. By generating mice with a liver specific deletion of LKB1, they demonstrated that both phosphorylation of AMPK in hepatocytes and the blood lowering effects of metformin was completely abolished (108). These results strongly suggest that in mice, metformin primarily decreases blood glucose concentrations by decreasing hepatic gluconeogenesis. Data from human studies have shown that besides the effects on the liver, metformin administration increases glucose disposal in skeletal muscle (22; 51; 65). This effect might also be mediated through AMPK as it has been demonstrated by Musi et al. that metformin treatment for 10 weeks significantly increases AMPK α 2 activity in skeletal muscles from human subjects with type 2 diabetes (86). Activation of AMPK therefore provides a unified explanation for the beneficial effects of metformin.

Thiazolidinediones, another group of anti-diabetic drugs, have also been found to activate glucose transport in an AMPK α 2 dependent manner in rat skeletal muscle *in vitro* (130). One of the beneficial effects of this class of drugs is the enhancement of insulin sensitivity. As described above, activation of AMPK enhances glucose transport in response to insulin stimulation. Therefore, it is likely that activation of AMPK is one of the mechanisms underlying the beneficial effects of this drug in the treatment of type 2 diabetes. Exactly how thiazolidinediones activate AMPK is unknown, but treatment of cultured muscle cells with these compounds increase the AMP:ATP ratio (32). Furthermore, treatment with thiazolidinediones increases synthesis of adiponectin, an adipokine that activates AMPK in liver (129) and muscle (117), which might in turn potentiate the effects of thiazolidinediones on AMPK *in vivo*.

Not only are metformin and thiazolidinediones beneficial in the treatment of type 2 diabetes, but treatment with both drugs have, similar to regular exercise, proven effective in the prevention of the disease in high risk populations (66; 67). If this effect

is due to activation of the AMPK system remains to be established, but the results suggest that targeting AMPK might be a strategy to oppose the increasing prevalence of type 2 diabetes.

SUMMARY

AMPK stimulates glucose transport when skeletal muscle is exposed to stimuli that lower intracellular energy charge. The efficacy of AMPK to activate glucose transport is comparable to that observed with insulin in muscle, but the proximal signals mediating insulin- and AMPK-regulated glucose transport are distinct. The finding that AMPK activation elicits a novel signaling mechanism leading to enhanced glucose transport in skeletal muscle has important clinical and therapeutic implications. AMPK also enhances insulin-sensitivity to glucose transport, and the mechanism for this effect is also not fully understood. To elucidate the expanding role of AMPK in the energy sensor hypothesis more comprehensively, it will be necessary to determine what signaling molecules are downstream of AMPK that are necessary for the activation of glucose transport. Furthermore, the recent data from the AMPK and LBK1 transgenic animal models have made it clear that mechanisms besides AMPK regulate insulin-independent glucose transport and the identification of these mediators will be an important future undertaking.

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FIGURE LGEND

Figure 1 Schematic illustrating the role of AMPK in glucose transport

Stimuli that cause an increase in [AMP]:[ATP] ratio activate AMPK and subsequently induce glucose transport. Hypoxia-induced glucose transport is probably regulated only by AMPK, whereas, exercise- and osmotic stress-induced glucose transport seem to be simultaneously regulated by both AMPK-dependent and AMPK-independent pathways. Metformin and thiazolidinediones, widely used drugs for the treatment of type 2 diabetes, also activate AMPK. Some reports suggest that metformin may activate AMPK in an [AMP]:[ATP] independent manner. Although several molecules have been suggested as intermediates of AMPK and glucose transport, the signaling mechanism remains largely undefined. The potential role of AMPK in enhancing insulin sensitivity is also poorly understood. Solid arrows illustrate more established relationships between stimuli, signals, and glucose transport, whereas dashed arrows are used for putative interactions.

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