Rapid Publication

Metabolic Stress and Altered Glucose Transport

Activation of AMP-Activated Protein Kinase as a Unifying Coupling Mechanism

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5′AMP-activated protein kinase (AMPK) can be activated in response to cellular fuel depletion and leads to switching off ATP-consuming pathways and switching on ATP-regenerating pathways in many cell types. We have hypothesized that AMPK is a central mediator of insulin-independent glucose transport, which enables fuel-depleted muscle cells to take up glucose for ATP regeneration under conditions of metabolic stress. To test this hypothesis, rat epitrochlearis muscles were isolated and incubated in vitro under several conditions that evoke metabolic stress accompanied by intracellular fuel depletion. Rates of glucose transport in the isolated muscles were increased by all of these conditions, including contraction (5-fold above basal), hypoxia (8-fold), 2,4-dinotrophenol (11-fold), rotenone (7-fold), and hyperosmolarity (8-fold). All of these stimuli simultaneously increased both **α**1 and **α**2 isoform-specific AMPK activity. There was close correlation between **α**1 (r^2 = 0.72) and **α**2 (r^2 = 0.67) AMPK activities and the rate of glucose transport, irrespective of the metabolic stress used, all of which compromised muscle fuel status as judged by ATP, phosphocreatine, and glycogen content. 5-Aminoimidazole-4-carboxamide ribonucleoside, a pharmacological AMPK activator that is metabolized to an AMP-mimetic ZMP, also increased both glucose transport and AMPK activity but did not change fuel status. Insulin stimulated glucose transport by 6.5-fold above basal but did not affect AMPK activity. These results suggest that the activation of AMPK may be a common mechanism leading to insulin-independent glucose transport in skeletal muscle under conditions of metabolic stress. *Diabetes* 49:XXX–XXX, 2000

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3MG, 3-O-methyl-D-glucose; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, 5′AMP-activated protein kinase; DNP, 2,4-dinitrophenol; KRB, Krebs-Ringer bicarbonate buffer; PI, phosphatidylinositol.

Under most conditions, glucose transport is the rate-limiting step in glucose utilization in skeletal muscle (1). While insulin is a potent stimulator of glucose transport in a number of tissues and cells (e.g., skeletal muscle, adipose cells, 3T3L1 adipocytes, L6 myocytes), transport can also be activated via insulin-independent mechanisms (2). In skeletal muscle, contraction can increase glucose transport in the absence of insulin via a wortmannin-insensitive and presumably phosphatidylinositol (PI) 3-kinase–independent mechanism (3). The combination of contraction and insulin can have additive or partially additive effects on glucose transport, providing further evidence for distinct mechanisms leading to glucose transport (4). Hypoxia is a potent stimulator of glucose transport in skeletal muscle (5–9), and the effects of hypoxia on transport are additive to the effects of insulin (7). In L6 myocytes and 3T3L1 adipocytes, pharmacological inhibition of oxidative phosphorylation using 2,4-dinitrophenol (DNP) (10–12) and rotenone (10) increases glucose transport by PI 3-kinase–independent mechanisms (10–12). It has long been known that hyperosmolarity can increase glucose transport in adipocytes and skeletal muscle (13), and more recent work in 3T3L1 adipocytes has demonstrated that this effect is wortmannin-insensitive (14). Although there is good evidence that the mechanism through which these stimuli increase glucose transport involves the translocation of the GLUT4 glucose transporter isoform to the cell surface (3,7,12,14), the signaling mechanism that leads to GLUT4 translocation has not been elucidated.

Recent studies from our laboratory (15,16) and others (17,18) have provided evidence that 5′AMP-activated protein kinase (AMPK) is a mediator of contraction-stimulated glucose transport in skeletal muscle. AMPK is a heterotrimeric protein consisting of one catalytic subunit (α) and two noncatalytic subunits (β and γ) (19). Two isoforms of the α-subunit have been identified (α1 and α2), which have broad tissue distribution, including skeletal muscle (20,21). In fact, in comparison to all other tissues, the highest expression level of the α2 isoform is found in skeletal muscle (20,21), suggesting a physiological role for AMPK in this tissue. Two different β-isoforms (β1 and β2) are both highly

expressed in skeletal muscle (22,23). AMPK is a member of a large kinase family that extends from plants to mammals and is the mammalian homolog of the SNF-1 protein kinase in *Saccharomyces cerevisiae*, which is critical for the adaptation of yeast to nutrient stress (24–26).

AMPK, in an isoform-specific way, is rapidly activated in tissues and cells under several conditions, including exercise/contraction (skeletal muscle) and ischemia (heart) (26). These in vivo observations are paralleled by in vitro data demonstrating that AMPK is activated by an increase in the AMP:ATP and creatine:phosphocreatine ratios via a complex mechanism that involves allosteric modification, phosphorylation by an AMPK kinase, and decreases in phosphatase activities (19,27). It has been proposed that AMPK acts as a fuel gauge in mammalian cells (19). When the cell senses low fuel (decreased ATP), AMPK acts to switch off ATP-consuming pathways and switch on alternative pathways for ATP regeneration.

Based on these observations, we have hypothesized that AMPK mediates signaling that leads to glucose transport when muscle cells sense low fuel. In the current investigation, we have studied several conditions that increase metabolic stress to ascertain whether they lead to an increase in isoform-specific AMPK activity in skeletal muscle, accompanied by alterations in glucose transport. Our results are consistent with the hypothesis that AMPK is central to the mechanism leading to glucose transport during metabolic stress accompanied by fuel depletion in skeletal muscle.

RESEARCH DESIGN AND METHODS

Materials. Male Sprague-Dawley rats weighing 120–140 g were purchased from Taconic (Germantown, MA). Radioactive reagents were from New England Nuclear (Boston, MA). Protein A/G agarose beads were from Pierce, and other reagents were purchased from Sigma (St. Louis, MO).

Muscle preparation. Rat epitrochlearis muscles were isolated and incubated as previously described (15), with some modifications. Briefly, muscles were preincubated in 10 ml Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, containing 2 mmol/l sodium pyruvate for 10 min. The muscles were then incubated for 40 min in KRB containing 2 mmol/l sodium pyruvate in the absence or presence of various agents. When added, insulin (1 µmol/l), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (2 mmol/l), rotenone (3 µmol/l), and sorbitol (120 mmol/l) were present during the entire incubation period, and DNP (500 µmol/l) was present during the last 20 min. For contraction treatment, muscles were stimulated during the last 10 min of the incubation period as described previously (15). The buffers were continuously gassed with 95% O_2–5% CO_2 and maintained at 37°C. For hypoxia studies, buffers were gassed with 95% N_2–5% CO_2. The maximal concentration of vehicle (dimethyl sulfoxide) was 0.1%, which did not affect any assay.

Isoform-specific AMPK activity. Muscles used to measure AMPK activity were immediately frozen in liquid nitrogen at the end of the incubation period. Muscles were weighed and then homogenized in ice-cold lysis buffer (1:100, wt/vol) containing 20 mmol/l Tris-HCl (pH 7.4), 1% Triton X-100, 50 mmol/l NaCl, 250 mmol/l sucrose, 50 mmol/l NaF, 5 mmol/l sodium pyrophosphate, 2 mmol/l dithiothreitol, 4 mg/l leupeptin, 50 mg/l trypsin inhibitor, 0.1 mmol/l benzamidine, and 0.5 mmol/l phenylmethylsulfonyl fluoride, and centrifuged at 14,000*g* for 20 min at 4°C. Supernatants (200 µg protein) were immunoprecipitated with a specific antibody against the $\alpha 1$ or $\alpha 2$ catalytic subunits (21) and protein A/G beads. Immunoprecipitates were washed two times in lysis buffer and two times in wash buffer (240 mmol/l Hepes and 480 mmol/l NaCl). The kinase reaction was carried out in 40 mmol/l Hepes (pH 7.0), 0.1 mmol/l synthetic peptide with sequence HMRSAM SGLHLVKRR (28), 0.2 mmol/l AMP, 80 mmol/l NaCl, 0.8 mmol/l dithiothreitol, 5 mmol/l $MgCl_2$, and 0.2 mmol/l ATP (2 µCi [γ-^{32}P]ATP) for 20 min at 30°C. Reaction products were spotted on Whatman P81 filter paper, the papers were extensively washed in 1% phosphoric acid, and radioactivity was assessed with a scintillation counter. Kinase activity was expressed by incorporated ATP (picomoles) per immunoprecipitated protein (milligrams) per minute.

3-O-methyl-D-glucose transport. Separate muscles were used to measure 3-O-methyl-D-glucose (3MG) transport. After the incubation period, muscles were incubated in 2 ml KRB containing 1 mmol/l 3-O-[^{3}H]methyl-D-glucose (1.5 µCi/ml) and 7 mmol/l D-[^{14}C]mannitol (0.3 µCi/ml) at 30°C for 10 min. Reagents (AICAR, DNP, rotenone, sorbitol, and insulin) were added to each buffer if present during the previous incubation period. Buffers were continuously gassed with 95% O_2–5% CO_2 or for the hypoxia treatment with 95% N_2–5% CO_2. Muscles were then processed, and the transport rate was determined as described previously (15).

Muscle glycogen, ATP, and phosphocreatine concentrations. Muscle glycogen was determined from muscle samples after acid hydrolysis (2 mol/l HCl) at 90°C for 2 h. The concentration of hydrolyzed glucose residues was measured with the Glucose HK reagent (Sigma, St. Louis, MO). ATP and phosphocreatine concentrations were determined as described previously (29).

Statistical analysis. Data are presented as means ± SE. Comparison of means was by one-way analysis of variance followed by post hoc comparison using the Fisher's protected least significant difference method. $P < 0.05$ was considered statistically significant.

RESULTS

Muscle fuel status after metabolic stress. Recent studies have demonstrated that AMPK activity is not only regulated by an increase in AMP concentrations, but also by an increase in the AMP:ATP and creatine:phosphocreatine ratios (19,27). Thus, it would be predicted that all metabolic stresses that alter muscle cell fuel status would be accompanied by an increase in AMPK activity. To address this hypothesis, we first determined that several metabolic stresses, namely contraction, hypoxia, DNP, rotenone, and sorbitol, all decreased ATP and/or phosphocreatine concentrations in the muscles (Table 1). Glycogen, another indicator of muscle fuel storage, was also significantly decreased by these treatments. AICAR and insulin, in contrast, were without effect on muscle concentrations of ATP, phosphocreatine, and glycogen.

Effects of metabolic stresses on glucose transport. Figure 1 shows the effects of the fuel-lowering metabolic stresses on rates of 3-O methylglucose transport in the isolated skeletal muscle preparation. Muscle contraction for 10 min increased glucose transport by fivefold above basal, while 50 min of hypoxia increased transport by eightfold. Pharmacological inhibition of oxidative phosphorylation using the chemical uncoupler DNP and the electron transport inhibitor rotenone each resulted in a very robust stimulation of glucose transport in the epitrochlearis muscles. Hyperosmolar stress, induced by incubation of muscles with 120 mmol/l sorbitol, was also effective in increasing glucose transport, as was insulin and AICAR (Fig. 1).

Effects of fuel-depleting metabolic stresses on isoform-specific AMPK activity. Both the $\alpha 1$ and $\alpha 2$ isoforms of the catalytic subunit of AMPK are expressed in skeletal muscle

TABLE 1
Muscle fuel status in treated skeletal muscle

	ATP (nmol/mg)	Phosphocreatine (nmol/mg)	Glycogen (µmol/g)
Basal	4.3 ± 0.1	17.7 ± 0.7	25.4 ± 1.0
AICAR	4.2 ± 0.3	18.0 ± 0.9	24.2 ± 1.9
Contraction	3.1 ± 0.2*	11.8 ± 1.5*	18.6 ± 0.8*
Hypoxia	2.3 ± 0.1*	2.0 ± 0.2*	5.9 ± 1.0*
DNP	ND	ND	2.1 ± 0.2*
Rotenone	3.9 ± 0.3	9.0 ± 1.0*	15.2 ± 1.2*
Sorbitol	3.6 ± 0.2†	12.9 ± 0.2*	15.1 ± 0.8*

Data are means ± SE. For DNP-treated muscles, ATP and phosphocreatine concentrations were below the assay limit of sensitivity. ND, not detectable. *$P < 0.01$ vs. basal; †$P < 0.05$ vs. basal. n = 4–11/group.

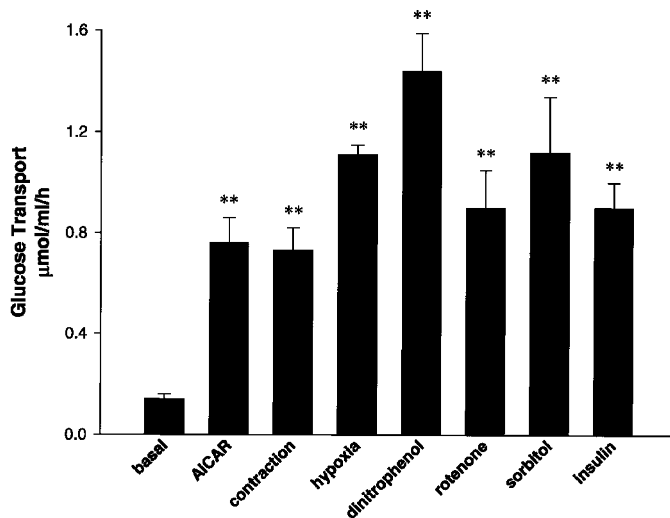

FIG. 1. Effects of fuel-depleting stimuli, AICAR, and insulin on glucose transport in rat skeletal muscle. Isolated epitrochlearis muscles were stimulated with AICAR (2 mmol/l), contraction (10 min), hypoxia (95% N_2–5% CO_2), DNP (0.5 mmol/l), rotenone (3 μmol/l), sorbitol (120 mmol/l), or insulin (1 μmol/l), followed by the measurement of 3MG transport, as described in RESEARCH DESIGN AND METHODS. Each treatment significantly increased the rate of glucose transport. Data are means ± SE. $^{**}P < 0.01$ vs. basal. n = 4–9/group.

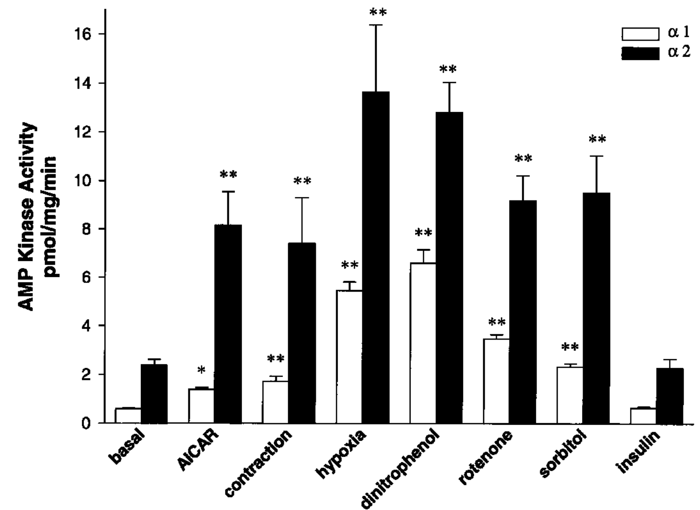

FIG. 2. Effects of fuel-depleting stimuli, AICAR, and insulin on isoform-specific AMPK activity in rat skeletal muscle. Isolated epitrochlearis muscles were treated as described in Fig. 1, followed by measurement of isoform-specific AMPK activity, as described in RESEARCH DESIGN ANDMETHODS. AICAR and all of the fuel-depleting stimuli significantly increased both **α**1 and **α**2 AMPK activity. Insulin did not change AMPK activity. Data are means ± SE. $^{**}P < 0.01$ vs. corresponding basal; $^{*}P < 0.05$. n = 5–11/group.

(20,21). Figure 2 shows that both α1 and α2 AMPK isoforms were significantly activated in response to all of the fuel-depleting stimuli (Fig. 2). The degree of activation of the two isoforms (expressed as fold increase above basal) was similar when muscles were stimulated by contraction, hyperosmolarity, and AICAR. In contrast, compared with α1 activity, α2 activity was greater in response to rotenone (~60%), hypoxia (~60%), and DNP (100%) treatments. Insulin did not change α1 or α2 AMPK activity in skeletal muscle, which was consistent with our previous study suggesting that insulin does not use AMPK to activate glucose transport (15).

Figure 3 demonstrates that the increase in AMPK activity above basal was closely correlated with the increased rate of glucose transport above basal, irrespective of the mode of AMPK stimulation. The linear relationship between the change in α2 activity and the change in glucose transport (r^2 = 0.67, $P < 0.05$) was similar to the relationship between the change in α1 activity and the change in glucose transport (r^2 = 0.72, $P < 0.05$). If α1 and α2 activities are directly correlated with glucose transport activity and the basal activities are included, the statistical relationships are even stronger (α1: $r^2 = 0.70$, $P < 0.02$; α2: $r^2 = 0.87$, $P < 0.02$).

DISCUSSION

There are numerous pharmacological treatments and physiological conditions that result in the diminution of cellular energy stores. A universal feature of fuel-depleted cells is a marked adaptive increase in glucose transport that can then result in an increase in ATP generation and a restoration of cellular energy. The increase in transport is associated with the recruitment of glucose transporter proteins to the cell surface (3,7,12,14). The intracellular signaling events that mediate glucose transporter translocation and glucose transport under these conditions have remained elusive. What is known is that the mechanism does not likely involve PI 3-kinase, since in skeletal muscle cells (5,6,12,15,30) and 3T3 L1 adipocytes (14), several fuel-depleting agents increase glucose transport through a wortmannin-insensitive pathway. Based on our current observations, we propose that AMPK activation is a common signaling mechanism involved in regulating glucose transport in response to fuel-depleting stimuli in skeletal muscle during metabolic stress.

AMPK has emerged as a critical signaling molecule that may mediate multiple cellular metabolic processes including β-oxidation, cholesterol synthesis, creatine phosphate synthesis, and most recently, glucose transport (26,31,32). We (15,16) and

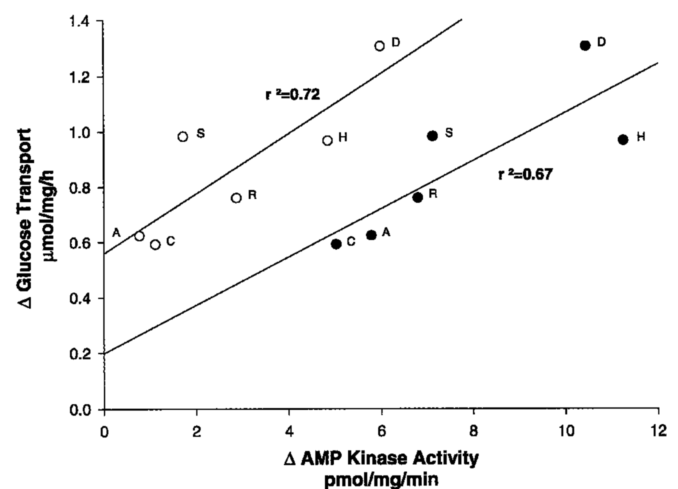

FIG. 3. Correlation between the change in AMPK activity and glucose transport in rat skeletal muscles. Averaged values of isoform-specific activity minus basal and 3MG transport rate minus basal are shown. A, AICAR; B, basal; C, contraction; D, DNP; H, hypoxia; R, rotenone; S, sorbitol. ○, **α**1 AMPK activity; ●, **α**2 AMPK activity. Regression curves were calculated by linear regression analyses (**α**1: $r^2 = 0.72$, $P < 0.05$; **α**2: $r^2 = 0.67$, $P < 0.05$).

others (17,18) first proposed AMPK as a mediator of cellular glucose transport, specifically functioning as a signaling intermediary in contraction-stimulated transport in skeletal muscle. This hypothesis is based on observations using AICAR, a compound that is taken up into skeletal muscle and metabolized by adenosine kinase to form ZMP, the monophosphorylated derivative that mimics the effects of AMP and activates AMPK (17,33,34). The effects of a maximal contraction stimulus in combination with maximal AICAR treatment does not result in an additive effect on glucose transport, and as with contraction, AICAR-stimulated transport is wortmannin-insensitive (15,18). Similar work has suggested that AMPK may also play a role in regulating glucose transport in cardiac muscle (35). In the current study, we have found that AMPK is significantly activated in response to numerous fuel-depleting stimuli in skeletal muscle. Our finding of a remarkably close correlation between increases in AMPK activity and increases in glucose transport suggests that AMPK is centrally involved in regulating glucose transport in contracting muscle during metabolic stress associated with intracellular fuel depletion.

There is now considerable evidence that AMPK can regulate fatty acid oxidation in skeletal muscle (36–38). This is accomplished through AMPK phosphorylation of the β-isoform of acetyl-CoA carboxylase. Phosphorylation by AMPK leads to acetyl-CoA carboxylase inactivation, a fall in malonyl-CoA content, and a subsequent increase in fatty acid oxidation (after deinhibition of carnitine palmitoyltransferase 1) (38). Our results demonstrate that AMPK activity is critical even when oxidative metabolic pathways do not function, such as in the presence of hypoxia or inhibitors of oxidative phosphorylation. Under these conditions, fatty acids cannot be used as a substrate for ATP regeneration, and glucose may become a major source for ATP regeneration via glycolysis, the non–oxygen-requiring pathway in skeletal muscle.

An important finding of our study is that all of the fuel-depleting stimuli significantly increased the activity of both the α1 and α2 isoform–containing AMPK heterotrimer. Furthermore, the highly significant correlation between glucose transport and enzyme activation existed for both α1 and α2, which was somewhat surprising because it was previously reported that in situ muscle contractions in anesthetized rats only increased α2 activity (38). We found that moderate-intensity exercise in vivo only increases α2 AMPK activity in rat and human skeletal muscle, but higher-intensity treadmill running of rats tends to also increase α1 activity (N.F., T.H., L.J.G., unpublished observations). Thus, it remains unclear whether one or both isoforms of the AMPK catalytic subunit might be involved in glucose transport regulation and will be an important area for future study.

The current study clearly demonstrates that hyperosmolar concentrations of sorbitol lead to AMPK activation, a previously unrecognized occurrence. Hyperosmolarity has long been documented as a potent stimulator of glucose transport in isolated skeletal muscle (13), but the signaling mechanism leading to glucose transport has not been revealed. In 3T3L1 adipocytes, osmotic shock increases GLUT4 translocation by a mechanism that is calcium-independent and wortmannin-insensitive, but this activation of translocation can be inhibited by the tyrosine kinase inhibitor genistein (39). These findings raise the possibility that a signaling pathway may exist involving AMPK and a tyrosine phosphoprotein.

In summary, we have demonstrated that conditions that cause reduction in cellular fuel status, including contraction, hypoxia, inhibition of oxidative phosphorylation, and hyperosmolar stress, increase AMPK activity and glucose transport in rat skeletal muscle. The close correlation between AMPK activity and glucose transport suggests that these metabolic stressors use the same AMPK-dependent signaling pathway leading to accelerated glucose transport, aimed at the restoration of cellular energy stores.

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