Glucose Autoregulates Its Uptake in Skeletal Muscle
Involvement of AMP-Activated Protein Kinase

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Preexposure to a low concentration of glucose upregulates glucose transport into skeletal muscle, whereas exposure to a high concentration of glucose has the opposite effect. This autoregulatory process occurs independently of insulin, and the mechanism by which it operates is incompletely understood. Activation of the energy-sensing enzyme AMP-activated protein kinase (AMPK) has been shown to increase insulin-independent glucose transport into skeletal muscle in response to such stimuli as exercise and hypoxia. In the present study, we examined whether AMPK could also mediate glucose autoregulation. The activity of the α2 isoform of AMPK and 2-deoxyglucose uptake were assessed in incubated rat extensor digitorum longus muscle after preincubation for 4 h in media containing 0, 3, 6, or 25 mmol/l glucose. The principal findings were as follows. First, AMPK activity was highest in muscles incubated with no added glucose, and it decreased as the concentration of glucose was increased. In keeping with these findings, the concentration of malonyl CoA was increased, and acetyl CoA carboxylase phosphorylation at serine 79 was decreased as the medium glucose concentration was raised. Second, decreases in AMPK activity at the higher glucose concentrations correlated closely with decreases in glucose transport (2-deoxyglucose uptake), measured during a subsequent 20-min incubation at 6 mmol/l glucose ($r^2 = 0.93$, $P < 0.001$). Third, the decrease in AMPK activity at the higher glucose concentrations was not associated with changes in whole-tissue concentrations of creatine phosphate or adenine nucleotides; however, it did correlate with increases in the rate of glycolysis, as estimated by lactate release. The results suggest that glucose autoregulates its own transport into skeletal muscle by a mechanism involving AMPK. They also suggest that this autoregulatory mechanism is not paralleled by changes in whole-tissue concentrations of creatine phosphate ATP, or AMP, but they leave open the possibility that alterations in a cytosolic pool of these compounds play a regulatory role. *Diabetes* 52:1635–1640, 2003

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ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase; EDL, extensor digitorum longus; MCD, malonyl CoA decarboxylase.

Studies in a wide variety of cultured cells have revealed that in the absence of added insulin, pre-exposure to a low concentration of glucose augments insulin-independent glucose transport, whereas after incubations with a high concentration of glucose, its transport is diminished in a dose-dependent manner (1). A similar phenomenon has been observed in incubated and perfused rat muscle (1–3). In these preparations, changes in glucose uptake were associated with alterations in the distribution of the GLUT4 glucose transporter (3); however, beyond this, the mechanism by which glucose autoregulates its uptake into muscle is incompletely understood. In this study, we examine the notion that the fuel-sensing enzyme AMP-activated protein kinase (AMPK) modulates this phenomenon.

AMPK is a ubiquitously expressed enzyme whose activity is increased when cells are exposed to such stresses as hypoxia, hyperosmolarity (4), fuel deprivation (5,6), reactive O_2 species (7), and, in muscle, contraction (exercise) (8–10). As first demonstrated by Hardie and Carling (4), a decrease in the energy state of a cell, as reflected by an increase in the AMP/ATP ratio, activates AMPK. They showed that such increases in the AMP/ATP ratio activate AMPK both allosterically and covalently—the latter by activating an AMPK kinase that phosphorylates AMPK on threonine 172. Activation of AMPK acutely produces a variety of changes that both increase ATP generation (e.g., increased fatty acid oxidation) and diminish its use for processes not immediately necessary for cell survival, such as the synthesis of fatty acids, cholesterol (4), triglycerides (11), and protein (12). In both yeast (13) and mammalian cells (5) deprived of glucose, it also causes genetic changes that enhance the ability of these cells to use glucose when it becomes available. In addition, AMPK activation in muscle during exercise and in response to hypoxia and certain pharmacological agents has been shown to correlate with increases in insulin-independent glucose transport (14).

In this present study, we examined the possibility that AMPK modulates the autoregulation of glucose uptake in rat extensor digitorum longus (EDL) muscle. Toward this end, muscles were preincubated for 4 h at different glucose concentrations, and the uptake of 2-deoxyglucose was assessed during a subsequent 20-min incubation. In addition, when the results revealed a relationship between changes in AMPK activity and 2-deoxyglucose uptake, the concentrations of adenine nucleotides and creatine phos-

phate (creatine-P) were determined in an effort to explain how these changes occurred.

RESEARCH DESIGN AND METHODS

Chemicals and materials. [γ-^{32}P]ATP was obtained from NEN, Protein A/G plus conjugate from Santa Cruz Biotechnology (Santa Cruz, CA), and total and phospho-AMPK and phospho–acetyl CoA carboxylase (ACC) antibodies from Cell Signaling. All other chemicals were purchased from either Sigma or Fisher.

Animal care. Male Sprague-Dawley rats weighing 55–65 g were purchased from Charles River Breeding Laboratories (Wilmington, MA). They were kept in an animal facility with a light-dark cycle of 6:00 P.M. to 6:00 A.M. and fed Purina rat chow ad libitum. All rats were fasted for 18–20 h before an experiment.

Muscle incubation. Rats were anesthetized with sodium pentobarbital (6 mg/100 g body wt i.p.). EDL muscles were isolated as described previously (14,15). Muscles were initially equilibrated in Krebs-Henseleit solution containing 6 mmol/l glucose for 20 min. They were then washed in Krebs-Henseleit solution containing no added glucose for 2 min and incubated in media containing 0, 3, 6, or 25 mmol/l glucose for 4 h. In selected experiments, after the 4-h preincubation, muscles were washed in a glucose-free medium, and 2-deoxyglucose uptake was determined during a subsequent 20-min incubation in media containing 2-[H^3]deoxyglucose and 6 mmol/l glucose as described previously (15). All muscles were frozen in liquid nitrogen and stored at −80°C until used for analyses.

AMPK activity. AMPK activity was measured in EDL muscle as described previously (9). In brief, frozen muscle was homogenized and muscle lysate containing 200 μg protein was immunoprecipitated with specific antibody to the α2 catalytic subunit of the AMPK and protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were washed five times, and the immobilized enzyme was assayed based on the phosphorylation of SAMS peptide (0.2 mmol/l) by 0.2 mmol/l ATP (containing 2 μCi [γ-^{32}P]ATP) in the presence and absence of 0.2 mmol/l AMP. Label incorporation into the SAMS peptide was measured on a Racbeta 1214 scintillation counter.

Western analysis. Protein homogenates (50 μg) were run on an 8% SDS polyacrylamide gel and transferred onto a PVDF (polyvinylidine fluoride) membrane. Blots were incubated with primary antibodies (total AMPK, phospho-AMPK, total ACC, and phospho-ACC) at a 1:1,000 dilution, except for phospho-AMPK, for which a 1:700 dilution was used. Blots were incubated with a secondary antibody conjugated to horse radish peroxidase at a 1:5,000 dilution. Membranes were then subjected to an enhanced chemiluminescence solution (Amersham), and multiple autoradiogaphs (to ensure linearity) were generated.

Other analyses. ATP, AMP, ADP, and phosphocreatine were measured spectrophotometrically as described previously (16,17). Malonyl CoA was determined radioenzymatically by a slight modification of the method of McGarry et al. (18,19). Malonyl CoA decarboxylase activity was assayed spectrophotometrically in the 500*g* supernatant of the muscle homogenate as described previously (20–23). Lactate release was determined spectrophotometrically using lactate dehydrogenase and NAD (24).

Statistical analysis. Results are expressed as means ± SE. Statistical differences between multiple groups were determined by ANOVA followed by the Student-Newman-Keuls multiple comparison test.

RESULTS

Effect of incubation with different glucose concentrations on AMPK activity and phosphorylation. Rat EDL muscles were incubated for 4 h in media to which 0, 3, 6, or 25 mmol/l glucose was added. As shown in Fig. 1, a progressive decrease in AMPK activity occurred as the glucose concentration was increased. Values at 3, 6, and 25 mmol/l glucose were both significantly different from those at 0 mmol/l glucose, as well as from each other. Phospho-AMPK abundance showed a similar decrease as the concentration of glucose was increased. No differences in AMPK abundance were observed in muscles preincubated for 4 h in media containing 0, 6, or 25 mmol/l glucose.

Malonyl CoA, ACC, and malonyl CoA decarboxylase. AMPK activation lowers the concentration of malonyl CoA in rat muscle by phosphorylating and inhibiting ACC (25,26) and phosphorylating and activating malonyl CoA decarboxylase (MCD) (27). Thus, with increasing concentrations of glucose (decreased AMPK activity), one would expect to find increases in the concentration of malonyl CoA and decreases in the phosphorylation of ACC. As shown in Fig. 2*A* and *B*, both of these changes were observed. The concentration of malonyl CoA was 60% higher in muscles incubated with 25 vs. 0 mmol/l glucose, and ACC phosphorylation was diminished by ~50%. In contrast, no difference in MCD activity was observed.

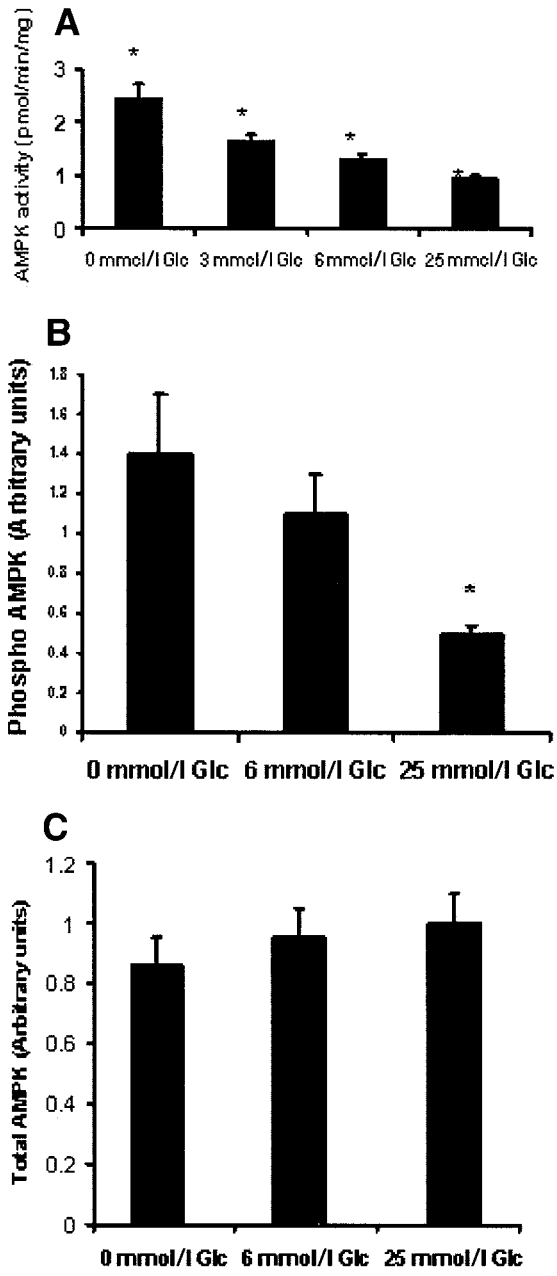

FIG. 1. Effect of preincubation with different glucose concentrations on AMPK activity, phosphorylation, and abundance. *A*: AMPK activity. EDL muscles were preincubated for 4 h in media containing 0, 3, 6, or 25 mmol/l glucose, at which time AMPK α2 activity was measured. *Values at all glucose concentrations were significantly different from each other ($P < 0.05$). Results are means ± SE ($n = 14$). For phospho-AMPK and total AMPK, EDL muscles were preincubated for 4 h in media containing 0, 6, or 25 mmol/l glucose. *B*: Mean phospho-AMPK abundance ± SE ($n = 6$). *$P < 0.02$ vs. values at 0 and 6 mmol/l glucose. *C*: Mean total (α1 and α2) AMPK abundance.

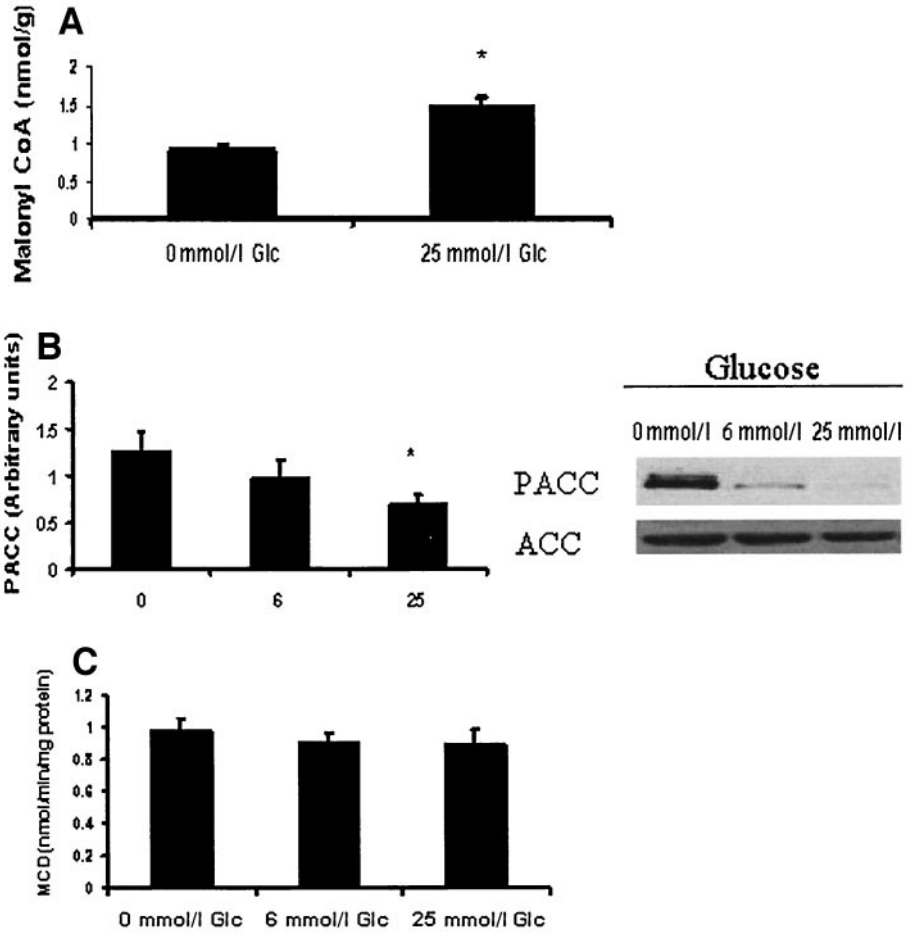

FIG. 2. Effect of preincubation with different glucose concentrations on malonyl CoA and enzymes that regulate its concentration. EDL muscles were preincubated for 4 h at the indicated concentrations after which the concentration of malonyl CoA (*A*) (n = 6), the phosphorylation of ACC (PACC) (*B*) (n = 11), and MCD activity (*C*) (n = 6) were determined. *P < 0.05 vs. value in muscles preincubated with no added glucose.

Glucose transport. Glucose transport in the absence of added insulin was measured on the basis of 2-deoxyglucose uptake in muscles that had been preincubated for 4 h with 0, 6, or 25 mmol/l glucose. As shown in Fig. 3, 2-deoxyglucose uptake was significantly lower in muscles preincubated with 25 mmol/l glucose than at the two lower glucose concentrations, confirming the results of Sasson et al. (1,2).

Creatine phosphate and adenine nucleotides. When the concentration of AMP relative to ATP is increased, AMPK kinase is activated and AMPK phosphorylation at threonine 172 and its activity are increased. As shown in Table 1, no differences in the whole-tissue concentrations of ATP, ADP, or AMP were observed in muscles incubated with 0, 6, or 25 mmol/l glucose for 4 h. Also shown in Table 1, we found no change in the concentration of creatine-P.

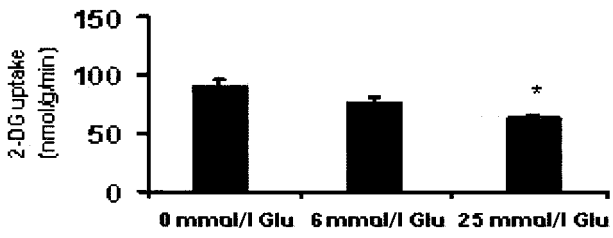

FIG. 3. Effect of preincubation with different glucose concentrations on subsequent uptake of 2-deoxyglucose (2-DG). EDL was preincubated for 4 h in 0, 6, or 25 mmol/l glucose. 2-DG uptake was measured during a subsequent 20-min incubation at 6 mmol/l glucose. *P < 0.05 vs. values at 0 and 6 mmol/l glucose. Results are means ± SE (n = 13).

Similar findings were observed when muscles incubated with 0 or 25 mmol/l glucose were studied after 30, 60, or 120 min of incubation (data not shown).

Lactate release. Although incubation of muscle with various glucose concentrations did not alter the concentrations of creatine phosphate, ATP, or AMP, the possibility remains that a pool of these compounds, not reflected by whole cell measurements, was altered. In this context, the release of lactate, the major fate of glucose taken up by the incubated EDL (15), was increased by two- and threefold in muscles incubated with 6 and 25 vs. 0 mmol/l glucose, respectively (Fig. 4). This result indicates that the magnitude of glycolytically generated ATP was substantially greater in muscles incubated at the higher concentrations of glucose.

TABLE 1
Effect of preincubation with different glucose concentrations on creatine phosphate and adenine nucleotide content in rat EDL muscle

	Glucose (mmol/l)		
	0	6	25
Creatine phosphate	13.3 ± 0.3	13.7 ± 0.6	13.0 ± 1.0
ATP	4.0 ± 0.1	4.1 ± 0.3	4.3 ± 0.2
AMP	0.035 ± 0.01	0.048 ± 0.02	0.05 ± 0.01
ADP	0.49 ± 0.05	0.65 ± 0.06	0.6 ± 0.03

Data are means ± SE (n = 6) and are expressed as micromoles per gram muscle.

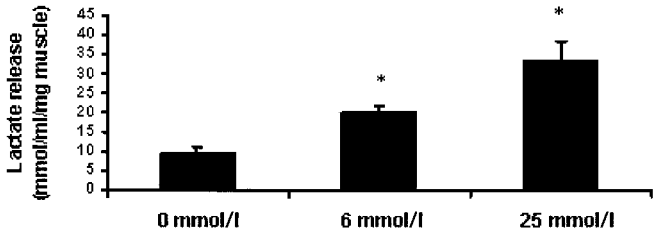

FIG. 4. Effect of preincubation for 4 h with 0, 6, or 25 mmol/l glucose on lactate release. Results are means ± SE (n = 8). *Results are significantly different from those at 6 mmol/l glucose and from each other (P < 0.01).

DISCUSSION

It has been long appreciated that glucose can acutely (2–3 h) autoregulate its own uptake by skeletal muscle (1,2). The results of the present study confirm these findings. In addition, they provide evidence that this autoregulatory mechanism is governed by AMPK. The data supporting this conclusion are as follows: First, AMPK activity diminished in parallel with increases in the ambient glucose concentration in EDL muscles during a 4-h preincubation. Second, these decreases in AMPK activity correlated closely with decreases in glucose transport (2-deoxyglucose uptake). Third, as reported elsewhere, activation of AMPK by a wide variety of means including contraction, hypoxia, hyperosmolarity (13,25), and incubation with the pharmacological agent AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) (25), or the adipocyte-derived hormone adiponectin (gACRP30) (28,29), are all associated with increased glucose transport in skeletal muscle. Furthermore, where studied, these increases in glucose transport in response to these studies were observed in the absence of insulin. Studies showing that specific AMPK inhibitors (30) or the expression of a dominant-negative AMPK (31) inhibit the autoregulation of glucose transport in muscle are needed to provide definitive evidence that this mechanism is regulated by AMPK.

Prior reports suggest that the acute autoregulation of glucose transport in skeletal muscle is AMPK-mediated glucose transport (14) and related to alterations in the distribution of the GLUT4 glucose transporter. Thus, Mathoo et al. (3) have shown that perfusion of rat hindlimbs with 20 vs. 6.5 mmol/l glucose (no added insulin) for 2 h results in a decrease in glucose clearance that is accompanied by a 20% decrease in the plasma membrane content of the GLUT4 glucose transporter and a 30% increase in the content of GLUT4 in the intracellular membrane fraction. Conversely, they found that perfusion with 2 mmol/l glucose (hypoglycemia) was associated with increases in glucose clearance and plasma membrane GLUT4 content. No change in the distribution of the GLUT1 glucose transporter was observed in either situation. AMPK activity and phosphorylation were not measured in this study; however, the responses of glucose transport in the perfused hindlimb preparation to changes in glycemia were comparable to those of incubated muscle (1,3). Thus, similar changes in AMPK would be expected.

Low and high glucose concentrations cause alterations in glucose transport in cultured L6 and L8 myocytes (32,33). In contrast to their effects in perfused muscle (3), in the myocytes, these changes were associated with alterations in the subcellular distribution, biosynthesis, and mRNA transcription of the GLUT1 glucose transporter (GLUT4 is much less abundant in these cells than in intact muscle) (34). Whether AMPK activity is also altered in response to changes in glucose concentration in cultured myocytes was not studied; however, increases in AMPK activity in response to hypoglycemia have been reported in cultured endothelium (6) and pancreatic β-cells (5). Likewise, studies in other cultured cells have implicated AMPK in GLUT1-mediated glucose transport (34,35). In rat skeletal muscle, chronic activation of AMPK has been shown to increase the abundance (36,37) or mRNA (38) of GLUT4 glucose transporters. It will clearly be of interest to determine the effect of AMPK on the two transporters in cultured cells.

An unanswered question is by what mechanism do changes in the ambient glucose concentration modulate the activity of AMPK. As already noted, the classic view (4) is that an AMPK kinase is activated by changes in the energy state of the cell, as reflected by the AMP/ATP ratio, and that it in turn phosphorylates and activates AMPK. In addition, increases in the AMP/ATP and creatine/creatine-P ratios can activate AMPK allosterically independent of their effects on its phosphorylation. In the present study, we found no changes in the concentrations of ATP, ADP, or total AMP after 4 h of preincubation at low versus high glucose. Likewise, the concentration of creatine-P, generally the first high-energy phosphate compound to decrease when the energy state of the muscle cell is diminished (39), was unchanged. It is possible that the concentration of free AMP, which comprises only a small fraction of total AMP, was elevated on incubation without glucose. The lack of change in creatine-P and ATP also indicates a lack of change in the free AMP concentration, since in muscle the latter is usually calculated from the concentration of ATP, creatine and creatine-P (40,41) and the equilibrium constants for certain kinase and myokinase. Because of the pH dependence of the creatine kinase equilibrium, the calculated free AMP concentration could also have been increased by a rise in cytoplasmic pH. Dudley and Terjung (41) calculated pH based on accumulation of lactate plus pyruvate in stimulated muscle. Based on the equation they used, if the normal lactate concentration of ~2 mmol/l in unstimulated muscle fell to 0 upon incubation without glucose, the calculated pH increase would then be 0.05 and the corresponding rise in free AMP only 12%.

Changes in the concentration of adenine nucleotides or creatine-P also were not observed after 30, 60, or 120 min of preincubation at a low glucose concentration, suggesting that the increased AMPK activity observed at 4 h was not caused by a decrease in the energy state of muscle at these earlier time points. It is still conceivable, however, that there was a very transient rise in AMP (energy state) that was rapidly normalized as a consequence of the AMPK activation. Another intriguing possibility stems from the observation that AMPK activity was increased in muscle that had a low glycolytic rate (low glucose concentration), and it was decreased when the glycolytic rate was high (high glucose concentration). Whether alterations in glycolytically generated ATP can selectively alter

AMPK activity is not known; however, the fact that much, if not most, of the AMPK in muscle is cytosolic at least makes this possibility worthy of consideration. In support of this notion, the amount of ATP generated by glycolysis in the EDL relative to that originating from oxidative metabolism is not trivial. For instance, in muscles incubated with 25 mmol/l glucose the rate of lactate release into the medium (3 ml) over 4 h (Fig. 4) translates into ~24 μmol ATP generated/g muscle/h. If one assumes rates of O_2 consumption by the EDL are comparable to those reported for the perfused hindquarter and other incubated muscles (~20 $\mu mol \cdot g^{-1} \cdot h^{-1}$) (42,43), ATP generation attributable to oxidative metabolism is 120 $\mu mol \cdot g^{-1} \cdot h^{-1}$. Finally, an alternative explanation for the changes in AMPK activity induced by glucose is that muscle contains an AMPK kinase that can be activated by factors other than a change in its energy state. Hardie (44) suggested the existence of such an AMPK kinase; however, this enzyme and the factors that regulate it have not yet been well characterized.

The physiological relevance of changes in AMPK in muscle due to sustained (>2 h) alterations in glucose availability remains to be determined. In isolated cells, AMPK activation in response to a limited glucose supply presumably has survival value, because it would both increase ATP generation by enhancing glucose transport and fatty acid oxidation and decrease the use of ATP for such processes as cholesterol, triglyceride, and possibly protein synthesis (4,10,11). Conversely, the decrease in AMPK activity induced by a surfeit of glucose, via effects on malonyl CoA concentration and perhaps GPAT (Sn-glycerol-3-phosphate acyltransferase) activity, would direct fatty acids into storage as triglyceride at the expense of its oxidation. Hypothetically, it could also allow an upregulation of other synthetic processes that are inhibited by AMPK. Less clear is how this scenario plays out in vivo where blood flow and hormonal and other factors might attenuate the effects of hyper- and hypoglycemia on the activity of AMPK. This question could be relevant to such clinical problems as glucose toxicity and the effects of acute hypoglycemia (which could upregulate glucose transporter translocation in skeletal muscle) on muscle glucose utilization and insulin action in type 1 diabetes (3). Its importance is underscored by recent studies showing that transient activation of AMPK in rat muscle either by prior exercise (46) or by the pharmacological agent AICAR (47,48) enhances the ability of insulin to stimulate glucose uptake. Conversely, incubation of muscle for 4 h with a hyperglycemic (25 mmol/l glucose) medium, as was done here, has been demonstrated to impair insulin action and signaling (15).

Finally, the AMPK-mediated glucose autoregulatory mechanism could hypothetically act in any cell. We would predict that, in vivo, it would operate predominantly in cells in which insulin-sensitive glucose transport or the presence of a high K_m hexokinase (i.e., glucokinase) make the cells sensitive to changes in the ambient glucose concentration. The latter would include liver, the pancreatic β-cell, the adipocyte, and some cells in the central nervous system.

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