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Involvement of Amp Kinase in Glucose Uptake and Palmitate Oxidation in L6 Muscle Cell Cultures

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Abstract

AICAR (5-aminoimidazole-4-carboxamide 1-ß-D-ribonucleoside) is known to activate the AMP-activated protein kinase (AMPK) in many cell types, including skeletal muscle. We showed that AICAR activated glucose uptake, fatty acid oxidation, and lactate formation by L6 skeletal muscle cell cultures. All of the actions were duplicated by adiponectin, another known activator of AMPK, demonstrating selectivity of AICAR. Moreover, AICAR and adiponectin similarly caused phosphorylation of AMPK, and its target proteins acetyl CoA carboxylase and S6Kinase. While creatine treatment of cells is known to greatly enhance creatine and creatine phosphate levels, it was proposed to paradoxically activate AMPK, and yet not affect glucose transport. However, we found creatine treatment to be without effect on glucose transport, fatty acid oxidation, or phosphorylation of AMPK or its target proteins. Unlike AICAR and adiponectin, creatine treatment stimulated glycogen formation from glucose and inhibited lactate formation. Consistent with this, we observed a diminution in phosphofructokinase expression. Thus, we conclude that creatine and creatine phosphate are not significant allosteric modulators of AMPK in intact cells. We investigated the role of AMPK in glycogen metabolism, using an inhibitor of glycogen phosphorylase. While the inhibitor enhanced glucose incorporation into glycogen, AICAR and adiponectin still diminished glycogen formation, supporting a direct action of AMPK on glycogen synthase. To probe the mechanism for adiponectin, we used siRNA constructs against adenyate kinase and LKB1 (the AMPK kinase). We concluded that adiponectin likely acts through an activation of LKB1, rather than indirectly increasing the turnover of ATP, involving adenylate kinase.

Keywords: Glucose metabolism; Fatty acid oxidation; AMP kinase; LKB1; mTOR

List of abbreviations: AICAR: 5-Aminoimidazole-4-Carboxamide 1-ß-D-Ribonucleoside; AMPK: AMP-Activated Protein Kinase; gAd: Globular Adiponectin; AK1: Adenylate Kinase 1; DMEM: Dulbecco's Modified Eagle Medium; α-MEM: Alpha-Minimum Essential Medium; KHB: Krebs-Henseleit Bicarbonate Buffer. PFK1: Phosphofructokinase 1; GSK: Glycogen Synthase Kinase

Introduction

Recent studies have implicated AMP-activated protein kinase (AMPK) in exercise-enhanced metabolism, and evidence suggests the enzyme may also account for metabolic changes resulting from hypoxia and ischemia [1-3]. AICAR (5-aminoimidazole-4-carboxamide 1-ß-D-ribonucleoside) is a commonly used activator of the AMPK [4,5]. AICAR induces glucose uptake in skeletal muscle by activating α2-AMPK, which is the main contributor of basal and AICAR-stimulated AMPK activity [6]. Chronic AICAR administration and long-term exercise increase insulin-stimulated glucose transport and GLUT-4 content in skeletal muscles [7]. AICAR-stimulated AMPK phosphorylation is dependent on LKB1, an upstream AMPK kinase, in cultured liver and muscle cells [8].

Despite the large number of publications that use AICAR for AMP Kinase activation, its specificity is uncertain. Whitters [9] refers to the compound as a "known, albeit nonspecific activator of AMPK". However, the supporting reference cited by that author [10] reported little evidence of non-selectivity. It has been shown that nonselective effects can result after several hours of incubation [11]. Moreover, in tissues such as muscle, it is possible that an AMP analog might also affect enzymes that are known (at least in vitro) to be affected by AMP, such as glycogen phosphorylase [12].

An alternative means of activating AMPK is available, as it is established that the adipocyte derived hormone, adiponectin, also activates AMPK in muscle [13]. However, the mechanism of action of this hormone remains unknown.

In skeletal muscle, creatine kinase, a near-equilibrium enzyme [14] could potentially alter adenine nucleotides and hence AMPK. Indeed it has been reported by one laboratory that creatine incubation can lead to an activation of AMPK; surprisingly this was suggested to occur without a corresponding increase in glucose transport [15]. Thus one goal of our investigation was to investigate this paradox with L6 cells. Our findings also suggest that creatine incubation does not actually affect AMPK; in fact, this condition has distinct metabolic actions on cells. In order to resolve the possible nonselectivity of AICAR, we compared this compound with the hormone adiponectin on metabolic events in the muscle cell cultures. Moreover, we examined how adiponectin might regulate AMPK by making use of ablation experiments using selective siRNA directed against adenylate kinase as well as the upstream kinase that regulates AMPK, the LKB1. Our results suggest that AICAR is, at least under our experimental conditions, a selective AMPK activator, as it is replicated by adiponectin.

Materials and Methods

AICAR, and most chemicals, including BSA (bovine serum

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albumin; 30%-essentially fatty acid free), were obtained from Sigma Chemical Co. (St. Louis, MO). Prior to use, BSA was dialyzed extensively against Krebs-Henseleit bicarbonate buffer (KHB) [16]. Palmitic acid was from Nu-Chek Prep Inc (Elysian, MN, U.S.A.). Dulbecco's Modified Eagle Medium (DMEM), alpha-Minimum Essential Medium (α-MEM), Hanks Balanced Salt Solution, fetal bovine serum (FBS), horse serum, and trypsin, were from Invitrogen (Grand Island, NY). Scintillation vials were obtained from Wheaton (Millville, NJ) and Scintisafe (30% advanced safety LSC cocktail) from Fischer Scientific (Fair lawn, NJ). Cell culture plates and flasks were obtained from Corning Inc. (Corning, NY). CP-91149 was kindly provided by Pfizer Inc. Recombinant rat globular adiponectin (gAd) was purchased from BioVision (Mountain View, CA). 2-Deoxy-D-[2,6-^{3}H] glucose, [1-^{14}C] palmitic acid, and D-[U-^{14}C]glucose were from Amersham Biosciences (Piscataway, NJ). Adenylate kinase 1 siRNA (siGenome duplex AK1), DharmaFECT 3 siRNA transfection reagent, and negative control siRNA (siCONTROL Non-Targeting siRNA), and transfection media were obtained from Dharmacon Inc. (Chicago, IL). AK1 antibody and LKB1 siRNA was from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies were from Cell Signaling Technology (Danvers, MA).

Cell culture

L6 rat skeletal muscle cells were cultured in growth medium composed of the proliferation medium DMEM supplemented with 10% FBS. Cells were converted to myotubes by switching to α-MEM with 2% horse serum for 1 day, and then switch to α-MEM with 1% horse serum for 5-8 days prior to use for experiments. Details of cell preparation are provided in a prior publication [16].

Glucose uptake assay

Glucose uptake was determined as the rate of 2-deoxy-d-[2, 6-^{3}H] glucose uptake. The cells were first incubated for 15 min with KHB, glucose, and other agents as indicated. At this point, the labeled deoxyglucose (0.6 µCi) was added to each well and the incubation continued for 45 min. The medium was aspirated, and the wells were washed three times with ice-cold KHB to remove exogenous label. The cells were lysed by the addition of 0.1% Triton X-100 (1 ml). Samples of each well were mixed with aqueous scintillation fluid and measured by liquid scintillation counting.

Palmitate oxidation assay

Palmitate oxidation was determined by a modification of quantitative measuring the rate of $^{14}CO_2$ production from ^{14}C-labeled palmitic acid. The KHB for these incubations was supplemented with fatty acid-poor albumin dialyzed against the same buffer (three changes). The final albumin concentration was 1%. After an initial 10 min of incubation, all samples received 2 mm carnitine and [1-^{14}C]palmitic acid (1 µCi/mmol), and other additions as noted, and incubations were continued for 3 h. Aliquots of 0.8 ml were taken from each well to an Eppendorf tube. Each tube had a circular piece of filter paper, produced with a paper punch, attached to the inside of the lid, to which 15 µl of 2 m NaOH was added. 200 µl of 3 m perchloric acid was carefully added to the 0.8 ml to ensure no acid was deposited on the side of the tube, and the lid was quickly closed. The tubes were incubated overnight to allow the [^{14}C] CO_2 to be absorbed into the wick. The caps were detached and placed in 10 ml of liquid scintillation fluid for counting.

Lactate formation

For lactate formation, cells were incubated for 4 h, after which 125 µl of incubation medium was taken for enzymatic end point assay using the HTS 7000 BioPlate Reader.

Glycogen synthesis

Glycogen synthesis was determined by a modification of the incorporation of d-[U-^{14}C]glucose into glycogen. After incubation, cells were washed with ice-cold KHB and lysed in 0.5 ml of KOH (30%). Aliquots of 0.2 ml were spotted onto Whatman 3T paper, and glycogen was precipitated by immersing the papers in ice-cold 66% (v/v) ethanol overnight. Whatman 3T paper containing precipitated glycogen was transferred to scintillation vials for radioactivity counting.

Glycogen content measurement

After aspiration of the medium, adherent cell monolayers were extracted with 400 µl of 30% (w/v) KOH and boiled for 15 min. Aliquots were spotted onto Whatman 3T paper, which was then immersed in ice-cold 66% (v/v) ethanol. The papers were washed twice with 66% ethanol, and dried papers containing precipitated glycogen were incubated in 2 ml of 0.4 m acetate buffer (pH 4.8) containing 25 units/ml of α-amyloglucosidase for 90 min at 37 °C. Glucose released from glycogen was measured by using an enzymatic end point assay for glucose using hexokinase.

Transfection of siRNA

L6 cells were transfected with 100 nM AK1 siRNA or 100 nM LKB1 siRNA for 3 days by following procedure: 1.0 ×10^{4} cells/well were seeded in 12 well plates that were incubated with DMEM with 10% FBS for 2 h before transfection. Prepare a 2 µM siRNA solution in 1X siRNA Buffer or another appropriate RNase-free solution. In separate tubes, dilute the appropriate volume of 2 µM siRNA and the appropriate transfection reagent with transfection medium. In tube 1 -Add 50 µL of 2 µM siRNA to 50 µL transfection medium. In tube 2 – Add 2 µL transfection reagent to 98 µL of transfection medium. Mix the contents of each tube gently by pipetting carefully up and down and incubate for 5 minutes at room temperature. Add the content of Tube 1 to Tube 2. Mix by pipetting carefully up and down and incubate for 20 minutes at room temperature. Add sufficient antibiotic-free complete medium (DMEM with 10% FBS) to the mix for the desired volume (1mL in total). Remove culture medium from the wells of the 12-well plate and add 1mL of the mixed transfection solution into each well. Incubate cells at 37°C in 5% CO_2 for 2 days. Change the medium to α-MEM with 1% horse serum with transfection solution for 1 day. And then switch the incubation medium into α-MEM with 1% horse serum without transfection solution for another 4 days. After that, the cells were incubated with serum free medium for 2 hours, treated with or without 2.5 µg/ml of g Ad or 10 mM metformin in the presence of 5 mM glucose for 2 hours, and then measured glucose uptake and the content of adenylate kinase 1 or LKB1 protein expression by Western blot.

Western blot

To extract total protein, cells were lysed with 100 µl of buffer containing 2% w/v SDS, 62.5 mM Tris (pH 6.8), 10% glycerol, 50 mM DTT, and 0.01% w/v bromophenol blue. The procedure of the commercial source of the antibody (Cell Signaling Technology) was essentially followed. The lysates were heated at 95-100°C for 5 minutes and cooled on ice. Aliquots of 20 µg of protein were loaded onto a 10% SDS-PAGE gel, and subsequently electroblotted onto nitrocellulose membranes. Membranes were incubated in 25 ml of blocking buffer (2.5 ml of 20mM Tris base and 140 mM NaCl, 1.25 g nonfat dry

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milk, and 25 μl 0.1% Tween-20) overnight at 4°C. Membranes were then incubated with 1:1000 dilutions of the appropriate antibodies in 10 ml of buffer (1 ml of 20mM Tris base and 140 mM NaCl, 0.5 g BSA, and 10 μl 0.1% Tween-20) for 2 h at room temperature. Then membranes were incubated with horseradish peroxidase -conjugated secondary antibody (1:2000) and horseradish peroxidase -conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 ml blocking buffer for 1 hour at room temperature. Immunoreactive proteins were revealed using enhanced chemiluminescence detection (LumiGLO™ , Cell Signaling). The signals on film were quantified using NIH Image 1.60 software.

Statistical analysis

Statistical evaluation was performed using student t-test or one-way ANOVA followed by the Student-Newman-Keuls post hoc test as appropriate. Data are expressed as mean ± S.E., and the level of significance was set at $p < 0.05$.

Results

Glucose transport

Figure 1A confirms that AICAR stimulates glucose transport by the L6 muscle cells [17]. The stimulation was unaffected by incubation with 0.5 mM or 5 mM glucose. The action of AICAR was replicated by the hormone adiponectin (abbreviated gAd to indicate the globular form) (Figure 1B), also known to activate AMPK [18,19]. Creatine incubation has been proposed to activate AMPK but not activate glucose transport [15]; we were able to confirm the second of those findings (Figure 1C).

Palmitate oxidation

Studies of palmitate oxidation matched those found with glucose transport: AICAR and gAd stimulated fatty acid oxidation at both high and low concentrations of the fatty acid (Figure 2A and 2B), but creatine treatment had no effect (Figure 2C).

Glycogen formation

AICAR (Figure 3A) inhibited the incorporation of radiolabeled glucose into glycogen, at both 0.5 mM as well as 5 mM glucose concentration in the medium. Since this result could be due to either an inhibition of glycogen formation (via glycogen synthase inhibition) or activation of glycogen breakdown (via glycogen phosphorylase activation), we examined the response to CP-91149, a known inhibitor of glycogen phosphorylase [20]. In the presence of CP-91149 alone (Figure 3B), incorporation of glucose into glycogen increased, as expected from inhibition of glycogen phosphorylase. Further addition of AICAR (compared with CP-91149 alone) again caused an inhibition of incorporation of glucose into glycogen. Thus, the result suggests a specific inhibitory action of AICAR on glycogen synthase. Similar to AICAR, gAd also inhibited glycogen formation at low and high concentrations of medium glucose (Figure 3C). However, the presence of creatine had the opposite effect. At both low and high concentrations of medium glucose, creatine stimulated glycogen formation (Figure 3D). Measurements of total glycogen content were performed for all of these conditions. While increased glucose itself did increase glycogen content from about 3 to 7 μmol/mg protein, there was no further effect with AICAR, gAd, or creatine (data not shown).

Lactate formation

The formation of lactate was stimulated by AICAR (Figure 4A) and by gAd (Figure 4B). On the other hand, incubation with creatine led

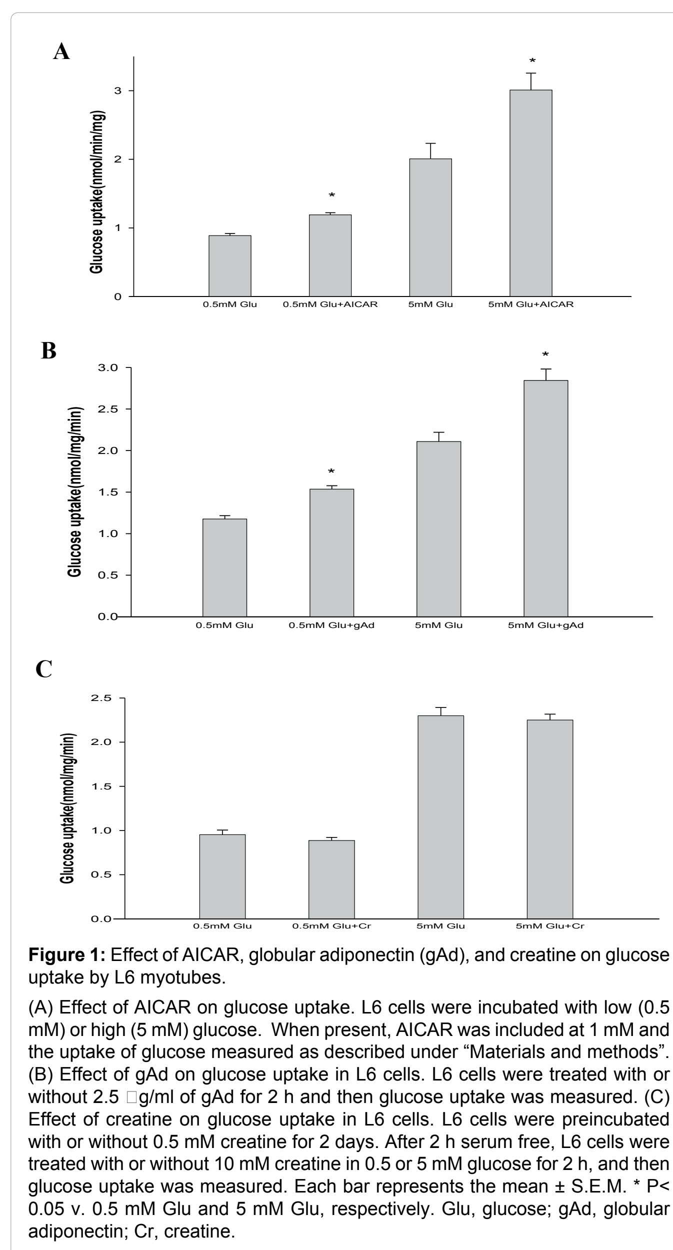

Figure 1: Effect of AICAR, globular adiponectin (gAd), and creatine on glucose uptake by L6 myotubes.

(A) Effect of AICAR on glucose uptake. L6 cells were incubated with low (0.5 mM) or high (5 mM) glucose. When present, AICAR was included at 1 mM and the uptake of glucose measured as described under "Materials and methods". (B) Effect of gAd on glucose uptake in L6 cells. L6 cells were treated with or without 2.5 μg/ml of gAd for 2 h and then glucose uptake was measured. (C) Effect of creatine on glucose uptake in L6 cells. L6 cells were preincubated with or without 0.5 mM creatine for 2 days. After 2 h serum free, L6 cells were treated with or without 10 mM creatine in 0.5 or 5 mM glucose for 2 h, and then glucose uptake was measured. Each bar represents the mean ± S.E.M. * $P < 0.05$ v. 0.5 mM Glu and 5 mM Glu, respectively. Glu, glucose; gAd, globular adiponectin; Cr, creatine.

to the opposite response: a decrease in lactate formation (Figure 5C).

Direct evidence of protein phosphorylation

As expected, AICAR and adiponectin led to an increase in the phosphorylated form of AMPK, leaving the total AMPK protein unaffected (Figure 5). However, there was no evidence for an effect of creatine on the phosphorylation of AMPK.

A time course for the phosphorylation of AMPK (Figure 6A,B) shows that this enzyme was phosphorylated earlier than its downstream target, acetyl CoA carboxylase (Figure 6C,D). The time course for the gAd-induced phosphorylation of AMPK was virtually to the AICAR-induced phosphorylation (data not shown).

As a further test for specific AMPK activation is the known inhibition of the phosphorylation of the p70 subunit of S6 Kinase [21]. We found that both AICAR and gAd attenuated this phosphorylation,

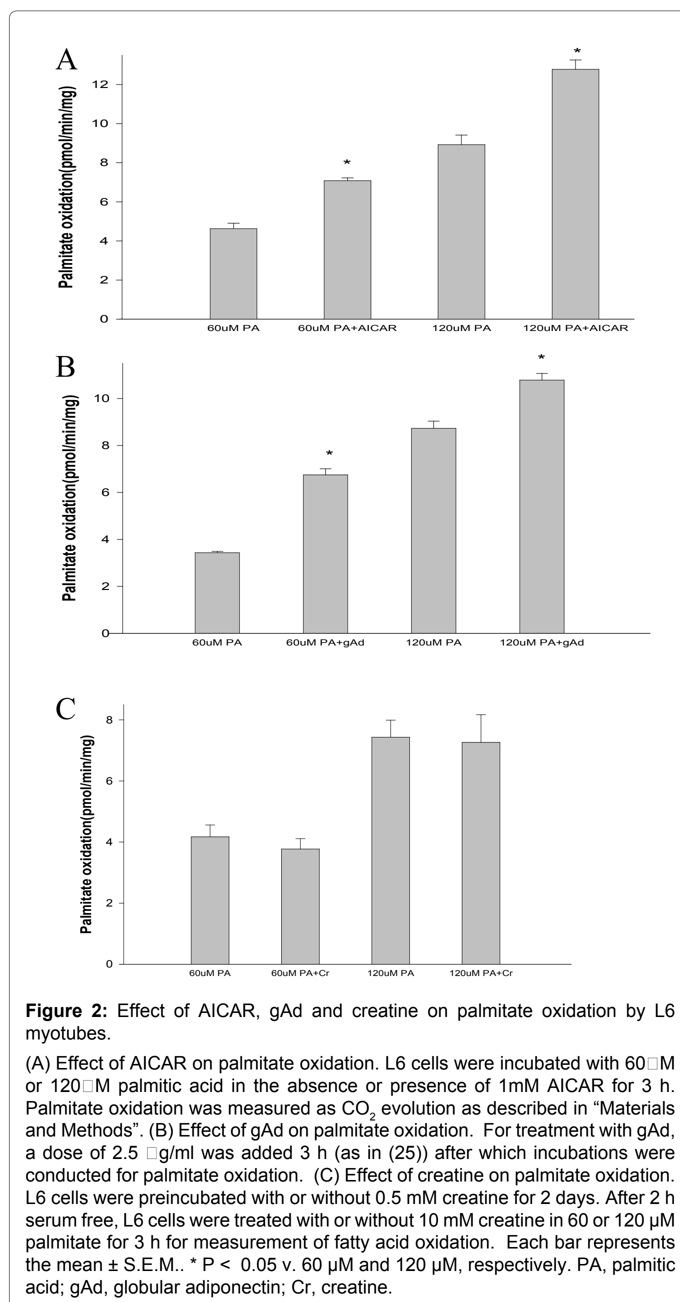

Figure 2: Effect of AICAR, gAd and creatine on palmitate oxidation by L6 myotubes.

(A) Effect of AICAR on palmitate oxidation. L6 cells were incubated with 60□M or 120□M palmitic acid in the absence or presence of 1mM AICAR for 3 h. Palmitate oxidation was measured as CO_2 evolution as described in "Materials and Methods". (B) Effect of gAd on palmitate oxidation. For treatment with gAd, a dose of 2.5 □g/ml was added 3 h (as in (25)) after which incubations were conducted for palmitate oxidation. (C) Effect of creatine on palmitate oxidation. L6 cells were preincubated with or without 0.5 mM creatine for 2 days. After 2 h serum free, L6 cells were treated with or without 10 mM creatine in 60 or 120 μM palmitate for 3 h for measurement of fatty acid oxidation. Each bar represents the mean ± S.E.M.. * $P < 0.05$ v. 60 μM and 120 μM, respectively. PA, palmitic acid; gAd, globular adiponectin; Cr, creatine.

while creatine had no effect (Figure 7).

Role of adenylate kinase in the action of adiponectin

Adiponectin is known to activate AMPK, and we used this to confirm the specific actions of AICAR. However, while the mechanism whereby AICAR activates AMPK is well known – the nucleoside is converted to an AMP analog [22]-the means by which adoponectin activates AMPK is not known. We considered the possibility that adiponectin increases ATP turnover, which should cause an increase in ADP concentration, and subsequently, through the adenylate kinase reaction, an increase in AMP concentration and thus of AMPK activity.

To assess this possibility, we used a specific siRNA for adenylate kinase, and found that this did strongly attenuate the immunoreactive protein content of adenylate kinase (Figure 8A). Nonetheless, this siRNA treatment did not affect basal glucose uptake nor did it affect the stimulation by gAd (Figure 8B). In order to provide further evidence that adenylate kinase was functionally suppressed, we experimentally established a condition that should definitely require adenylate kinase: glucose transport stimulated by potassium depolarization. In preliminary studies, we observed that addition of KCl did not reproducibly increase glucose uptake (data not shown), probably because of the transport of Cl^-. On the other hand, potassium gluconate at high concentrations stimulated glucose uptake (Figure 9).

When cells which had been treated with siRNA for adenylate kinase were subsequently K-depolarized, the stimulation of glucose uptake was eliminated; in fact, we observed that K-depolarization inhibited glucose transport (Figure 10). At the 40 mM concentration of potassium gluconate – conditions where there was no effect on basal conditions – there was no effect of adenylate kinase depletion. However, at higher concentrations, where control cells showed a stimulation of glucose transport in response to K-depolarization, the siRNA treated cells showed a diminished glucose transport compared to control.

Role of LKB1 in the action of adpionectin

We next turned to the question of whether an upstream kinase might be responsible for the action of adiponectin. In studies in which the AMPK Kinase, LBK1, was diminished by pretreatment with cognate siRNA (Figure 11A), the stimulation of glucose transport by gAd was abolished. Indeed, application of gAd led to a diminution of glucose uptake in the case of siRNA treated cells, compared to the absence of gAd (Figure 11B).

Effect of creatine on phosphofructokinase

Since we found that creatine treatment – which did not affect AMPK, or its downstream targets – caused a stimulated glycogen synthesis, and an inhibition of lactate formation, we considered the possibility that this condition led to a diminution of the expression of phosphofructokinase. Indeed, upon creatine treatment, we observed a decrease in the amount of phosphofructokinase expressed in the L6 cells (Figure 12).

Discussion

AICAR is well established to stimulate glucose uptake in many cell types [6]. Acute and chronic treatment with AICAR in obese and diabetic mice decreases blood glucose concentrations by activating the AMPK pathway, which increase glucose uptake [23]. While this conclusion is widely accepted, there are underlying uncertainties.

For example, Ceddia et al. found that creatine incubation activates AMPK phosphorylation but does not increase glucose uptake or GLUT 4 translocation [15]. The result would suggest that turnover of adenine nucleotides through their link with creatine phosphokinase can lead to AMPK activation, and yet not be causally connected with glucose transport. In another study, Sweeney et al. [24] demonstrated that intracellular delivery of phosphatidylinositol (3,4,5)-trisphosphate causes GLUT 4 translocation from cytoplasm into the plasma membrane of muscle and fat cells without increasing glucose uptake. It is also possible that AICAR may act as a nonselective agonist, activation sites other than AMPK, such as glycogen phosphorylase [25].

Beyond these findings, there has been little effort to collect in single study observations of the major pathways of muscle to determine if a consistent and selective set of actions can be ascribed to AMPK. For each of the major pathways we have analyzed here-glucose transport,

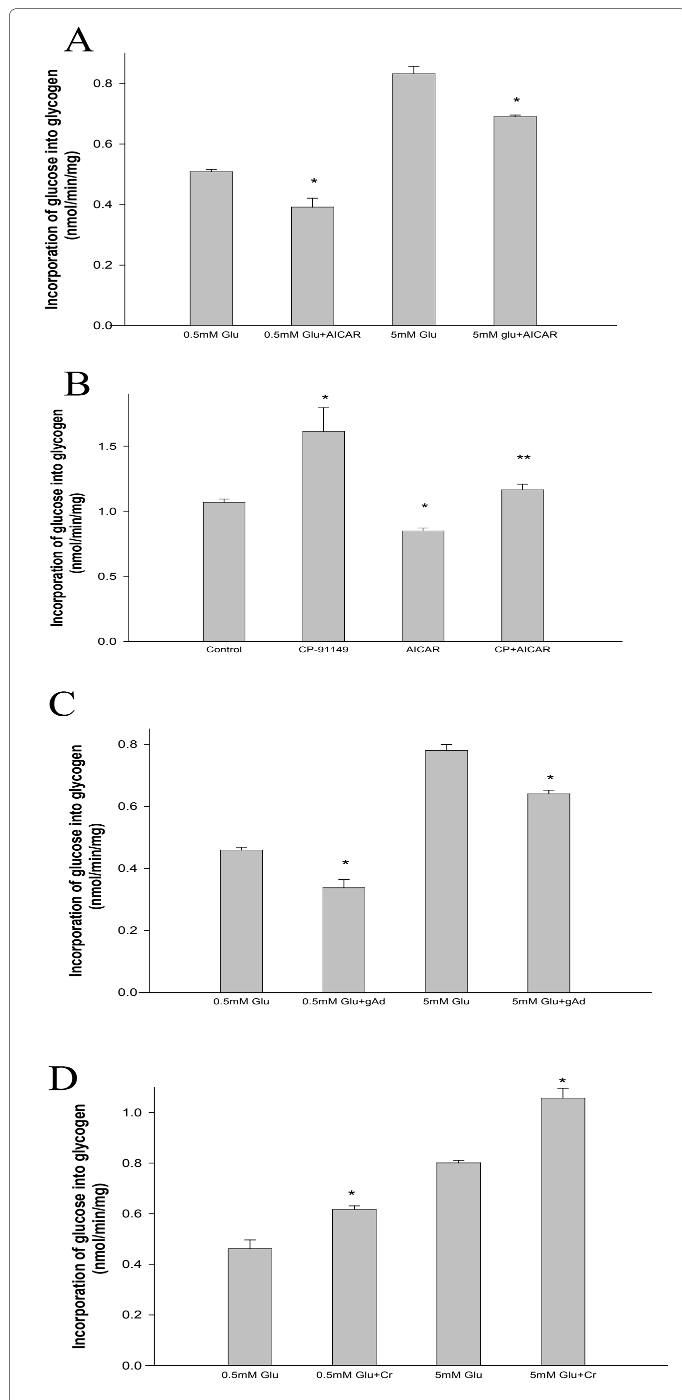

Figure 3: Effect of AICAR, CP-91149, gAd and creatine on glycogen synthesis by L6 myotubes

(A) Effect of AICAR on glycogen synthesis. When present, AICAR was included at 1 mM. Incubations contained 0.5 mM or 5 mM glucose and incubated 2 h, with glycogen synthesis measured as incorporation of D-[U-^{14}C] glucose into glycogen. (B) Effect of CP-91149. CP-91149 (10 μM) was added with or without AICAR, in the presence of 5 mM glucose, and incubations conducted for glycogen synthesis as in Fig. 3A. (C) Effect of gAd on glycogen synthesis. The incubation with gAd was conducted as in Fig. 1B, and measurement of glycogen synthesis conducted as above. (D) Effect of creatine on glycogen synthesis. Creatine pre-treatment was performed as in prior experiments (e.g., Fig. 1F). Glycogen synthesis was evaluated as in Fig. 3A. Each bar represents the mean ± S.E.M. * $P < 0.05$ v. 0.5 mM Glu and 5 mM Glu, respectively; ** P 0.05 v. CP-91149 alone. Glu, glucose; CP, CP-91149; gAd, globular adiponectin; Cr, creatine.

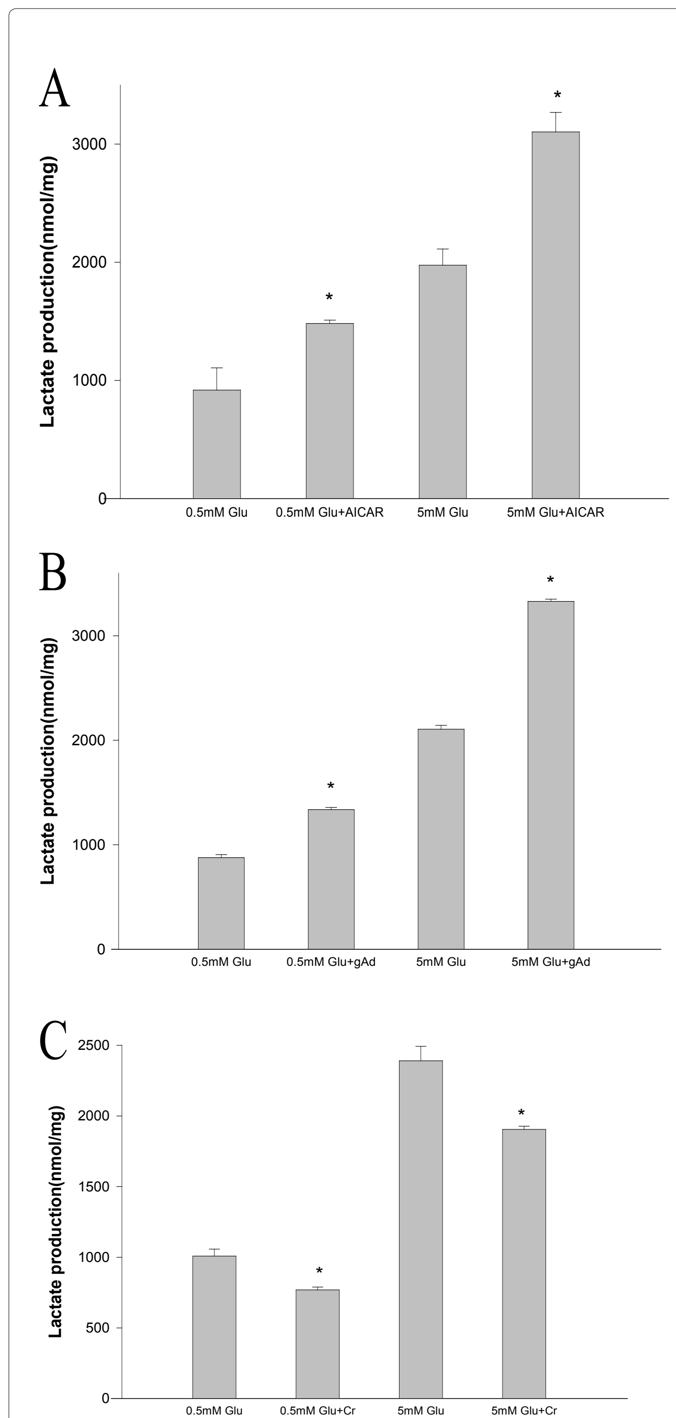

Figure 4: Effect of AICAR, gAd and creatine on lactate production by L6 myotubes

(A) Effect of AICAR on lactate formation. Cells were incubated as indicated for 4 h and the content of lactate was determined; no lactate was found at zero time (data not shown). (B) Effect of gAd on lactate formation. Cells were incubated with gAd and assayed for lactate formation as above. (C) Effect of creatine on lactate formation by L6 cells. L6 cells were preincubated with or without 0.5 mM creatine for 2 days. After 2 h serum free, L6 cells were treated with or without 10 mM creatine in 0.5 or 5 mM glucose for 4 h, for determination of lactate formation. Each bar represents the mean ± S.E.M.. * $P < 0.05$ v. 0.5 mM Glu and 5 mM Glu, respectively. Glu, glucose; gAd, globular adiponectin; Cr, creatine.

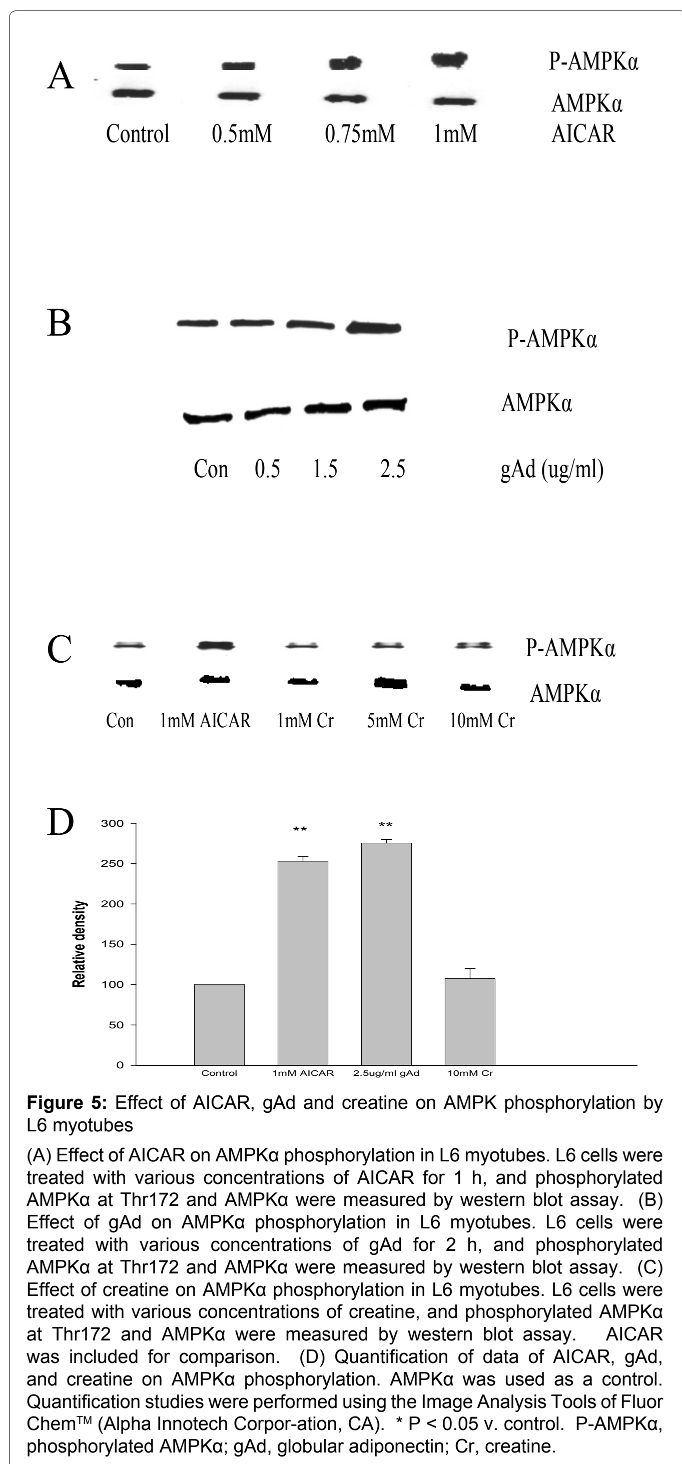

Figure 5: Effect of AICAR, gAd and creatine on AMPK phosphorylation by L6 myotubes

(A) Effect of AICAR on AMPKα phosphorylation in L6 myotubes. L6 cells were treated with various concentrations of AICAR for 1 h, and phosphorylated AMPKα at Thr172 and AMPKα were measured by western blot assay. (B) Effect of gAd on AMPKα phosphorylation in L6 myotubes. L6 cells were treated with various concentrations of gAd for 2 h, and phosphorylated AMPKα at Thr172 and AMPKα were measured by western blot assay. (C) Effect of creatine on AMPKα phosphorylation in L6 myotubes. L6 cells were treated with various concentrations of creatine, and phosphorylated AMPKα at Thr172 and AMPKα were measured by western blot assay. AICAR was included for comparison. (D) Quantification of data of AICAR, gAd, and creatine on AMPKα phosphorylation. AMPKα was used as a control. Quantification studies were performed using the Image Analysis Tools of Fluor Chem™ (Alpha Innotech Corpor-ation, CA). * $P < 0.05$ v. control. P-AMPKα, phosphorylated AMPKα; gAd, globular adiponectin; Cr, creatine.

fatty acid oxidation, glycogen metabolism, and glycolysis-there are separate studies which would indicate that either AMPK does not regulate them, does not control them in a consistent direction, or that there is a lack of correspondence between AICAR treatment, AMPK activation, a subsequent downstream target such as ACC, and a metabolic event [26-29].

Our studies were designed primarily to determine how AMPK affects the major pathways of metabolism in the muscle, using the L6 skeletal muscle cell cultures. We examined specificity of action for AICAR by comparison with the known activator of AMPK, adiponectin. In addition, we examined role of adenylate kinase in the action of adiponectin using siRNA ablation. We also examined the effects of creatine incubation as performed by Ceddia [15], and correlated the metabolic results with downstream phosphorylation events known to be elicited by AMPK. Our findings led us to consider new implications for AMPK regulation of muscle cell metabolism.

Adiponectin and AICAR

Our results indicate that, despite suggestions of non-selectivity, AICAR stimulated glucose transport, fatty acid oxidation, lactate formation, and inhibited glycogen formation in a manner indistinguishable from adiponectin. AICAR is known to activate AMPK directly as it is metabolized to an AMP analog which directly activates the enzyme [22]. However, the mechanism for adiponectin activation of AMPK remains unknown. In order to provide a new insight into this question, we turned to selective enzyme ablation using siRNA.

One means by which adiponectin could activate AMPK is through an increased ATP turnover, which might subsequently increase ADP and then AMP levels through the near-equilibrium enzyme adenylate kinase. However, when we pretreated cells with siRNA against adenylate kinase 1 (AK1), the main isoform of adenylate kinase in skeletal muscle cells, the response to adiponectin was unaffected. The western blot of AK1 protein demonstrated that the knockout was nearly complete; only trace amounts of protein were observed. However, in order to prove that this enzyme was functionally removed, we conducted experiments with potassium depolarization.

When muscle cells are depolarized, they are known to increase their glucose transport, the result of an increased ATP turnover, adenylate kinase, and subsequently AMP activation of AMPK. Cells incubated with potassium gluconate showed an increased glucose transport. However, this was prevented by preincubation with siRNA for AK1. Thus, the trace amount of AK1 revealed by western blotting was apparently functionally insignificant. A further finding from the depolarization control experiment was that this process leads to *less* glucose uptake when AK1 was suppressed (Figure 10). This is likely the result of an increase energy demand from the depolarization that cannot be met by signaling an increase in glucose uptake.

Knockout of the AMPK kinase, LKB1, by selective siRNA prevented the ability of adiponectin to stimulate glucose transport. This strongly suggests that it is the upstream kinase that is required for adiponectin signaling, the single finding that differentiates adiponectin from AICAR. Thus, the results presented here provide two separate means for AMPK activation: AICAR, which activates AMPK directly, similar to muscle excitation; and adiponectin, which activates the upstream kinase. The data also show that when the LKB1 is ablated, adiponectin decreased glucose transport rates to below control values. Thus it would appear that the hormone activates energy-depleting steps prior to its activation of LKB1. In a manner analogous to the depolarization experiment discussed above, since signaling to increase glucose uptake was blocked, no energy substrate could be provided to replenish the cell. The controls of the experiments of (Figure 8, 10, and 11) indicated that AK1 depletion *per se* did not alter glucose uptake.

Metabolic actions correlating to expected enzyme activations

The metabolic actions found here for AICAR correlated to the phosphorylation of AMPK, as well as the phosphorylation of the AMPK

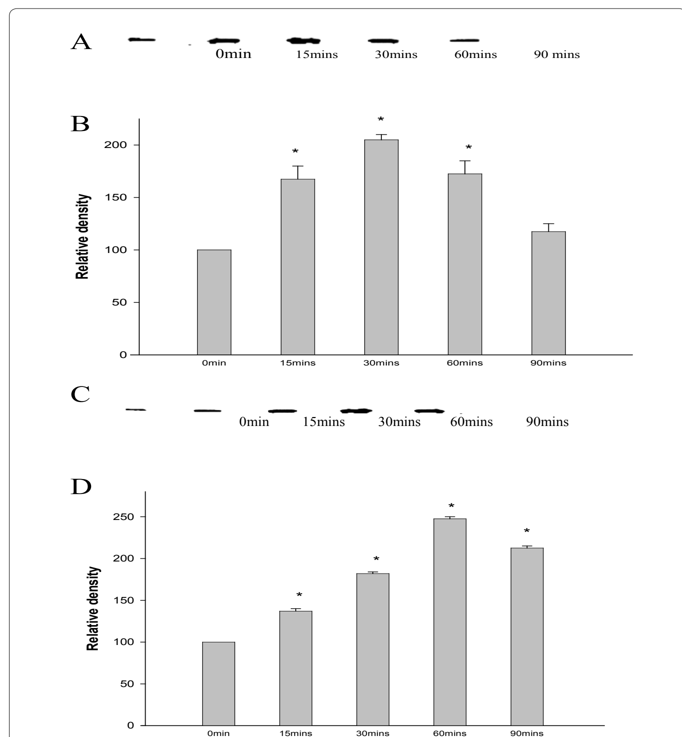

Figure 6: Effect of AICAR on the time course of AMPKα and ACC phosphorylation

(A) Cells were incubated with 1 mM AICAR for various times and the amount of phosphorylated AMPKα was measured as above. (B) Quantification of data of 6A, with at least three independent experiments. *P < 0.05 v. 0 min. (C) Time course of ACC phosphorylation at Ser79 treated with 1mM AICAR for various time periods. (D) Quantification of data of 6C, with at least three independent experiments. *P < 0.05 v. 0 min.

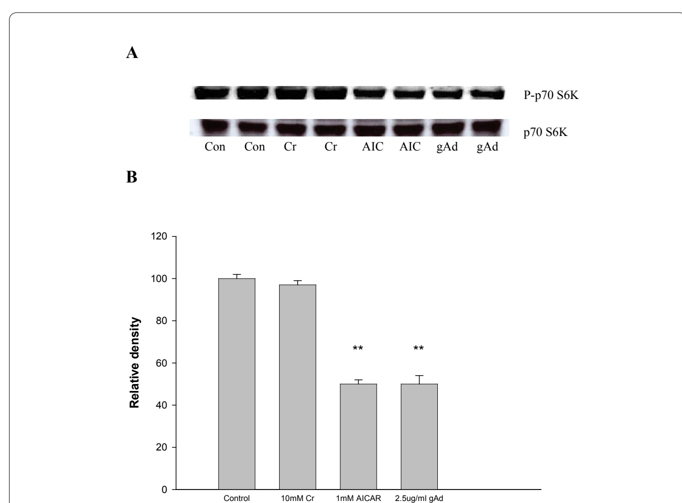

Figure 7: Effect of AICAR, gAd, and creatine on p70 S6 kinase phosphorylation in L6 myotubes.

(A) Effects of AICAR, gAd, and creatine on p70 S6 Kinase phosphorylation in L6 myotubes. L6 cells were incubated with 1 mM AICAR for 1 hour, 2.5 μg/ml gAd for 2 hours, and 10mM creatine for 2 hours (0.5 mM creatine preincubation for 2 days), and then p70 S6 kinase phosphorylation at Thr389 were measured by western blot, and p70 S6K was used as a control; P-p70 S6K; phosphorylated p70 S6 Kinase. Con: control; Cr: creatine; AIC: AICAR; gAd: globular adiponectin. (B) Quantification of data of AICAR, gAd, and creatine on p70 S6 Kinase phosphorylation in L6 myotubes. ** P < 0.01 v. control.

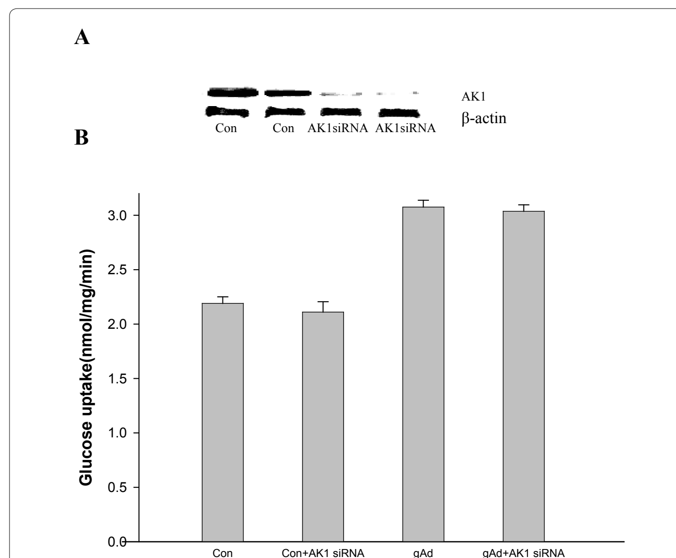

Figure 8: Role of adenylate kinase in the action of gAd

(A) Adenylate kinase 1 (AK1) protein expression treated with AK1 siRNA in L6 myotubes. L6 cells were incubated with or without 100 nM AK1 siRNA for 3 days and then protein expression was measured by western blot described under "Materials and methods". β-actin was used as a control; Con: control; AK1: AK1 siRNA. (B) Effect of AK1 siRNA on gAd induced glucose uptake in L6 myotubes. After transfected by AK1 siRNA, L6 cells were incubated with 5 mM glucose with or without 2.5 μg/ml gAd for 2 hours, and then glucose uptake was measured as described under "Materials and methods".

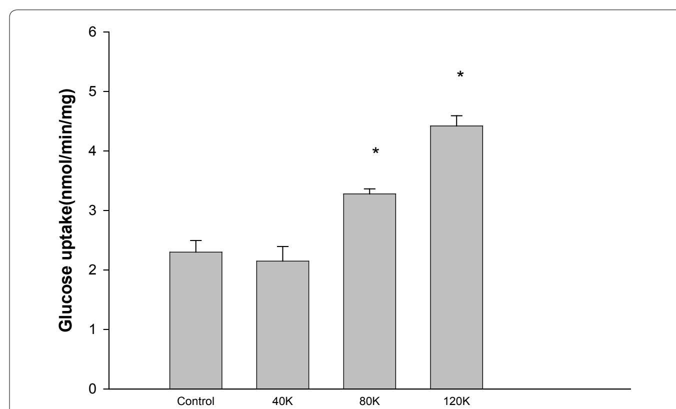

Figure 9: Effect of potassium gluconate on glucose uptake in L6 myotubes

L6 cells were incubated with 5 mM glucose in different concentration of potassium gluconate for 1 hour, and then glucose uptake was measured as described under "Materials and methods". ** p < 0.01 compared to control; K: potassium gluconate.

target, ACC. Moreover, the ACC phosphorylation was kinetically distinct from AMPK itself; appropriately, ACC phosphorylation appeared subsequent to AMPK phosphorylation [30]. These results confirm AICAR functionally stimulated AMPK activation in a manner indistinguishable from adiponectin, a hormone known to activate the enzyme. Not only that, we also found AICAR led to an inhibition of mTOR, an expected finding subsequent to AMPK activation [21].

Creatine effects

Whereas it had been suggested that a pre-incubation with creatine can increase AMPK but not glucose transport [15], our findings do not support this conclusion. We did find a lack of effect of creatine

on glucose transport, but there was none on AMPK either. Not only that, we found no increase in AMPK phosphorylation, nor any effect on mTOR: both of these were clearly demonstrated for AICAR and for adiponectin. Thus, we suggest that creatine and creatine phosphate levels do not affect AMPK.

We also found no effect of the creatine treatment on fatty acid oxidation. There was an effect of creatine treatment on glycogen formation and lactate formation: however, these were in opposite directions to the results obtained with AMPK activation. That is, creatine incubation led to a stimulation of glycogen formation and an inhibition of lactate formation, the reverse of our findings for AICAR and adiponectin. Thus, we propose that this perturbation led to a situation unrelated to AMPK. One possibility is that the incubation procedure, which is a period of two days (acute creatine incubation produced no effects in experiments not shown), lead to a down regulation of the rate limiting step of glycolysis, namely phosphofructokinase. Indeed, directly analysis of phosphofructokinase 1 (PFK1) protein (Figure 12) supported that view: we found a diminution of expressed protein. Thus, it is possible that the effects of creatine treatment were secondary to suppression of PFK1, which would build up glucose phosphates, increasing incorporation of glycogen, and decreasing lactate formation, explaining a lack of effect on glucose uptake by the cell. One interesting side point from this observation is that the Randle hypothesis posits that an inhibition of phosphofructokinase (in this case relayed indirectly through increased fatty acid oxidation) should lead to a decrease glucose uptake, through buildup of glucose phosphates [31]. However, no alteration at all was observed on glucose uptake in the situation we established, suggesting that it is not merely the signaling by glucose phosphates that controls glucose uptake. This suggests, as has been pointed out elsewhere [32], that the details of regulation need to be reconsidered, not surprising for a hypothesis that was posited 40 years ago and still serves as the organizing principle for understanding fundamental metabolic processes in muscle.

It has been found that the purified enzyme AMPK is altered when the ratio of these creatine compounds is varied. In vitro, creatine and creatine phosphate can be shown to be modulators of AMPK, and this has formed the basis of a regulation system for these compounds of AMPK [33]. However, we found no effect whatever on the AMPK phosphorylation state when cells were preincubated with creatine, conditions that have been established to markedly alter both these compounds and their ratio. It would therefore also seem that AMPK is not affected by these intermediates either. In retrospect, this would be a complication if it were true, since muscle creatine and creatine phosphate can vary tremendously depending upon the rapidly fluctuating energy status.

Overall metabolic actions of AMPK

Our results suggest that activators of AMPK stimulate glucose transport, inhibit glycogen synthesis, stimulate glycolysis (lactate formation), and fatty acid oxidation. The increased glycolytic flux is likely due to the increase uptake of glucose into the cell and inhibition of glycogen formation. While most studies of the effects of AMPK on glycogen metabolism have focused on the isolated enzyme, glycogen synthase [34], there have been reports of stimulation of glycogen synthase, secondary to the inhibition of the glycogen synthase kinase, GSK-3 [27]. However, a possible role for the breakdown enzyme of glycogen, the phosphorylase, has not been examined in the context of AMPK.

It is possible that a direct action of AMP may lead to activation

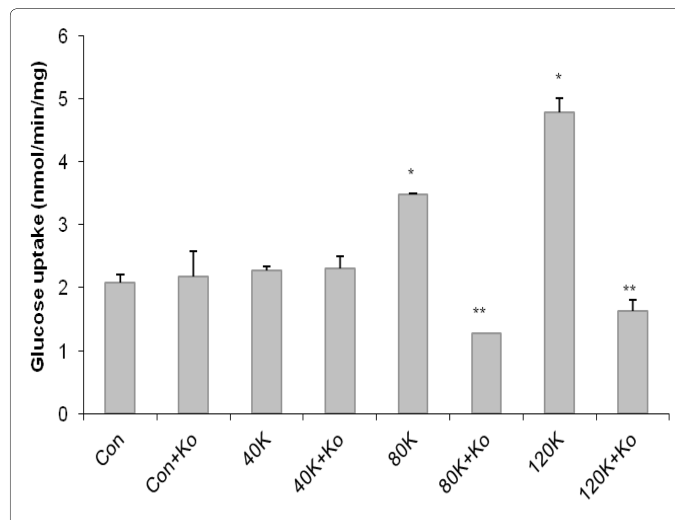

Figure 10: Effect of AK1 siRNA on potassium gluconate induced glucose uptake in L6 myotubes

After transfected by AK1 siRNA, L6 cells were incubated with 5 mM glucose with or without different concentration of potassium gluconate for 1 hour, and then glucose uptake was measured as described under "Materials and methods". C: control; Ko: AK1 siRNA; 40K: 40 mM potassium gluconate; 80K: 80 mM potassium gluconate; 120K: 120 mM potassium gluconate; * $p < 0.05$ versus control; ** $p < 0.01$ versus 80K and 120K, respectively.

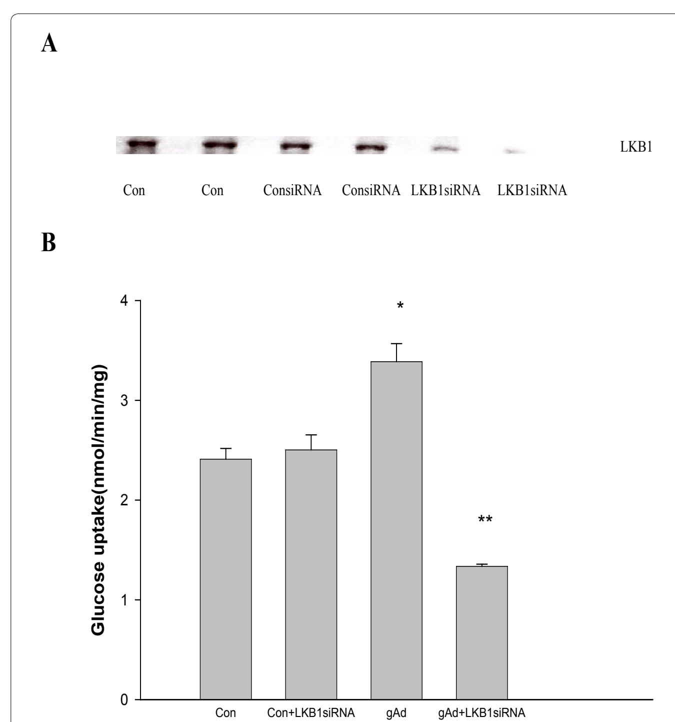

Figure 11: Role of LKB1 in the action of gAd in L6 myotubes

(A) LKB1 protein expression treated with LKB1 siRNA in L6 myotubes. L6 cells were incubated with or without 100 nM LKB1 siRNA for 3 days and then protein expression was measured by western blot described under "Materials and methods". ConsiRNA was used as a control; Con: control; ConsiRNA: control siRNA (non-targeting siRNA). (B) Effect of LKB1 siRNA on gAd induced glucose uptake in L6 myotubes. After LKB1 siRNA transfection, L6 cells were incubated with 5 mM glucose with or without 2.5 μg/ml of gAd for 2 hours, and then glucose uptake was measured as described under "Materials and methods". * $p < 0.05$ versus control (Con); ** $p < 0.01$ versus gAd.

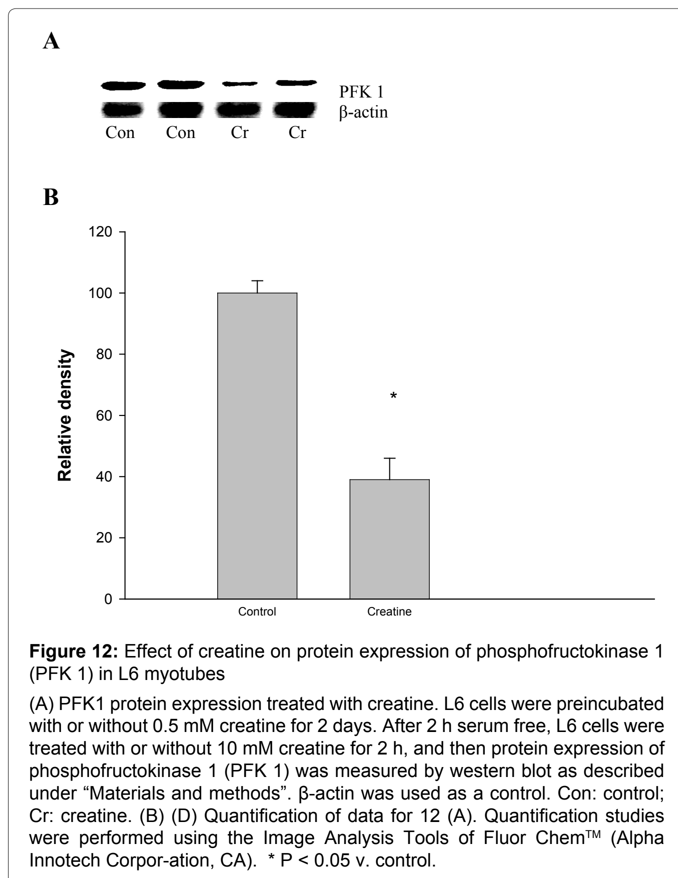

Figure 12: Effect of creatine on protein expression of phosphofructokinase 1 (PFK 1) in L6 myotubes

(A) PFK1 protein expression treated with creatine. L6 cells were preincubated with or without 0.5 mM creatine for 2 days. After 2 h serum free, L6 cells were treated with or without 10 mM creatine for 2 h, and then protein expression of phosphofructokinase 1 (PFK 1) was measured by western blot as described under "Materials and methods". β-actin was used as a control. Con: control; Cr: creatine. (B) (D) Quantification of data for 12 (A). Quantification studies were performed using the Image Analysis Tools of Fluor Chem™ (Alpha Innotech Corpor-ation, CA). * P < 0.05 v. control.

of glycogen phosphorylase [35]; this might lead to a similar finding of decreased label from glucose incorporated into the glycogen fraction. To explore involvement of glycogen phosphorylase, we used the inhibitor CP-91149. By itself, CP-91149 caused an increase in labeled glucose incorporation to glycogen. However, in the presence of AICAR, we nonetheless found an inhibition of glycogen synthesis, rendering the conclusion that inhibition of glycogen synthase as the basis for the glycogen effects of AMPK more likely.

There are few studies of how AMPK activation causes lactate production. AICAR treatment increases net hepatic lactate output in hypoglycemic dogs [36]. AICAR stimulates net lactate release at a basal concentration of insulin in rat soleus muscle cells, an action attributed to stimulation of glycogen phosphorylase activity [37]. However, as we have just seen, glycogen phosphorylase is not likely for AMPK activation; thus, the lactate production probably reflects an increased glucose uptake and decreased glycogen synthesis. Smith et al. [38] found that AICAR leads to a pyruvate-independent activation of the pyruvate dehydrogenase complex. This would not explain an increase in lactate formation, nor would it be consistent with the known increase in fatty acid oxidation. However, it may result from a situation where fatty acids are low in concentration, and further oxidation of glucose may provide further cellular energy. Such an effect requires further investigation. Finally, the new finding that AICAR can be considered "exercise in a pill" [39] should be tempered by the fact that a full accounting for the metabolic actions in different situations of AMPK activation are still not entirely clear. It is true that the results of the present study do support AICAR as a relatively selective drug, and can activate several of the metabolic pathways related to exercise. However, despite the support we provide here for selectivity of AICAR activation of AMPK, sole activation of that enzyme in the absence of an increase in Ca^{2+} ions, or of myosin ATPase that would be the consequence of exercise clearly differentiate the physiological situation from the drug action of AICAR. Rather, the recent study shows that AICAR represents "partial exercise in a pill"; even then, it should be co-administered with a grain of NaCl.

Acknowledgements

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