AMP-activated Protein Kinase Suppresses Protein Synthesis in Rat Skeletal Muscle through Down-regulated Mammalian Target of Rapamycin (mTOR) Signaling*

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AMP-activated protein kinase (AMPK) is viewed as an energy sensor that acts to modulate glucose uptake and fatty acid oxidation in skeletal muscle. Given that protein synthesis is a high energy-consuming process, it may be transiently depressed during cellular energy stress. Thus, the intent of this investigation was to examine whether AMPK activation modulates the translational control of protein synthesis in skeletal muscle. Injections of 5-aminoimidazole-4-carboxamide 1-β-D-ribonucleoside (AICAR) were used to activate AMPK in male rats. The activity of α1 AMPK remained unchanged in gastrocnemius muscle from AICAR-treated animals compared with controls, whereas α2 AMPK activity was significantly increased (51%). AICAR treatment resulted in a reduction in protein synthesis to 45% of the control value. This depression was associated with decreased activation of protein kinases in the mammalian target of rapamycin (mTOR) signal transduction pathway as evidenced by reduced phosphorylation of protein kinase B on Ser273, mTOR on Ser2448, ribosomal protein S6 kinase on Thr389, and eukaryotic initiation factor eIF4E-binding protein on Thr7. A reduction in eIF4E associated with eIF4G to 10% of the control value was also noted. In contrast, eIF2B activity remained unchanged in response to AICAR treatment and therefore would not appear to contribute to the depression in protein synthesis. This is the first investigation to demonstrate changes in translation initiation and skeletal muscle protein synthesis in response to AMPK activation.

Considerable attention has focused on understanding the role of AMP-activated protein kinase (AMPK) in monitoring the energy status of the cell and mediating subsequent metabolic events. AMPK has been referred to as an energy-sensing/signaling protein within the cell that responds to changes in the ratio of ATP/AMP as well as phosphocreatine/creatine (1, 2). Changes in the cellular energy state activate AMPK through various mechanisms involving allosteric regulation of AMPK, activation by an upstream AMPK kinase, and diminished activity of phosphatases (3). AMPK activation increases glucose uptake and fatty acid oxidation in muscle (4) as well as up-regulates expression of various metabolic genes (e.g. the glucose transporter, GLUT4, uncoupling protein-3, and cytochrome c) (5–7). Consequently AMPK serves as a sensor/modulator of intermediary metabolism by directing cellular events to increase energy availability and sustain high energy phosphate levels.

Research using in vitro systems has shown that AMPK can be activated under artificial conditions such as treatment with high fructose or 2-deoxyglucose, heat shock, and inhibitors of oxidative phosphorylation (3). Pharmacological use of 5-aminoimidazole-4-carboxamide 1-β-D-ribonucleoside (AICAR) has been commonly utilized to directly activate AMPK without altering cellular concentrations of ATP, ADP, and AMP (8). Additionally, starvation and endurance exercise result in increased activity of AMPK in skeletal muscle (9–11). Exercise alters the adenine nucleotide ratios and serves as a physiological context for AMPK activation. Recently specific catalytic isoforms of AMPK (α1 and α2) have been shown to be differentially regulated by exercise intensity with α2 AMPK exhibiting greater metabolic sensitivity compared with the α1 isoform (10, 12).

The concept of AMPK acting as an energy sensor suggests that cellular processes that utilize ATP, and are not vital to short term survival, are potential control points for regulation by the protein kinase (13). Thus, a hierarchy may exist for ensuring sufficient energy availability during an energetic stress and the anabolic process of protein synthesis may be diminished to support that dominant function. The acute control of global rates of protein synthesis is predominantly executed at the level of translational initiation with the modulation of various eukaryotic initiation factors (eIFs) (14).

The protein kinase referred to as the mammalian target of rapamycin (mTOR), which serves as a convergence point for signaling by growth factors and amino acids to the mRNA binding step of translation initiation is involved in modulation of the phosphorylation of the binding protein for the eukaryotic initiation factor 4E, i.e. 4E-BP1. It also acts to control the phosphorylation status of the 70-kDa ribosomal protein S6 kinase (S6K1).

Modulation of these translation initiation events allows for more immediate control of protein synthesis and is responsive to changes associated with acute metabolic or nutritional alterations. Therefore, the present investigation examined whether or not activation of AMPK by treatment with AICAR would depress translational initiation. We hypothesized that during an apparent cellular energy stress induced by AICAR,
increased AMPK activity would diminish translation initiation and attenuate protein synthesis.

**EXPERIMENTAL PROCEDURES**

**Animals**—Animal facilities and the experimental protocol were reviewed and approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University College of Medicine. Male Sprague-Dawley rats (~175 g) were kept on a 12-h light:dark cycle with food (Harlan-Teklad Rodent Chow, Madison, WI) and water provided freely.

**AICAR Injections**—Rats were injected subcutaneously with AICAR (1 mg/g of body weight) in sterile 0.9% NaCl, or controls were given an equivalent volume of 0.9% NaCl (n = 6–10 animals per group). A flooding dose (1.0 ml/100 g body of weight) of 1-[3,4,5,6-3H]Hepialiannine (150 mmol/liter) was injected via the tail vein 50 min after the subcutaneous injections for the measurement of rates of synthesis of total mixed muscle protein (15). Rats were sacrificed by decapitation 1 h after receiving the subcutaneous injection. Previous research has demonstrated that AMPK activity peaks between 1 and 2 h following injection with specific antibodies to the α1 or α2 catalytic subunits of AMPK and BioMag goat anti-rabbit beads, and AMPK activity was measured in the immunoprecipitate as described under "Experimental Procedures" (n = 6 per group). † †, p < 0.001 versus control group.

**Results**

The experimental model utilized in the studies reported herein is based on the use of the chemical AICAR to artificially activate AMPK. AICAR is internalized by the cell and subsequently phosphorylated to form an AMP analog, termed ZMP, that acts as a metabolic activator of both AMPK and AMP kinase without altering the adenine nucleotide ratios within the cell (8). To assess the effectiveness of AICAR treatment in the present study, the activity of the α1 and α2 isoforms of AMPK were measured following immunoprecipitation of the kinase from skeletal muscle of rats administered either AICAR or vehicle alone 1 h before analysis. As shown in Fig. 1, activation of the AMPK α1 isoform was unchanged in AICAR-treated rats compared with rats administered vehicle alone (control rats). In contrast, α2 AMPK activity was significantly elevated (p < 0.001) in AICAR-treated animals compared with controls.

To determine whether or not activation of α2 AMPK in response to AICAR treatment was associated with a change in protein synthesis, in vivo rates of the synthetic process were measured using the flooding dose technique (15). The results show that in AICAR-treated rats, protein synthesis in skeletal muscle was depressed to 55% of the value (p < 0.02) observed in control animals (Fig. 2). To examine potential mechanisms regulating the reduction in protein synthesis associated with increased α2 AMPK activity, several key regulatory steps in translation initiation were investigated.

The regulation of eIF2 is an important event during translation initiation in maintaining global rates of protein synthesis. The first step in translation initiation is the binding of methionyl-tRNA, to eIF2-GTP to form a ternary complex that subsequently binds to the 40 S ribosomal subunit. Formation of the ternary complex can be modulated by phosphorylation of eIF2 on the α-subunit by converting it into a competitive inhibitor of eIF2B activity (21). In the present study the phosphorylation state of eIF2α was examined by protein immunoblot analysis using an anti-phosphopeptide antibody that only recognizes eIF2α when it is phosphorylated on Ser51 (2). The results show that the relative phosphorylation of eIF2α was reduced to 80% of the control value (p < 0.05) in AICAR-treated rats. Because the activity of eIF2B can be modulated by the phosphorylation state of eIF2α, the guanine nucleotide exchange activity of eIF2B was measured in extracts of muscle from AICAR-treated and control animals. However, the activity of eIF2B was not significantly different between the two groups (0.063 ± 0.009 and 0.056 ± 0.008 pmol of GDP exchanged/min, respectively).
To further examine potential mechanisms involved in the depression of protein synthesis associated with AMPK activation, the effect of AICAR treatment on eIF4G association with eIF4E was examined. As shown in Fig. 3A, the amount of eIF4G associated with eIF4E was decreased to ~10% of the control value (p < 0.01) in muscle from AICAR-treated rats. One mechanism for regulating the binding of eIF4G to eIF4E involves phosphorylation of 4E-BP1, which releases eIF4E from the inactive 4E-BP1-eIF4E complex and allows it to bind to eIF4G. In muscle from AICAR-treated rats, phosphorylation of 4E-BP1 on Thr37, a priming event for phosphorylation of the protein on additional residues that promote its dissociation from eIF4E, was significantly reduced (p < 0.01) (Fig. 3B). Phosphorylation of Thr37 on 4E-BP1 is mediated by a protein kinase referred to as mTOR (22). Another downstream target of mTOR is the 70-kDa S6K1. Similar to its effect on 4E-BP1 phosphorylation, AICAR treatment reduced phosphorylation of Thr389 on S6K1 to 5% of the value (p < 0.01) observed in control rats (Fig. 3C). Together the changes in 4E-BP1 and S6K1 phosphorylation suggest that the activity of mTOR was repressed in skeletal muscle of AICAR-treated rats.

The stimulation of the protein kinase activity of mTOR by growth factors such as insulin or insulin-like growth factor-I is mediated in part by phosphorylation of protein kinase B on Ser473, which results in its activation (23). PKB subsequently phosphorylates a residue (Ser2448) on mTOR that is present in a domain that normally acts to repress mTOR protein kinase activity (24). To examine whether or not activation of AMPK might result in changes in PKB activity and thereby alter phosphorylation of mTOR, protein immunoblot analysis was performed using antibodies specific for mTOR phosphorylated on Ser2448 and PKB phosphorylated on Ser473. As shown in Fig. 4A and B, the relative phosphorylation of both mTOR on Ser2448 and PKB on Ser473 in AICAR-treated rats was proportionately decreased to ~40% of the control value (p < 0.05).

**DISCUSSION**

AMPK is recognized as having a well established role in the regulation of energy production and nutrient flux within skeletal muscle during periods of energetic stress. The present study provides the first in vivo evidence that AMPK activation directly affects translational initiation and protein synthesis in skeletal muscle and that these responses are mediated through the mTOR signaling pathway. The results also further establish AMPK as a unique energy sensor that not only modulates glucose and fatty acid metabolism but also appears to regulate,
ampolyte exists that the diminished eIF2/Thr37 on 4E-BP1 and Thr389 on S6K1 was significantly
sis observed in the present study was mediated by alterations
energetic stress.
acting as an energy sensor is reinforced by the present inves-
ment results in significant suppression of the synthesis of total
protein synthesis, such as fasting, exercise, anorexia, and cachexia, have all been shown to depress protein
changes in mRNA translation and protein synthesis under these
conditions has remained elusive. The concept of AMPK
acting as an energy sensor is reinforced by the present presen-
tivation by establishing increased AMPK activity as a regula-
tor of skeletal muscle protein synthesis under conditions of
an energetic stress.

The dramatic suppression of skeletal muscle protein synthesis observed in the present study was mediated by alterations in several key steps in translation initiation. Phosphorylation of Thr37 on 4E-BP1 and Thr389 on S6K1 was significantly reduced with AICAR, indicative of a decrease in global rates of protein synthesis as well as the synthesis of specific proteins involved with the translational apparatus (mRNAs containing a terminal oligopyrimidine tract adjacent to the m7GTP cap, i.e. TOP mRNA), respectively. The hypophosphorylated 4E-BP1 would be expected to sequester eIF4E and prevent its association with eIF4G (30). Indeed the profound decrease in eIF4E associated with eIF4G observed in AICAR-treated rats corroborates this idea. Because the binding of eIF4E/mRNA complex to the ribosome requires association of eIF4F with eIF4G, the decreased binding of eIF4F to eIF4E observed in AICAR-treated rats would contribute to the depression in protein synthesis noted.

The role of eIF2 in regulating mRNA translation with AMPK activation appears less clear. A reduction in eIF2B activity would be suggestive of a decrease in global rates of protein synthesis, but eIF2B activity remained unchanged with AICAR treatment. Moreover, the small reduction in eIF2α phosphorylation is somewhat paradoxical given that such a change should be associated with enhanced ternary complex formation, MetRNA binding to ribosomes, and protein synthesis. The possibility exists that the diminished eIF2α phosphorylation was part of a response that represented an attempt by the cell to maintain a basal level of protein synthesis to prevent extensive catabolism. Nonetheless the function of eIF2 may not be crucial to regulating translation initiation under these conditions.

This study represents the first investigation to demonstrate alterations in mTOR phosphorylation and accompanying changes in 4E-BP1 and S6K1 using an in vivo model. Our results suggest that AMPK may signal through PKB to down-regulate the activity of mTOR and its downstream effectors. The exact mechanism(s) by which AMPK modulates PKB/mTOR phosphorylation is unknown; however, in addition to Ser172 phosphorylation of Ser208 on PKB was also reduced suggesting kinases upstream of PKB (e.g. PDK1 or PDK2) may be targets for AMPK. Moreover, the possible involvement of changes in phosphatase activity (e.g. PP1 or PP2A) cannot be ruled out.

A recent investigation has proposed that mTOR serves as an energy sensor by monitoring changes in ATP concentrations (31). However, significant decreases in ATP concentrations in vivo are difficult to demonstrate under physiological conditions. Additionally, AMPK activation appears to be more sensitive to alterations in the ratio of AMP/ATP and phosphocreatine/creatine than to absolute changes in ATP concentrations.

Therefore, AMPK may act as a molecular signal to control mRNA translation depending on the cellular adenine nucleotide ratios.

In conclusion, this research identifies a new cellular function for AMPK by its ability to modulate skeletal muscle protein synthesis and the phosphorylation state of translation initiation factors upon activation. Given the high energy consumption associated with protein synthesis in the cell, this anabolic function may be suppressed while cellular energy is either conserved or partitioned to maintain ATP concentrations. Furthermore, gaining a mechanistic appreciation for the regulation of skeletal muscle protein synthesis during an acute or chronic energy stress may provide nutritional and/or therapeutic strategies for the treatment of metabolic diseases. Ultimately AMPK should be viewed with an integrated approach by understanding its role in cellular function and how these events can potentially influence whole body metabolism of carbohydrate, fat, and protein.

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