Time course changes in signaling pathways and protein synthesis in C2C12 myotubes following AMPK activation by AICAR


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The AMP-activated protein kinase (AMPK) has been shown to be a sensor of cellular energy status, regulated by reciprocal changes in the levels of AMP and/or ATP (18, 19, 26). Increases in the AMP-to-ATP ratio such as those observed in response to starvation, exercise, muscle contraction, or other conditions involving skeletal muscle catabolism lead to activation of AMPK, which in skeletal muscle causes a repression of the growth-promoting mammalian target of rapamycin (mTOR) signaling pathway (16). Activation of AMPK in experimental model systems is often achieved by administration of the AMPK activator 5-aminoimidazole-4-carboxamide-1-β-d-ribonucleoside (AICAR), which is metabolized to ZMP, an AMP mimic (55). Previous studies from our laboratory (2, 44) and others’ (21) have demonstrated an association between an AICAR-induced reduction in protein synthesis and a repression of mTOR-related signaling.

Signal transduction through mTOR is modulated in part by the regulatory associated protein of mTOR (Raptor) (15, 25, 28, 49). Under conditions of cell stress, Raptor tightly associates with mTOR, thereby reducing signaling to the downstream targets eukaryotic initiation factor 4E (eIF4E) and ribosomal protein (rp)S6 kinase (S6K1) (15, 41). As a consequence of reduced mTOR signaling, eIF4E is hypo-phosphorylated, resulting in binding and sequestration of eIF4E, thereby decreasing the availability of eIF4E (30, 31) for assembly of the eIF4E complex, which is required for the mRNA binding step in translation initiation (38). Reduced mTOR signaling also results in dephosphorylation of S6K1 on Thr389 (51), which is associated with decreased translation of mRNAs containing 5’-oligopyrimidine tracts (36, 54). Dephosphorylation of S6K1 also represses translation elongation through inhibition of the eukaryotic elongation factor (eEF)2 kinase (4, 8). Thus AICAR-induced repression of mTOR signaling can lead to a reduction in global rates of protein synthesis as well as changes in the selection of mRNAs for translation.

Two mechanisms have been proposed through which AMPK might repress signaling through mTOR. In the first case, phosphorylation of mTOR on Ser2448 by AMPK correlates with reduced phosphorylation of downstream mTOR targets (9). In that study, it was proposed that rather than directly inhibiting mTOR activity, phosphorylation of mTOR on Ser2448 instead acts to prevent phosphorylation of Ser2461, a residue whose phosphorylation often correlates with increased signaling through mTOR (2, 10). The second mechanism through which AMPK might inhibit mTOR function involves AMPK-mediated phosphorylation of the tuberous sclerosis complex 2 (TSC2) gene product Tuberin on Thr1462 and Ser1345 (23). Tuberin, in a complex with Hamartin (aka TSC1), acts as a GTPase activator protein (GAP) for the Ras homolog enriched in brain (Rheb), resulting in an increase in GDP bound to Rheb (35, 60). Because Rheb-GDP binds to and
inhibits mTOR, AMPK-mediated activation of Tuberin indirectly results in a reduction in mTOR activity. Conversely, the GAP activity of the Tuberin-Hamartin complex is repressed by extracellular signal-regulated protein kinase (ERK)1/2-mediated phosphorylation of Tuberin on Ser664 (37). Furthermore, phosphorylation of Tuberin on Ser1210 and Ser798 by 90-kDa ribosomal protein S6 kinase (p90RSK) also represses Tuberin-Hamartin function (1, 48). The finding that AICAR-induced AMPK phosphorylation is associated with activation of the ERK1/2 pathway suggests that regulation of mTOR signaling by AMPK is a balance between the repressive effect of ERK1/2 signaling and the stimulatory effect of AMPK-mediated phosphorylation of Tuberin.

We (2) showed previously that AICAR administration to rats in vivo inhibits protein synthesis and suppresses signaling through the mTOR pathway in skeletal muscle. However, in that study, plasma insulin concentrations (and probably other hormones) were also affected by the treatment protocol. The present study was therefore performed to examine the direct effect(s) of AICAR on protein synthesis and signaling pathways controlling mRNA translation with cultures of C2C12 myotubes as an experimental model. Collectively, the results from the present study demonstrate that AICAR induces inhibition of protein synthesis through the simultaneous repression of the mTOR- and activation of the ERK1/2-signaling pathways modulating translation initiation and elongation.

**EXPERIMENTAL PROCEDURES**

**Materials.** AICAR was purchased from Toronto Research Chemicals. Polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences. 35S-labeled Easytag Express protein labeling mix was purchased from PerkinElmer. Anti-4E-BP1, anti-Raptor, and anti-S6K1 antibodies were purchased from Bethyl Laboratories. The anti-Hamartin antibody and the anti-Tuberin antibody used for Tuberin immunoprecipitation were purchased from Santa Cruz Biotechnology. The eIF4G antibody was produced in the authors’ laboratory (32). All other antibodies were obtained from Cell Signal Technology. Enhanced chemiluminescence (ECL) detection kits were purchased from Amersham Biosciences, and the donkey anti-rabbit and sheep anti-mouse horseradish peroxidase-conjugated IgG were purchased from Bethyl Laboratories.

**Cell culture.** C2C12 myoblasts (American Type Culture Collection) were seeded in six-well (35-mm) plates or 100-mm culture dishes in DMEM supplemented with 10% fetal calf serum (Atlas Biologicals, Fort Collins, CO) and 1% penicillin and streptomycin. Cells were grown to ~95% confluence and then induced to differentiate into myotubes by incubation in serum- and antibiotic-free DMEM supplemented with ITS Liquid Media Supplement (Sigma, St. Louis, MO) for 3 days. Before the start of the experiments, the myotubes were incubated in serum- and antibiotic-free DMEM for 1 h. AICAR, when present, was added to the medium at a final concentration of 2 mM for the times indicated in the figure legends. For Western blot analysis, cells were harvested by scraping in 150 μl of 1× SDS sample buffer. For polysome aggregation analysis, cells were harvested by scraping in a buffer consisting of 50 mM Hepes (pH 7.4), 250 mM KCl, 5 mM MgCl2, 250 mM sucrose, 1% Triton X-100, 1.3% deoxycholate, 100 μg/ml cycloheximide, and 5 μl/ml SUPERase-in (Ambion). The resulting cell homogenate was mixed on a platform rocker for 10 min at 4°C, clarified by a 3,000 g centrifugation (4°C) for 15 min and then layered onto a sucrose density gradient for polysome aggregation analysis. When protein synthesis measurements were being performed, cells were incubated in six-well dishes, and 1.5 μl of 35S-Easytag Express protein labeling mix (14 μCi/ml) were added to the cell culture medium either 5 min prior to harvest (for the 15-min time point) or 30 min prior to harvest (for the 30- and 60-min time points). The myotubes were then harvested by scraping in a buffer containing 20 mM Hepes (pH 7.4), 2 mM EGTA, 50 mM NaF, 100 mM KCl, 0.2 mM EDTA, 50 mM β-glycerophosphate, 1% Triton X-100, 1% deoxycholate, 0.1 mM phenylmethylsulfonlfyl fluoride (PMSF), 1 mM benzamidine, 1 mM DTT, and 0.5 mM sodium vanadate. The resulting cell homogenate was mixed on a platform rocker for 30 min at 4°C and then clarified by a 1,000-g centrifugation (4°C) for 3 min. Aliquots of the supernatant were used for protein synthesis measurements, and Western blot analysis, or stored at −80°C until analyzed.

**Analysis of Western blots.** Protein immunoblots were visualized via ECL, as described previously (33), and then quantified by measuring the luminescent signal using a GeneGnome Bio-Imaging System (SynGene).

**Quantitation of phosphorylated and total AMPK, MEK1/2, ERK1/2, eIF4E, PKB, S6K1, rpS6, eIF2, and eIF4G.** Following harvest in 1× SDS sample buffer, total cell lysate was resolved by electrophoresis on 7.5% (S6K1, eIF4G), 10% (AMPK, eIF2, PKB), 12.5% (MEK1/2, ERK1/2), or 15% (rpS6, eIF4E) polyacrylamide gel. The proteins were transferred to PVDF membranes, which were then incubated with anti-phosphopeptide antibodies directed against Thr389 and Thr422/Ser424 for S6K1, Ser1108 for eIF4G, Thr172 for AMPK, Thr36 for eEF2, Ser73 for PKB, Thr172/212 for MEK1/2, Thr202/Tyr204 for ERK1/2, Ser235/236 and Ser240/254 for rpS6, or Ser209 for eIF4E. The immunoblots were developed and analyzed as described above. The blots were then stripped and reprobed with antibodies that recognize S6K1, eIF4G, AMPK, eEF2, PKB, MEK1/2, ERK1/2, rpS6, or eIF4E independently of phosphorylation state. Results obtained using phosphospecific antibodies were corrected for total protein expression, and results are presented as a percentage of the control condition. No change in expression level in response to AICAR treatment was observed for any of the proteins analyzed.

**Immunoprecipitation of eIF4E complexes.** The association of 4E-BP1 or eIF4G with eIF4E was determined by the use of previously described methodology (28). Briefly, eIF4E was immunoprecipitated from the supernatant fraction using a monoclonal anti-eIF4E antibody. Proteins in the immune complexes were resolved by SDS-PAGE and subjected to Western blot analysis for 4E-BP1, eIF4G, or eIF4E. The

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**Fig. 1.** Effect of 5-aminoimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR) treatment on phosphorylation of AMPK on Thr172 in C2C12 myotubes. Cells were incubated in serum-free medium for 1 h, followed by a 15-, 30-, or 60-min treatment with the AMPK activator AICAR (2 mM). Treated and control (for each time point) cells were harvested in 1× SDS sample buffer, and the resulting homogenate was assessed for the phosphorylation status of AMPK on the Thr172 site by immunoblot analysis (see **EXPERIMENTAL PROCEDURES for further description**). Representative Western blots are shown. CON, control; AIC, AICAR; P, phosphorylated; T, total. *P < 0.05 vs. control conditions.
ratios of eIF4G to eIF4E or 4E-BP1 to eIF4E were calculated and expressed as a percentage of the control value.

**Immunoprecipitation of Tuberin and mTOR.** Cells were harvested in CHAPS buffer (40 mM HEPES, pH 7.5, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM β-glycerophosphate, 40 mM NaF, 1.5 mM sodium vanadate, 0.3% CHAPS, 0.1 mM PMSF, 1 mM benzamidine, and 1 mM DTT). The resulting homogenate was mixed on a platform rocker for 20 min at 4°C and then clarified by a 1,000 g centrifugation for 3 min (4°C). Then, an aliquot from the resulting supernatant containing either 500 or 700 µg of protein was combined with 5.8 µl of anti-Tuberin antibody or 1.4 µl of anti-mTOR antibody, respectively, and mixed on a platform rocker overnight at 4°C. Prior to analysis, the immune complexes were isolated with a goat anti-mouse BioMag IgG (PerSeptive Diagnostics) bead slurry. Prior to use, the beads were blocked with 0.1% nonfat dry milk in CHAPS buffer, washed in CHAPS buffer, and then incubated with the sample for 1 h at 4°C. The beads were collected using a magnetic stand, washed twice with CHAPS buffer, and once in CHAPS buffer containing 200 µl of 3% nonfat dry milk in CHAPS buffer.

**Fig. 2.** Effect of AICAR treatment on incorporation of [35S]methionine/cysteine into protein in C2C12 myotubes. Cells were incubated as described in the legend to Fig. 1. [35S]-labeled Easytag Express label was added to culture medium 10 or 30 min before the end of the treatment period, depending on the time point, and cells were then harvested in lysis buffer containing protease and phosphatase inhibitors. The resulting homogenate was analyzed for incorporation of radiolabel into cellular protein, as described in EXPERIMENTAL PROCEDURES. *P < 0.05 vs. control conditions.

**Fig. 3.** Effect of AICAR treatment on polyosome aggregation in C2C12 myotubes. Cells were incubated in serum-free medium for 1 h, followed by a 15- (A), 30- (B), or 60-min treatment (C) with 2 mM AICAR. Treated and control cells were harvested in 1× SDS sample buffer containing cycloheximide, SUPERasin, and detergent. The resulting homogenate was clarified, layered onto a 20–47% sucrose density gradient, and then centrifuged at 34,000 g for 160 min. After centrifugation, the gradient was displaced upward through a spectrophotometer and optical density at 254 nm was continuously recorded (chart speed 150 cm/h) to determine nonpolyosome, subpolyosome, and polyosome fractions.
mM instead of 120 mM NaCl and 60 mM instead of 40 mM HEPES. The precipitates were eluted in 1× SDS sample buffer and then boiled for 5 min. The beads were pelleted by centrifugation, and the supernatant was collected and subjected to SDS-PAGE. The proteins were transferred to PVDF membranes, which were then incubated in anti-RXXRxpsT motif antibody, anti-Tuberin antibody, anti-Hamartin antibody, anti-Raptor antibody, or anti-mTOR antibody overnight at 4°C. The blots were visualized by ECL, and then the ratios of RXXRxpsT motif and Hamartin to Tuberin or Raptor to mTOR were calculated.

Analysis of 4E-BP1 phosphorylation. After harvest in 1× SDS sample buffer, the total cell lysate was resolved by electrophoresis on a 15% polyacrylamide gel. The proteins were transferred to PVDF membrane, the membrane was incubated with anti-4E-BP1 polyclonal antibody, and the blot was visualized by ECL. Typically, upon phosphorylation 4E-BP1 resolves into multiple forms following electrophoresis on SDS-polyacrylamide gels. It is generally accepted that the electrophoretic mobility of 4E-BP1 is inversely proportional to the degree of its phosphorylation, whereby the fastest migrating, hypophosphorylated forms of the proteins are designated the α-band. The relative phosphorylation of 4E-BP1 phosphorylation was estimated as the proportion present in the hyperphosphorylated γ-band relative to the total amount of the protein.

Measurement of protein synthesis. Global rates of protein synthesis were estimated by the incorporation of [35S]methionine and [35S]cysteine into the total amount of the protein.

Polysome aggregation. Sucrose density gradient centrifugation was employed to separate mRNA not present in polysomes (designated subpolysomal) from polysome-associated mRNA (polysomal). For this analysis, cells were harvested by scraping in HEPES buffer and clarified (see Cell culture for details). The resulting supernatant (600 μl) was layered onto a 20 – 47% linear sucrose density gradient (50 mM HEPES, pH 7.4, 75 mM KCl, 5 mM MgCl2) and centrifuged in a SW41 rotor at 34,000 rpm for 160 min at 4°C. After centrifugation, the gradient was displaced upward (2 ml/min) using Fluorinert (Isco, Lincoln, NE) through a spectrophotometer, and the optical density at 254 nm was continuously recorded (chart speed, 150 cm/h).

Statistics. The results represent the means ± SE for three experiments and are presented as a percentage of the control value. Within each experiment, three cultures were individually analyzed. All comparisons were made versus control conditions of the respective time for each variable (two-tailed t-test analysis; Prism v. 3.0, GraphPad Software). The significance level was set at P < 0.05.

RESULTS

We showed previously (2) that AICAR administration to rats in vivo inhibits protein synthesis and suppresses signaling through the mTOR pathway in skeletal muscle. However, in that study, plasma insulin concentrations (and probably other hormones) were also affected by the treatment protocol. The current study was therefore performed to examine the direct effect(s) of AICAR on protein synthesis and signaling pathways controlling mRNA translation by use of cultures of C2C12 myotubes as an experimental model. Initially, the effect of AICAR on AMPK phosphorylation was evaluated over time. As shown in Fig. 1, AICAR treatment caused a rapid (within 15 min) and significant elevation of AMPK phosphorylation on Thr172 that was maintained over the 60-min time course. To assess whether activation of AMPK was associated with an inhibition of protein synthesis, global rates of synthesis were measured as the incorporation of [35S]methionine/cysteine into protein. AICAR-treated myotubes showed a small but significant reduction in [35S]methionine/cysteine incorporation into protein at the 15-min time point with a larger decline to ~60% of the control value observed at the 30- and 60-min time points (Fig. 2). Thus the maximal effects of the AICAR-stimulated AMPK phosphorylation on Thr172 preceded the decline in global rates of protein synthesis.

To identify the step(s) in mRNA translation that was affected by AICAR, polysome aggregation was examined by sucrose density gradient centrifugation. Under conditions where the rate of translation initiation is reduced relative to elongation, polysomes become disaggregated and 40S and 60S ribosomal subunits and/or 80S monomers accumulate. In contrast, if elongation is reduced relative to initiation, polysome aggregation increases and 40S and 60S subunits and 80S monomers decline. In the present study, AICAR treatment of 15 min duration had no detectable effect on polysome aggregation (Fig. 3A), suggesting that elongation and initiation contributed equally to the reduction in protein synthesis. However, after 30 min of treatment, a shift of ribosomes from polysomes to 80S monomers/monosomes was evident (Fig. 3B).
and the effect was more pronounced after 60 min (Fig. 3C). This result suggests that, after 30 or 60 min of AICAR treatment, translation initiation was relatively more inhibited than elongation.

One mechanism through which translation initiation is regulated involves the reversible association of eIF4E with 4E-BP1 and eIF4G (53). Therefore, the effects of AICAR treatment on the association of eIF4G with eIF4E and 4E-BP1 with eIF4E were examined. As shown in Fig. 4, AICAR treatment caused a reduction in the amount of eIF4G in eIF4E immunoprecipitates (Fig. 4A) while simultaneously increasing the amount of 4E-BP1 in the eIF4E immunoprecipitates (Fig. 4B). Such a result is consistent with a reduction in translation initiation.

Our previous studies showed that AICAR-induced inhibition of protein synthesis in vivo was associated with repressed signaling through the mTOR pathway (2). Therefore, the effect of AICAR treatment on phosphorylation of the mTOR targets S6K1, 4E-BP1, and eIF4G was examined in C2C12 myotubes. AICAR treatment resulted in a rapid and sustained dephosphorylation of 4E-BP1 (Fig. 5A) and the site (Thr389) on S6K1 directly phosphorylated by mTOR (Fig. 5B). In contrast, dephosphorylation of the Thr421/Ser424 site on S6K1 (Fig. 5C) and of the rapamycin-sensitive site on eIF4G (Ser1108) (Fig. 5D) was not observed until 60 min after exposure to AICAR.

To establish whether or not the AICAR-induced change in S6K1 phosphorylation had functional consequences, phosphorylation of two downstream targets of S6K1 action, rpS6 and eEF2, was assessed. As shown in Fig. 6A, AICAR treatment promoted a rapid and sustained reduction in rpS6 phosphorylation on Ser240/244. Conversely, reduced phosphorylation of rpS6 on Ser235/236 was observed only after 30 min of AICAR treatment (Fig. 6B). S6K1 phosphorylates, and thereby inhibits, eEF2 kinase (4, 8). Therefore, AICAR-induced dephosphorylation of S6K1 would be expected to promote increased phosphorylation of eEF2. As shown in Fig. 6C, AICAR treatment significantly enhanced phosphorylation of eEF2 on Thr56 at 15 and 30 min; however, phosphorylation returned to the control value by the 60-min time point. Because phosphorylation of eEF2 inhibits translation elongation (6, 39), the observed transient increase in eEF2 phosphorylation was consistent with the suggestion that a decrease in the rate of translation elongation contributed to the inhibition of global rates of protein synthesis observed at early times after exposure to AICAR.

An important mechanism for regulating signaling through mTOR involves changes in its association with the regulatory protein Raptor (15, 25, 28, 49). To assess whether AICAR induces changes in mTOR·Raptor association, the amount of Raptor present in mTOR immunoprecipitates was measured by Western blot analysis. As illustrated in Fig. 7, AICAR treatment resulted in an increase in the amount of Raptor recovered in the mTOR immunoprecipitate. To further define the mechanism through which AICAR treatment represses signaling through mTOR, AICAR-mediated changes in the assembly of the mTOR-inhibitory complex Tuberin·Hamartin were assessed. As shown in Fig. 8A, association of Hamartin with Tuberin increased approximately twofold after addition of AICAR to the culture medium. Moreover, AICAR treatment caused a significant increase in Tuberin phosphorylation, as assessed by Western blot analysis using an anti-phospho-RXXRXXpS/T motif antibody (Fig. 8B) that has been previously shown to recognize sites on Tuberin phosphorylated by two different kinases, PKB (43) and p90RSK (1, 48). However, as shown in Fig. 8, C and D, AICAR treatment had no effect on
PKB phosphorylation or phosphorylation of Tuberin on Thr1462, a site directly phosphorylated by PKB in vitro (43). In contrast, AICAR treatment caused a rapid and sustained phosphorylation of eIF4E on Ser209 (Fig. 9C). Thus, although AICAR treatment repressed signaling through mTOR, components of the ERK1/2 pathway were simultaneously activated.

DISCUSSION

In the present study, the hypothesis that AICAR-induced repression of global rates of protein synthesis is a result of inhibition of mTOR signaling was tested in cultures of C2C12 myotubes. The results confirm previous studies (2, 21) showing that activation of AMPK by AICAR treatment results in an inhibition of global rates of protein synthesis. In addition, the present study extends the previous ones by showing that, as assessed by sucrose density gradient centrifugation, AICAR-induced activation of AMPK rapidly inhibits both translation initiation and elongation but that inhibition of initiation predominates with time. The conclusions drawn from the results of sucrose density gradient centrifugation are supported by the time course of changes in phosphorylation of 4E-BP1, rpS6, and eEF2. Thus the rapid dephosphorylation of 4E-BP1 and rpS6 following AICAR administration is consistent with an inhibition of translation initiation occurring during the first 15 min of treatment, whereas the increase in eEF2 phosphorylation would be expected to repress elongation. The changes in 4E-BP1 and rpS6 phosphorylation are maintained for at least 60 min, a result consistent with continued inhibition of translation initiation. However, increased phosphorylation of eEF2 was transient and returned to the control value by 60 min, suggesting that the AICAR-induced decrease in elongation is not maintained.

The transient increase in phosphorylation of eEF2 on T56 in response AICAR treatment suggested that eEF2 kinase (aka calcium/calmodulin-dependent protein kinase III) was activated (21). The activity of eEF2 kinase is modulated through multiple phosphorylation events mediated by a number of protein kinases including AMPK, S6K1, and p90RSK (3, 34, 58). Both S6K1 and p90RSK phosphorylate eEF2 kinase on Ser366, resulting in inactivation of the kinase and subsequent dephosphorylation and inactivation of eEF2 (34, 58). In contrast, AMPK phosphorylates eEF2 kinase on Ser398 (3), an event associated with activation of the kinase, increased phosphorylation of eEF2, and inhibition of translation elongation. Thus the presumptive early increase in eEF2 kinase activity could have been mediated through dephosphorylation of Ser366, increased phosphorylation of Ser398, or both. However, although S6K1 phosphorylation on Thr389 phosphorylation was reduced in AICAR-treated compared with control cells, no change in eEF2 kinase phosphorylation on Ser398 was observed in response to AICAR treatment (data not shown), suggesting that AMPK-mediated phosphorylation of Ser398 might be responsible for the change in eEF2 phosphorylation. Unfortunately, reagents to examine potential changes in eEF2 kinase phosphorylation on Ser398 were not available at the time this study was performed. Regardless, the finding that eEF2 phosphorylation returns to control values by 60 min despite continued elevation of AMPK phosphorylation suggests that the mechanism(s) regulating eEF2 kinase activity may be more complex than phosphorylation by a single upstream kinase and
in addition could also involve altered protein phosphatase activity.

The finding that phosphorylation of both 4E-BP1 and S6K1 was reduced in response to treatment with AICAR strongly supports the conclusion that signaling through mTOR was repressed. The function of mTOR is controlled, in part, through its interaction with regulatory proteins such as Raptor (15, 25, 28, 49). Raptor binds to 4E-BP1 and S6K1 through a domain referred to as a TOR-signaling (TOS) motif that is common to both proteins (40, 50) and thereby serves to recruit them to the Raptor-mTOR complex for phosphorylation. The association of Raptor with mTOR is modulated by hormones and nutrients (27–29). For example, in cells deprived of amino acids, the Raptor-mTOR complex is thought to adopt a closed, less active conformation that is relatively insensitive to dissociation by the detergent CHAPS (28). In contrast, repletion of amino acids is proposed to induce a conformational change resulting in an open, more active Raptor-mTOR complex that more easily dissociates in solutions containing CHAPS. In the present study, the amount of Raptor present in mTOR immunoprecipitates washed with buffer containing CHAPS was increased in AICAR-treated cells compared with controls, suggesting that AICAR promotes formation of the closed, less active conformation and may explain, in part, the decreased signaling through mTOR.

Another mechanism through which mTOR function is regulated involves modulation of the Rheb GTPase activity of...
Tuberin, whereby increased GTPase activity represses signaling through mTOR by increasing the proportion of Rheb present in an inhibitory complex with GDP (7, 22, 35). Tuberin activity is regulated through phosphorylation by a number of protein kinases, and the multiple phosphorylation events represent a point of convergence of inhibitory signaling from PKB (43), p90RSK (48), and ERK1/2 (37) with stimulatory signaling from AMPK (11, 20, 23, 52). However, recent studies suggest that Tuberin does not function by itself to enhance Rheb GTPase activity. In this regard, overexpression of Tuberin alone does not repress mTOR signaling (56). Instead, coexpression of Tuberin with Hamartin is required to inhibit mTOR function (56), suggesting that Tuberin acts in a complex with Hamartin to modulate Rheb activity. In the present study, AICAR treatment enhanced the assembly of the Tuberin-Hamartin complex, a result consistent with the observed decrease in phosphorylation of mTOR substrates. The mechanism through which AICAR acts to promote the association of Tuberin with Hamartin is unknown. Previous studies have shown that phosphorylation of Tuberin by PKB causes dissociation of the Hamartin-Tuberin complex (43). However, in the present study, no change in phosphorylation of PKB on Ser17 (or Tuberin on Thr1462 (a PKB-mediated event) was observed, suggesting that changes in Tuberin phosphorylation by PKB do not explain the observed changes in Hamartin binding to Tuberin. In contrast, the AICAR-induced increase in Tuberin phosphorylation measured using an anti-RXRXXpS/T motif antibody positively correlated with assembly of the Hamartin-Tuberin complex. The anti-RXRXXpS/T antibody recognizes both residues phosphorylated by PKB as well as phosphorylation at the p90RSK-directed phosphorylation site Ser1798 (48). Because phosphorylation of Tuberin by both PKB and p90RSK is typically associated with increased signaling through mTOR (47), the increase in Tuberin phosphorylation observed using the anti-RXRXXpS/T antibody is not consistent with the decrease in 4E-BP1 and S6K1 phosphorylation shown in Fig. 5. A possible explanation for the discrepancy is that the effect of phosphorylation of Tuberin by AMPK is dominant to the effect of phosphorylation by PKB or p90RSK. In such a model, phosphorylation of Tuberin by AMPK would promote the binding of Hamartin to Tuberin even if PKB- or p90RSK-directed phosphorylation was present. In this regard, a recent study (14) shows that activation of AMPK by treatment with 5-thioglucone overcomes the inhibitory effect of insulin-induced PKB activation on Tuberin function.

The rapid dephosphorylation of rpS6 on Ser240/244 observed in AICAR-treated cells is consistent with the observed change in S6K1 phosphorylation on Thr389 and suggests that S6K1 activity is also rapidly repressed. However, phosphorylation of rpS6 on Ser235/236 was sustained for at least 15 min after AICAR addition to the culture medium, suggesting that S6K1 activity was still active at that time. This apparent contradiction may be explained by results showing that, whereas phosphorylation of Ser240/244 is predominantly catalyzed by S6K1 and S6K2, phosphorylation of Ser235/236 can also be phosphorylated by p90RSK (42). The finding that phosphorylation of both MEK1/2 and ERK1/2 is rapid and sustained in AICAR-treated myotubes suggests that p90RSK also would be rapidly activated by AICAR. Unfortunately, we were not able to detect changes in p90RSK phosphorylation by Western blot analysis using commercially available antibodies (data not shown); therefore, alternative explanations, such as a change in activity of another protein kinase and/or phosphatase, cannot be excluded.

In addition to regulating p90RSK, ERK1/2 also phosphorylates, and thereby activates, MNK1/2, which subsequently phosphorylates elf4E (12, 59). MNK1/2 is also phosphorylated by p38 MAP kinase (12, 59). However, although phosphorylation of both ERK1/2 and elf4E was increased in AICAR-treated cells, p38 MAPK phosphorylation on Thr180/Y182 did not change (data not shown), indicating that the

Fig. 9. Effect of AICAR treatment on phosphorylation of MEK1/2, ERK1/2, and elf4E in C2C12 myotubes. Cells were incubated as described in the legend to Fig. 1 and then assessed for phosphorylation status of MEK1/2 on Ser217/221 (A), ERK1/2 on Thr202/Tyr204 (B), and elf4E on Ser209 (C) by immunoblot analysis. *P < 0.05 vs. control conditions.

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observed AICAR-induced changes in eIF4E phosphorylation were mediated by activation of ERK1/2 rather than p38 MAP kinase.

Protein synthesis is one of the major energy-consuming processes within cells (46). Under conditions wherein energy consumption is increased (e.g., enhanced utilization of ATP during acute endurance exercise), energy consuming processes that are not immediately required for cell survival are repressed (17). Because many housekeeping proteins possess relatively long half-lives, a temporary reduction in their synthesis should have minimal effect on cell viability. Therefore, it is not surprising that global rates of protein synthesis are repressed during acute treadmill exercise (13, 45). However, other proteins turn over much more quickly (i.e., within minutes), and even a relatively short reduction in their synthesis would result in a substantial decrease in abundance. In general, proteins with short half-lives, e.g., cyclin D1 (5) and ornithine decarboxylase (24), play important roles in cell cycle and division as well as the cellular response to stress. Hence, maintenance of the translation of such mRNAs is important for cell survival. However, little is known about possible mechanisms through which the selection of mRNAs for translation is regulated during conditions of energy deficit. The results of the present study are consistent with a model wherein activation of AMPK by AICAR simultaneously represses signaling through mTOR and enhances signaling through the MEK1/2/ERK1/2 pathway. In this regard, it is tempting to speculate that activation of the MEK1/2-ERK1/2 pathway may result in changes in the pattern of gene expression through alterations in transcription as well as through modulation of the translation of mRNAs encoding specific proteins (e.g., through phosphorylation of eIF4E). The combination of altered gene transcription and mRNA translation would, together, alter muscle size. In this regard, a recent report (57) shows that activation of AMPK in overloaded plantar fascia muscle represses muscle hypertrophy. Future studies will be required to address those possibilities.

In summary, upon activation of AMPK by AICAR, C2C12 myotubes exhibited a reduction in protein synthesis and mRNA translation. These changes were associated with an AMPK-induced increase in the association of Raptor with mTOR. Likewise, there was a rapid dephosphorylation of 4E-BP1 and S6K1 (Thr389) and a delayed phosphorylation of the Thr421/Ser424 site of S6K1 and the Ser1108 site on eIF4G. Similarly, AMPK activation induced a rapid dephosphorylation of rpS6 on Ser240/244, whereas dephosphorylation of rpS6 on Ser235/236 was more protected. AMPK activation stimulated phosphorylation of eEF2; it also elicited an increase in MEK-ERK signaling. Last, there was increased association of Hamartin with Tuberin. Overall, the results suggest that activation of AMPK inhibits protein synthesis through changes in both translation initiation and elongation and that such regulation involves modulation of multiple intracellular signaling pathways.

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REFERENCES


