Repression of protein synthesis and mTOR signaling in rat liver mediated by the AMPK activator aminimidazole carboxamide ribonucleoside


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Reiter, Ali K., Douglas R. Bolster, Stephen J. Crozier, Scot R. Kimball, and Leonard S. Jefferson. Repression of protein synthesis and mTOR signaling in rat liver mediated by the AMPK activator aminimidazole carboxamide ribonucleoside. Am J Physiol Endocrinol Metab 288:E980–E988, 2005. First published December 21, 2004; doi:10.1152/ajpendo.00333.2004.—The studies described herein were designed to investigate the effects of 5-aminimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), an activator of the AMP-activated protein kinase (AMPK), on the translational control of protein synthesis and signaling through the mammalian target of rapamycin (mTOR) in rat liver. Effects of AICAR observed in vivo were compared with those obtained in an in situ perfused liver preparation to investigate activation of AMPK in the absence of accompanying changes in hormones and nutrients. AMPK became hyperphosphorylated, as assessed by a gel-shift analysis, in response to AICAR both in vivo and in situ; however, increased relative phosphorylation at the Thr172 site on the kinase was observed only in perfused liver. Phosphorylation of AMPK either in vivo or in situ was associated with a repression of protein synthesis as well as decreased phosphorylation of a number of targets of mTOR signaling including ribosomal protein S6 kinase 1, eukaryotic initiation factor (eIF)4G, and eIF4E-binding protein (4E-BP)1. The phosphorylation changes in eIF4G and 4E-BP1 were accompanied by a reduction in the amount of eIF4E present in the active eIF4E-eIF4G complex and an increase in the amount present in the inactive eIF4E-eIF4E-eIF4E complex. Reduced insulin signaling as well as differences in nutrient availability may have contributed to the effects observed in vivo as AICAR caused a fall in the serum insulin concentration. Overall, however, the results from both experimental models support a scenario in which AICAR directly represses protein synthesis and mTOR signaling in the liver through an AMPK-dependent mechanism.

THE AMP-ACTIVATED PROTEIN KINASE (AMPK) acts as an energy sensor that responds to changes in the intracellular ratio of AMP:ATP (13). Activation of AMPK results in the stimulation of a variety of cellular processes involved in ATP production, e.g., glucose uptake (36, 44, 54) and fatty acid oxidation (7, 16, 37, 39), and a repression of energy-consuming processes, e.g., fatty acid (16) and protein (3, 8, 18) synthesis. The repression of protein synthesis by AMPK is associated with decreased signaling through the protein kinase referred to as the mammalian target of rapamycin (mTOR) (8, 20, 30, 31). Decreased signaling through mTOR leads to downregulated phosphorylation of several proteins that play important roles in regulating the initiation phase of mRNA translation, including the eukaryotic initiation factor (eIF)4E-binding protein, 4E-BP1 (6), and the 70-kDa ribosomal protein (rp)S6 protein kinase, S6K1 (6). It also downregulates mRNA elongation through modulation of eukaryotic elongation factor (eEF)2 kinase activity (4, 55). Downregulated phosphorylation of 4E-BP1 leads to its association with the mRNA cap binding protein eIF4E, which prevents eIF4E from associating with a second initiation factor, eIF4G (33), thereby inhibiting the mRNA binding step in translation initiation (12). Downregulated phosphorylation of S6K1 decreases phosphorylation of its substrate, rpS6, and correlates with a relative reduction in the translation of mRNAs containing a 5′-terminal oligopyrimidine tract (TOP) adjacent to the m7GTP cap (22). Such mRNAs include those encoding eEF1A and eEF2 as well as those encoding many of the ribosomal proteins (23, 32, 35).

Activation of AMPK in skeletal muscle, whether through endurance exercise or administration of 5-aminimidazole-4-carboxamide-1-β-D-ribonucleoside (AICAR), results in repressed protein synthesis and decreased signaling through mTOR (3, 11, 51). Endurance exercise is also associated with a repression of protein synthesis (15) and decreased signaling through mTOR (unpublished observations) in the liver. However, it is unclear whether endurance exercise-induced activation of AMPK is sufficient to explain the observed changes in the liver. An alternative explanation for the repressed protein synthesis and decreased signaling through mTOR in the liver in response to endurance exercise might be a change in hormone or nutrient availability. In this regard, a decrease in either plasma insulin or amino acid concentrations or an increase in glucocorticoid levels might repress protein synthesis and decrease signaling through mTOR (48, 50) in an AMPK-independent manner.

To define more clearly a possible link between activation of AMPK and repression of protein synthesis and decreased signaling through mTOR in the liver, AICAR was administered to rats in vivo or, in a separate study, was added to the medium of in situ perfused rat liver preparations. In both the in vivo and in situ systems, activation of AMPK by AICAR was associated with repressed protein synthesis and decreased phosphorylation of proteins downstream of mTOR, suggesting that AMPK-independent signaling was not necessarily involved in the previously observed effects of endurance exercise on these processes in the liver.

MATERIALS AND METHODS

Animals. The animal facilities and experimental protocols used for the studies described herein were reviewed and approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University, in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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State University College of Medicine. Male Sprague-Dawley rats weighing 100–140 g for the in situ perfusion studies and 200–260 g for the in vivo studies were obtained from Charles River Laboratory. They were housed on a 12:12-h light-dark cycle and allowed free access to food (Harlan-Teklad Rodent Chow, Madison, WI) and water. Before experimentation, the rats used in the in situ perfusion studies were anesthetized with pentobarbital sodium (1 ml/100 g body wt). For the in vivo studies, rats used for analysis of the phosphorylation status of AMPK were also anesthetized with pentobarbital sodium (1 ml/100 g body wt), and one lobe of the liver was rapidly frozen in place between two aluminum blocks precooled to the temperature of liquid nitrogen. For other analyses in the in vivo studies, rats were killed via decapitation as described below.

**AICAR administration in vivo.** On the day of the experiment, rats used for the in vivo studies were injected subcutaneously with AICAR (1 mg/g body wt) in sterile 0.9% NaCl, a dose previously used to activate AMPK in skeletal muscle of rats (3, 17). Control rats were administered an equivalent volume of sterile 0.9% NaCl as previously described (3). A flooding dose (1.0 ml/100 g body wt) of L-[2,3,4,5,6-3H]phenylalanine (150 mM) was injected via the tail vein 50 min following the subcutaneous injection of AICAR for measurement of the rate of synthesis of total mixed liver protein (10). Ten minutes following the injection of radiolabeled phenylalanine, the rats were killed via decapitation, blood was collected, and the liver was excised for analysis of the rate of protein synthesis. The remainder of the liver was processed for analysis of mTOR-mediated signaling and translation control mechanisms. All serum and tissue samples were stored at −70°C until analyzed.

**Liver perfusion.** The liver was perfused in situ essentially as previously described (9). Briefly, it was perfused through the portal vein for a period of 30 min at a flow rate of 7 ml/min with nonrecirculating buffer consisting of Krebs-Henseleit buffer (pH 7.4) containing 11 mM glucose, 3% (wt/vol) bovine serum albumin (MP Biomedicals, Aurora, OH), 30% washed bovine erythrocytes, amino acids at 5× the concentrations found in the arterial plasma of normal fasted rats, and when present, 2 mM AICAR (Toronto Chemicals, Tororo, ON, Canada) (29). For the final 10 min of the perfusion period, radiolabeled L-[4,5-3H]leucine (6.7 μCi) was added while the total concentration of leucine was maintained at 5× the plasma value. Following the 30-min perfusion period, the liver was removed and a portion was immediately clamped between two aluminum blocks precooled to the temperature of liquid nitrogen for analysis of AMPK phosphorylation. The remaining unfrozen portion of liver was used for analysis of protein synthesis and biomarkers of translation initiation or for analysis of polysomal aggregation as described below.

**Sample preparation.** A portion (~0.5 g) of the liver was weighed and homogenized using a Polytron PT10 homogenizer in 7 vol of buffer A consisting of (in mM) 20 HEPES (pH 7.4), 100 KCl, 0.2 EDTA, 2 EGTA, 50 NaF, 50 β-glycerophosphate, 0.1 phenylmethylsulfonyl fluoride, 1 benzamidine, and 0.5 sodium vanadate. An aliquot of the homogenate was used for measurement of protein synthesis, as described below. The remaining homogenate was centrifuged at 10,000 g for 10 min at 4°C. The resulting supernatant was used for analysis of the phosphorylation status of eEF2, eIF2α, eIF2β, eIF4G, 4E-BP1, and S6K1 and of the association of 4E-BP1 and eIF4G with eIF4E as described below. For the in vivo studies, a second portion (~0.3 g) of the liver was weighed and homogenized in 5 vol of buffer B consisting of (in mM) 45 HEPES (pH 7.4), 0.375 magnesium acetate, 0.075 EDTA, 95 potassium acetate, 2.03 digitonin, 0.003 micromycin, and 10% glycerol. Buffer B homogenates were centrifuged at 10,000 g for 10 min at 4°C. Resulting supernatants were analyzed for eIF2B activity as described below. For the in situ liver perfusion studies, a second portion (~1 g) of perfused liver was weighed and homogenized using a Dounce homogenizer in 4 vol of buffer C consisting of (in mM) 40 HEPES (pH 7.5), 100 KCl, and 5 magnesium chloride. The homogenate was centrifuged at 3,000 g for 15 min, and the resulting supernatant was used for analysis of polysomal aggregation.

**Analysis of polysomal aggregation.** Polysome profile analysis was performed as previously described (1). Briefly, 1 vol of detergent (10% Triton X-100, 0.24 M deoxycholate sodium salt) was added to 9 vol of the supernatant from livers homogenized in buffer C and an aliquot (2 ml) of the sample was layered over a 10–70% linear sucrose density gradient and centrifuged at 90,000 g in a Beckmann SW28 rotor for 3 h. After centrifugation, the gradient was fractionated on an Isco gradient fractionator while the absorbance at 254 nm was continuously recorded.

**Measurement of protein synthesis.** Following in vivo AICAR administration, the fractional rate of protein synthesis was assessed as the rate of incorporation of i-[2,3,4,5,6-3H]phenylalanine into total mixed liver protein using the specific radioactivity of serum phenylalanine as representative of the precursor pool during the incorporation time, i.e., the time elapsed from injection until homogenization (2). In the in situ liver perfusion studies, protein synthesis was assessed as the rate of incorporation of [3H]leucine into total mixed liver protein (49) using the specific radioactivity of leucine in the perfusate as representative of the precursor pool during the incorporation time, i.e., the 10-min period of perfusion with the radiolabeled amino acid.

**Measurement of elf2B activity.** The guanine nucleotide exchange activity of elf2B in the liver homogenate was assessed as previously described (26). Briefly, the rate of exchange of [3H]GDP bound to elf2B for free, nonradioactively labeled GDP was measured, and the activity of elf2B was calculated as the slope of the nearest fit line with DPM as the dependent variable and time as the independent variable. The slope indicated the rate of GDP exchanged as picomoles per minute.

**Measurement of protein phosphorylation status.** All antibodies used were obtained from Cell Signaling Technology (Beverly, MA) unless otherwise noted. Samples were analyzed by Western blot analysis for relative phosphorylation of the α-subunit of elf2B employing an anti-phospho-elf2α (Ser51) antibody (BioSource International, Hopkinton, MA); the blots were normalized for total elf2α content using a monoclonal antibody that recognizes the protein irrespective of its phosphorylation status (47). elf2B phosphorylation was also analyzed using an anti-phospho-elf2Bα (Ser535) (BioSource International) and normalized to total elf2B content using a monoclonal anti-elf2Bα antibody (28). The phosphorylation status of elf2B (Thr56) and elf4G (Ser1108) was analyzed as the signal intensity from a phosphospecific antibody normalized to that of an antibody that recognizes the protein independent of phosphorylation state. Hyperphosphorylation of AMPK was assessed using an anti-AMPK antibody and analyzed as the percent of the signal displaying reduced mobility relative to the total signal. Phosphorylation of AMPK was also assessed using a polyclonal anti-phospho-AMPK (Thr172) antibody and normalized to total content of AMPK α using a polyclonal anti-AMPK α-antibody (3). Phosphorylation of S6K1 was assessed using a polyclonal anti-phospho-S6K1 (Thr389) antibody. Finally, phosphorylation of 4E-BP1 was assessed as a change in electrophoretic mobility during SDS-PAGE using an anti-4E-BP1 antibody. The phosphorylation status of the proteins with eIF4E from the immunoprecipitates.

**λ-Protein phosphatase treatment.** Frozen liver samples were homogenized in 7 vol of buffer consisting of 20 mM HEPES, pH 7.4, 2 mM EGTA, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, 0.1 mM DTT.
PMSE, 1 mM benzamidine, 1 μl/ml leupeptin, and 10 μg/ml aprotinin. Aliquots (65 μl) of homogenates were incubated with 6.5 μl 10× λ-protein phosphatase buffer (New England Biolabs), 2 μl λ-phosphatase (400,000 U/ml; New England Biolabs), and 6.5 μl magnesium acetate and incubated at 37°C for 15 min.

Statistical analysis. All data were analyzed by InStat 3.0 (GraphPad Sofware, San Diego, CA). Unless otherwise stated, differences between groups were analyzed using a paired t-test comparing AICAR treatment with the control. The level of significance was set at P < 0.05 for all statistical tests.

RESULTS

The relative phosphorylation status of AMPK was assessed by gel-shift analysis as well as through the use of a phosphospecific antibody for residue Thr172, phosphorylation of which allows for maximal kinase activation (14). As shown in Fig. 1, AICAR treatment in either the in vivo experimental model or the in situ liver perfusion model resulted in reduced mobility of AMPK on SDS-PAGE compared with untreated controls following Western blot analysis using an antibody that recognizes the α-subunit of AMPK independent of phosphorylation status. The reduced mobility of AMPK was most likely due to increased phosphorylation of the kinase because treatment of samples with λ-phosphatase before Western blot analysis resulted in a collapse of the signal into the fastest migrating band (Fig. 1A). Assessment of the phosphorylation status of AMPK, quantitated as the proportion of the protein present in the slower migrating forms divided by the total intensity of the signal, demonstrated AICAR induced hyperphosphorylation of the kinase (Fig. 1, B and C). Although no significant change in AMPK phosphorylation at Thr172 was observed in the in vivo experimental model (Fig. 2A), a significant increase at this site was observed in the in situ liver perfusion model comparing AICAR-treated to control livers (Fig. 2B). Additionally, Western blot analysis using the phosphospecific antibody for AMPK revealed a similar reduction on mobility to that observed using the anti-AMPK-α antibody, suggesting that the slower migrating bands were phosphorylated on Thr172 in addition to other sites. Thus both experimental models exhibited an increase in hyperphosphorylation of AMPK in response to AICAR, as assessed by a gel-shift analysis, whereas an increase in phosphorylation at the Thr172 site was only observed in the perfused liver.

After AICAR treatment, the rate of synthesis of total mixed liver protein was repressed to 18 and 45% of the control values in the in vivo (Fig. 3A) model and in situ liver perfusion model (Fig. 3B), respectively. To assess whether translation initiation and/or elongation was repressed in response to AMPK activation, polysomal aggregation in the in situ model was examined by sucrose density gradient centrifugation. In this analysis, if translation initiation is inhibited relative to elongation, the relative amount of free ribosomes, i.e., ribosomes not associated with mRNA, should increase and the number of ribosomes associated with mRNA in polysomes should decrease (53). On the other hand, if the rate of elongation is repressed relative to initiation, the relative amount of free ribosomes should decrease and polysomal aggregation should increase due to stalling of ribosomes on mRNA following initiation. As shown in Fig. 4A, no change in polysomal aggregation was observed following perfusion of liver with AICAR, suggesting that inhibition of both translation initiation and elongation contributed to the repression of protein synthesis. To evaluate more directly the effect of AICAR treatment on elongation, the relative phosphorylation of eEF2 was evaluated. As shown in Fig. 4, B and C, eEF2 phosphorylation at Thr56 was unaltered in either experimental model, suggesting that phosphorylation of this translation factor did not contribute to the reduction in the rate of elongation.

One mechanism involved in the regulation of global rates of protein synthesis involves the binding of met-tRNA{\textsubscript{i}} to the 40S ribosomal subunit, a process mediated by the initiation factors eIF2 and eIF2B (25, 56). As discussed further below, the best characterized mechanisms for regulating the met-tRNA{\textsubscript{i}} binding step involve phosphorylation of eIF2 on Ser51 of its α-subunit and phosphorylation of eIF2B on Ser535 of its e-subunit. As shown in Fig. 5, A and B, eIF2α phosphorylation at Ser51 did not change in the in vivo model but increased in the perfused livers in response to AICAR. eIF2Bε phosphorylation at Ser535 was unchanged in the liver following in vivo...
AICAR administration and was undetectable in either control or AICAR-treated, perfused liver (Fig. 5, C and D). Furthermore, eIF2B activity did not change in response to AICAR administration in the in vivo model (data not shown). Hence, the data suggest that modulation of eIF2B activity is not involved in the marked repression of protein synthesis in the liver following administration of AICAR.

Effects of AICAR on mTOR-mediated signaling were assessed in both experimental models by quantitation of eIF4G phosphorylation at Ser1108, 4E-BP1 phosphorylation at Thr37, and S6K1 phosphorylation at Thr389, and 4E-BP1 as assessed by gel-shift analysis. A significant reduction in eIF4G phosphorylation at Ser1108 to 25% of the control value in the in vivo model and 56% of the control value in the in situ liver perfusion model was observed in response to AICAR administration (Fig. 6, A and B). A reduction in 4E-BP1 phosphorylation, as assessed by the intensity of the γ-band divided by the intensity of all three bands, was observed in both experimental models following AICAR treatment (Fig. 6, C and D) as was a reduction in 4E-BP1 phosphorylation at Thr389 (Fig. 6, E and F). Finally, phosphorylation of S6K1 on residue Thr389 was undetectable in either the control or AICAR-treated liver in the in vivo model (Fig. 6G); however, following perfusion of liver with AICAR, a decrease in S6K1 phosphorylation at Thr389 to 15% of the control value was observed, an observation made possible by the higher basal rate of mTOR-mediated signaling (presumably due to the relatively high concentration of amino acids in the perfusate) in the in situ model compared with the in vivo model (Fig. 6H). Overall, the results suggest that phosphorylation of several downstream targets of mTOR-mediated signaling is repressed in the liver in response to AICAR administration.

Phosphorylation of 4E-BP1 promotes its dissociation from eIF4E allowing eIF4E to bind to eIF4G (33). Because phosphorylation of 4E-BP1 decreased in response to AICAR administration, the association of eIF4E with either 4E-BP1 or eIF4G was evaluated in eIF4E immunoprecipitates. As illustrated in Fig. 7, A and B, the association of 4E-BP1 with eIF4E increased to 140% of the control value in the in vivo model and 280% of the control value in the in situ perfusion model in response to AICAR treatment. Furthermore, following in vivo AICAR administration, the increase in eIF4E association with 4E-BP1 was correlated with a marked reduction in the relative amount of eIF4E-eIF4G association (Fig. 7C); however, the reduction in eIF4E-eIF4G association was not significant in the liver perfusion model (P = 0.16; Fig. 7D). Thus the results of both experimental models suggest that AICAR treatment leads to an increase in eIF4G-eIF4E association and a reduction in eIF4F complex assembly in the liver.

DISCUSSION

In the present study, administration of AICAR to rats in vivo or addition of the AMP mimetic to isolated, perfused rat liver preparations resulted in hyperphosphorylation of AMPK, decreased global rates of protein synthesis, and reduced phosphorylation of several biomarkers of the mRNA binding step with no change in phosphorylation of eEF2 or two biomarkers of the met-tRNAi, binding step in translation initiation. Three of the biomarkers of the mRNA binding step examined are pictured in Fig. 3. AICAR treatment reduces global rates of liver protein synthesis. Rates of protein synthesis were assessed using the flooding dose technique as described in MATERIALS AND METHODS and are expressed as a percentage of the respective controls. Values represent the means ± SE for each group. A: light gray bar: control rats (n = 6); dark gray bar: AICAR-treated rats (n = 8). *P < 0.0192 vs. control. B: light gray bar: livers perfused without AICAR (n = 8); dark gray bar: livers perfused with AICAR (n = 7). *P < 0.001 vs. control.
downstream targets of mTOR signaling, 4E-BP1, S6K1, and eIF4G (6, 41, 42). The finding that phosphorylation of all three proteins is reduced by AICAR treatment in vivo and in situ strongly suggests that activation of AMPK represses signaling through mTOR. This suggestion is supported by previous studies in isolated hepatocytes (8, 30). In those studies, activation of AMPK with AICAR was shown to prevent the amino acid-induced activation of S6K1. Because amino acids promote S6K1 activation through an mTOR-dependent signaling pathway (reviewed in Ref. 52), the previous studies concluded that activation of AMPK in hepatocytes represses signaling through mTOR. Moreover, activation of AMPK by fructose or glycerol (8) or anoxia or oligomycin (30) similarly attenuates activation of S6K1 by amino acids, and expression of a constitutive active AMPK variant in human corneal epithelial (HCE)-T cells represses S6K1 activity in the absence of activators of AMPK (29). Combined, the results demonstrate that activation of AMPK per se and not an indirect effect of AICAR, e.g., mediated through altered hormonal or nutrient availability, is responsible for the effect of the AMP mimetic in repressing signaling through mTOR. Although AICAR represses signaling through mTOR in liver (present study) and
isolated hepatocytes (8, 30), the effect may not be universal. Thus, although AICAR inhibits S6K1 activity in HCE-T cells and H4IIE hepatocytes, it has no effect on S6K1 activity in CHO-IR or human embryonic kidney (HEK)-293 cells (29). However, in HEK-293 cells, 2-deoxyglucose both activates AMPK and inhibits S6K1 activity, suggesting that the lack of S6K1 inhibition by AICAR in those cells is not due to a lack of AMPK but instead may be a result of insufficient phosphorylation of AICAR by adenosine kinase to generate enough 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranosyl-5′-monophosphate, the actual activator of AMPK (45).

The question of how activation of AMPK results in decreased signaling through mTOR is unresolved. One study reports that activation of AMPK in C2C12 myotubes using AICAR results in enhanced phosphorylation of IRS-1 on Ser789 and that AMPK directly phosphorylates IRS-1 on Ser789 in vitro (21). Moreover, phosphorylation of this residue reportedly correlates with a reduction in IRS-1-associated phosphatidylinositol (PI) 3-kinase activity (21). Because PI3-kinase is upstream of mTOR (5), AMPK-mediated phosphorylation of IRS-1 may explain, in part, the reduction in mTOR signaling. A second study reports an inhibition of Akt

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**Fig. 6.** AICAR treatment represses mammalian target of rapamycin (mTOR)-mediated signaling as assessed by eIF4G phosphorylation at Ser1108, eIF4E-binding protein (4E-BP1) hyperphosphorylation, 4E-BP1 phosphorylation at Thr37, and S6 protein kinase (S6K1) phosphorylation at Thr389.

**A, C, E, and G:** results using the in vivo model. B, D, F, and H: results from isolated liver perfusion preparations. A: eIF4G phosphorylation at Ser1108 was assessed in liver of control rats (light gray bar, n = 6) or AICAR-treated rats (dark gray bar, n = 6) by performing Western blot analysis using an anti-phospho-eIF4G (Ser1108) antibody and then normalized to the total amount of eIF4G. Inset: representative blot of eIF4G phosphorylation. Values are expressed as means ± SE for each group.

B: eIF4G phosphorylation at Ser1108 in livers perfused in the absence (light gray bar, n = 7) or presence (dark gray bar, n = 7) of AICAR. Inset: representative blot.

C: hyperphosphorylation of 4E-BP1 was measured by Western blot analysis in liver of control rats (light gray bar, n = 10) and AICAR-treated rats (dark gray bar, n = 10) as described in MATERIALS AND METHODS. Results are expressed as the percent of the protein present in the hyperphosphorylated γ-form (i.e., slowest migrating) relative to total. Inset: representative blot.

D: hyperphosphorylation of 4E-BP1 in livers perfused in the absence (light gray bar, n = 8) or presence (dark gray bar, n = 8) of AICAR. Inset: representative blot.

E: 4E-BP1 phosphorylation at Thr37 was measured by Western blot analysis in liver of control rats (light gray bar, n = 10) and AICAR-treated rats (dark gray bar, n = 10) using an anti-phospho-4E-BP1(Thr37) antibody (note that this antibody cross-reacts with 4E-BP1 phosphorylated on Thr45). Inset: representative blot.

F: 4E-BP1 phosphorylation at Thr37 in livers perfused in the absence (light gray bar, n = 8) or presence (dark gray bar, n = 7) of AICAR. Inset: representative blot.

G: S6K1 phosphorylation at Thr389 was measured by Western blot analysis in liver of control rats (n = 10) and AICAR-treated rats (n = 10) using an anti-phospho-S6K1 (Thr389) antibody. Inset: representative blot.

H: S6K1 phosphorylation at Thr389 in livers perfused in the absence (light gray bar, n = 8) or presence (dark gray bar, n = 7) of AICAR. Inset: representative blot.
phorylation on Ser473 in skeletal muscle following in vivo AICAR administration (3). However, in HCE-T cells, activation of AMPK by AICAR has no effect on Akt phosphorylation, an event downstream of PI 3-kinase, even though AICAR clearly represses S6K1 phosphorylation (29). Thus phosphorylation of IRS-1 on Ser789 may not explain completely the repression of mTOR signaling by AMPK. An alternative explanation is provided by results from a recent study showing that AMPK directly phosphorylates tuberin and that such phosphorylation enhances tuberin function (20). Tuberin is a GTPase-activator protein that negatively regulates a protein referred to as ras-homolog enriched in brain (Rheb) (19, 57). Because Rheb is a positive regulator of mTOR signaling (46), activation of tuberin by AMPK and subsequent inhibition of Rheb would be expected to repress mTOR-dependent signaling. Whether AICAR treatment results in tuberin phosphorylation in liver is unknown.

In the present study, the inhibition of signaling through mTOR by AICAR was associated with a decrease in the global rate of protein synthesis. However, in liver mTOR signaling does not appear to have an acute effect on global rates of protein synthesis. In this regard, administration of the specific mTOR inhibitor rapamycin to rats in vivo strongly attenuates phosphorylation of both 4E-BP1 and S6K1 (43). In addition, rapamycin prevents completely the activation of S6K1 by amino acids in isolated rat hepatocytes (8, 30). However, when assessed over a relatively short period of time, rapamycin does not inhibit global rates of protein synthesis in liver in vivo (43) and does not prevent the stimulation of protein synthesis by amino acids in hepatocytes (8). Instead, inhibition of signaling through mTOR correlates with decreased translation of a subset of mRNAs such as those containing a TOP sequence (43). Thus AMPK-mediated repression of mTOR-dependent signaling may have a preferential, rather than global, effect on mRNA translation, which over an extended period of time would result in a reduction in ribosome biogenesis and thus a decrease in the protein synthetic capacity of the tissue.

Other steps in mRNA translation that could contribute to global changes in mRNA translation include those involved in the binding of met-tRNA\textsubscript{i} to the 40S ribosomal subunit and those that mediate translation elongation. The binding of met-tRNA\textsubscript{i} to the 40S ribosomal subunit is mediated by the eIF2-GTP complex (25, 56). During a late step in the initiation process, the GTP bound to eIF2 is hydrolyzed to GDP and eIF2 is released from the 40S ribosomal subunit as an eIF2-GDP binary complex. For eIF2 to bind to met-tRNA\textsubscript{i}, the GDP bound to eIF2 must be exchanged for GTP, a process catalyzed by the guanine nucleotide exchange protein eIF2B. Mechanisms for regulating eIF2B activity include phosphorylation of eIF2 on Ser51 of its \(\alpha\)-subunit and phosphorylation of eIF2B on Ser535 of its \(\epsilon\)-subunit, both of which repress the activity of the protein (25, 56). However, in the present study, no change in eIF2\(\alpha\) (Ser51) or eIF2Be (Ser535) phosphorylation or eIF2B activity was observed in liver of AICAR-treated rats, suggesting that inhibition of the met-tRNA\textsubscript{i} binding step is not responsible for the global inhibition of protein synthesis caused by AICAR.

The best characterized mechanism for regulating translation elongation is phosphorylation of eEF2 on Thr56 (38, 40). Phosphorylation of eEF2 on Thr56 is associated with decreased rates of elongation. However, no changes in eEF2 phosphorylation at Thr56 were observed in the present study, suggesting that AICAR does not promote phosphorylation of eEF2 on Thr56 in the liver under the conditions employed in the present study. This result is in contrast to a report that activation of AMPK by AICAR, oligomycin, or carbonyl cyanide \textit{m}-chlorophenylhydrazone increased eEF2 phosphorylation at Thr56 in rat ventricular myocytes (34). In that study, eEF2 phosphor-
ylation at Thr56 was maximal within 5 min of the start of treatment and returned to control values within 20 min. In the present study, livers were analyzed 60 min after the administration of AICAR to rats in vivo or after 30 min of the start of perfusion. Thus eEF2 phosphorylation at Thr56 may have increased soon after administration of AICAR but returned to control values before the livers were harvested for analysis. A previous study demonstrated an inhibition of TNF-α production independent of an increase in phosphorylation of AMPK at Thr172 following AICAR treatment (24). Therefore, the possibility also exists that AICAR exerts its effects on translation elongation and protein synthesis independent of a change in eEF2 phosphorylation.

An apparent discrepancy between the in vivo and in situ models used herein is the finding that AICAR promoted dephosphorylation of S6K1 on Thr389 in isolated, perfused rat livers, but this result was not observed in livers of animals administered AICAR in vivo. The different results obtained using the two models are likely a result of animals used for the in vivo study being in a postabsorptive state before administration of AICAR, whereas for the in situ study, livers were perfused with medium containing amino acids at five times the level measured in plasma of fasted rats and 11 mM glucose. Thus, in the in vivo model, S6K1 phosphorylation at Thr389 was undetectable in control animals, whereas S6K1 phosphorylation at Thr389 in control perfused livers was enhanced as a result of provision of amino acids. The finding that 4E-BP1 hyperphosphorylation was greater in livers perfused in the absence of AICAR compared with livers from control rats in vivo likewise supports this conclusion. Additionally, the animals used in the in vivo study were older than those used in the in situ study, and the age of the animals could have led to the in vivo animals being more sensitive to the postabsorptive state (8, 30).

Overall, the results of the present study extend earlier observations in isolated rat hepatocytes to show that activation of AMPK in liver represses signaling to multiple downstream targets of mTOR. AICAR administration also inhibits global rates of protein synthesis in liver, and results from sucrose in situ study, and the age of the animals could have led to the in vivo likewise supports this conclusion. Additionally, the animals used in the in vivo study were older than those used in the in situ study, and the age of the animals could have led to the in vivo animals being more sensitive to the postabsorptive state (8, 30).

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