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AMPK Represses TOP mRNA Translation But Not Global Protein Synthesis in Liver

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Abstract

The AMP-activated protein kinase (AMPK) represses signaling through the mammalian target of rapamycin complex 1 (mTORC1). In muscle, repression of mTORC1 leads to a reduction in global protein synthesis. In contrast, repression of mTORC1 in the liver has no immediate effect on global protein synthesis. In the present study, signaling through mTORC1 and translation of specific mRNAs such as those bearing a 5'-terminal oligopyrimidine (TOP) tract and were examined in rat liver following activation of AMPK after treadmill running. Activation of AMPK repressed translation of the TOP mRNAs encoding rpS6, rpS8, and eEF1 α . In contrast, neither global protein synthesis nor translation of mRNAs encoding GAPDH or β -actin was changed. Basal phosphorylation of the mTORC1 target 4E-BP1, but not S6K1 or rpS6, was reduced following activation of AMPK. Thus, in liver, AMPK activation repressed translation of TOP mRNAs through a mechanism distinct from downregulated phosphorylation of S6K1 or rpS6.

Keywords

mammalian target of rapamycin; mTOR; AMP-activated protein kinase; terminal oligopyrimidine tract; liver; mRNA translation

INTRODUCTION

The process of mRNA translation is tightly regulated, with a variety of signaling pathways modulating the function of over a dozen translation initiation, elongation and termination factors. Arguably, one of the most important regulators of mRNA translation is the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway which controls the phosphorylation of eukaryotic initiation factor (eIF)4E binding protein-1 (4E-BP1) and the ribosomal protein S6 kinase, S6K1 [1]. Depending upon conditions, the mTORC1 signaling pathway can mediate rapid changes in either global rates of protein synthesis and/or the translation of mRNAs encoding certain proteins, e.g. mRNAs bearing an oligopyrimidine tract adjacent to the m⁷GTP cap (referred to as a TOP motif). For example, following repression of mTORC1 signaling by rapamycin, varying degrees of inhibition of global rates of protein synthesis are observed, ranging from little or no change to greater than 50%, depending upon

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cell type or tissue. Even under conditions where little or no change in global rates of protein synthesis are observed, however, the mTORC1 signaling pathway may mediate control of the translation of specific mRNAs [2].

The mTORC1 signaling pathway is repressed in liver [3], skeletal muscle [4], and in cells in culture [5;6] in response to activation of the AMP-activated protein kinase (AMPK) by administration of the AMP mimetic, 5-aminoimidazole-4-carboxamide 1- β -D-ribose (AICAR). Under such conditions, AMPK activation in liver leads not only to decreased mTORC1 signaling, but also to inhibition of global rates of protein synthesis. This finding is surprising because inhibition of mTORC1 with rapamycin has no measurable effect on global rates of protein synthesis in liver [2]. A possible explanation for this apparent discrepancy is that AICAR may modulate translation through a pathway other than AMPK-mTORC1, as suggested by the observation that in some cases AICAR acts independently of AMPK [e.g. 7]. Therefore, in the present study, a more physiologically relevant mechanism for activating AMPK in liver was utilized, i.e. endurance exercise [8]. It was found that exercise acutely repressed mTORC1 signaling, with no change in global protein synthesis. In contrast, exercise-induced AMPK phosphorylation was accompanied by downregulated translation of TOP mRNAs. Furthermore, the changes in mRNA translation occurred in the absence of detectable changes in phosphorylation of either S6K1 or rpS6. Overall, the results are consistent with a specific downregulation of mRNA translation in liver in response to AMPK phosphorylation independent of changes in global rates of protein synthesis or S6K1 phosphorylation.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 200-260g (Charles River Laboratory) were kept on a 12-h light:dark cycle with free access to food and water. The experimental protocol utilized in the studies reported herein was approved by the Institutional Animal Care and Use Committee of The Pennsylvania State College of Medicine and was conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Exercise Protocol

On the three days prior to an experiment, rats assigned to an exercise group were acclimated to the protocol by allowing them to run on a treadmill (Exer-6M-Treadmill, Columbus Instrument) at a pace of 16, 18, and 18 m/min respectively for 10 min. Food was removed from the cages 12 hours prior to the start of the study for rats in both control and exercise groups to avoid acute metabolic and/or hormonal responses resulting from food intake. On the day of the study, rats ran at a pace of 26 m/min with a 10% incline for 60 min. Immediately following the exercise period, rats were anesthetized with Isoflurane, and one portion of the liver was frozen in situ between aluminum blocks pre-cooled to the temperature of liquid nitrogen for analysis of AMPK phosphorylation. The remaining portion of the liver was rapidly excised and processed as described below. All tissue samples were stored at -70°C until analyzed.

Sample Preparation

A portion of liver was weighed and homogenized in 7 volumes of buffer A consisting of 20 mM HEPES (pH 7.4), 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 50 mM NaF, 50 mM β -glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 0.5 mM sodium vanadate. An aliquot (250 μ l) of the homogenate was used to assess incorporation of [3 H]phenylalanine into protein as described below. The homogenate was then centrifuged at 10,000 $\times$ g for 10 min at 4°C, and the resulting supernatant was used for analysis of S6K1, rpS6, and 4E-BP1 phosphorylation, as well as the association of eIF4E with 4E-BP1 as described below. Another portion of liver was weighed and homogenized in 4 volumes of

buffer B containing 40 mM HEPES (pH 7.5), 100 mM KCl, and 5 mM magnesium chloride. The homogenate was centrifuged at $3,000 \times g$ for 15 min and the resulting supernatant was used for the analysis of polysomal aggregation.

Polysomal Aggregation

One volume of detergent (10% Triton X-100, 0.24 M deoxycholate) was added to nine volumes of buffer B supernatant and the sample was layered over a 10-70% linear sucrose density gradient. The gradients were centrifuged at $90,000 \times g$ for 3 h at 4°C in a Beckman SW28 rotor. The gradients were then fractionated on an ISCO gradient fractionator while the absorbance at 254 nm was recorded.

RNA Extraction and Analysis

Total RNA was extracted from sucrose gradient fractions as described previously [9], and reverse transcribed using a kit (Superscript One-Step) from Invitrogen. The primers used for rpS6, rpS8, eEF1 α , and GAPDH were described previously [10] and the primers used for β -actin were: upper-5'-TAGGCACCAGGTGTGATGG-3', lower-5'-GCAGCTCATTGTAGAAAGTGT-3'.

Measurement of Phosphorylation Status

S6K1, 4E-BP1, and AMPK phosphorylation was assessed as reduced migration during electrophoresis using antibodies that recognize the proteins independent of phosphorylation state (S6K1 and AMPK, Cell Signaling Technology; 4E-BP1, Bethyl Laboratories). Phosphorylation of rpS6 was evaluated using antibodies that recognize either the Ser 235/236 or Ser 240/244 phosphorylation sites on rpS6 (Cell Signaling Technology).

Measurement of eIF4G and 4E-BP1 Association with eIF4E

The association of eIF4G and 4E-BP1 with eIF4E was evaluated by Western blot analysis of eIF4E immunoprecipitates as described previously [11]. Results were normalized to the amount of eIF4E in the immunoprecipitates.

Measurement of Protein Synthesis

Global rates of protein synthesis were assessed by the flooding dose technique using L-[2,3,4,5,6- ^3H]phenylalanine (150 mM containing 3.70 GBq/L) as previously described [12].

Statistical Analysis

All data were analyzed by InStat 3.0 (GraphPad Software). Unless otherwise stated, data were analyzed using a paired t-test comparing endurance exercise values to control values. The level of significance was set at $p < 0.05$ for all statistical analyses.

RESULTS

Previous studies have shown that intense, but not moderate endurance exercise promotes activation of AMPK in liver [8;13]. To confirm that the exercise protocol used in the present study was sufficiently intense to cause activation of AMPK, the phosphorylation state of the protein was assessed by changes in its rate of migration during electrophoresis [10]. This method was chosen because the activity of AMPK is regulated through phosphorylation on multiple residues [14;15], and reagents are not commercially available to examine each individually. As shown in Fig. 1A, the electrophoretic migration of AMPK was reduced immediately after exercise compared to nonexercised control animals. Incubation of the liver homogenate with λ phosphatase prior to electrophoresis resulted in a collapse of the pattern into a single, rapidly migrating form, demonstrating that the slower migrating forms were

attributable to increased phosphorylation of the protein (Fig. 1B). These results suggest that the exercise protocol was of sufficient intensity to activate AMPK.

To assess changes in mTORC1 signaling, phosphorylation of the downstream targets, 4E-BP1 and S6K1 was measured. Exercise-induced activation of AMPK was associated with a shift in 4E-BP1 from the hyperphosphorylated γ -form to the faster migrating α - and β -forms (Fig. 2A), indicating a decrease in 4E-BP1 phosphorylation. In addition, the amount of 4E-BP1 associated with eIF4E in liver from exercised rats was approximately 2-fold greater than the control value (Fig. 2B), whereas eIF4G binding to eIF4E was significantly reduced to 26% of the control value (Fig. 2C). In contrast to the observed changes in 4E-BP1 phosphorylation, the bulk of S6K1 in the control state was detected in the faster migrating α - and β -bands and no further reduction in this relatively hypophosphorylated state was observed following exercise-induced AMPK activation (Fig. 2D). To further assess potential changes in S6K1 activity, the phosphorylation state of the S6K1 substrate, rpS6, was examined. As shown in Figs. 2E and 2F, there was no change in the phosphorylation of either the Ser235/236 or the Ser240/244 sites, respectively, following exercise-induced AMPK activation. This result is consistent with the lack of change in S6K1 phosphorylation, and suggests that exercise-induced AMPK activation causes no reduction in S6K1 activity beyond that engendered by an overnight fast.

To assess the effect of exercise-induced AMPK activation on the global rate of protein synthesis in the liver immediately after cessation of treadmill running, the flooding dose technique [12] was employed. As illustrated in Fig. 3, global rates of protein synthesis in the liver following exercise-induced AMPK activation were unchanged, being $93.1 \pm 4.5\%$ of the control value.

Many of the mRNAs that are translationally regulated in response to changes mTORC1 signaling have a TOP motif [16]. To elucidate the effect of exercise-induced AMPK activation on the translation of TOP mRNAs, the distribution of the mRNAs encoding rpS8, rpS6, and eEF1A between the polysomal (i.e. actively translated) and nonpolysomal (i.e. nontranslated) fractions of livers was compared to the distribution of two non-TOP mRNAs, GAPDH and β -actin. No significant change in polysome aggregation was observed in response to exercise-induced AMPK activation (Fig. 4A and 4B), a result consistent with the finding of no change in the global rate of protein synthesis. In control rats, the proportion of the mRNAs encoding GAPDH and β -actin present in the polysomal fraction compared to the nonpolysomal fraction was greater than 2:1, and exercise-induced AMPK activation had no significant effect on their distribution (Fig. 4C). In contrast, the polysome:nonpolysome ratios of eEF1A, rpS8, and rpS6 mRNAs were 0.65, 0.55, and 0.55 respectively in livers from control rats (Fig. 4D), and the values decreased significantly to 0.45, 0.43, and 0.39 respectively in response to exercise. Thus, the shift in distribution of eEF1A, rpS8, and rpS6 mRNAs from polysomal to nonpolysomal fractions demonstrates that the translation of these three messages was specifically repressed in the liver in response to exercise-induced AMPK activation.

DISCUSSION

In the present study, exercise-induced AMPK activation in the liver diminished the translation of TOP mRNAs in conjunction with reduced phosphorylation of the mTORC1 effector 4E-BP1, increased in association of 4E-BP1 with eIF4E, and decreased eIF4E association with eIF4G. In contrast, S6K1 did not manifest a detectable change in phosphorylation, likely due to the rats being fasted for 12 h prior to the start of the study. A similar finding in skeletal muscle of fasted rats subjected to a single bout of treadmill running was reported previously [17]. In that study, S6K1 was hypophosphorylated in both fasted control and exercised rats. The finding of a relatively small proportion of 4E-BP1 in the hyperphosphorylated γ -form in the unexercised control rats is also consistent with this suggestion. Overall, the results are in

agreement with previous studies [18;19] showing that signaling through mTORC1 is partially repressed by fasting. The results also extend earlier studies to show that immediately after a single bout of endurance exercise, AMPK activation parallels a repression in signaling through mTORC1. The finding that 4E-BP1, but not S6K1, phosphorylation was reduced after exercise-induced AMPK activation suggests that S6K1 may be more sensitive to fasting-induced changes in phosphorylation compared to 4E-BP1, and/or is controlled through both mTORC1-dependent and -independent signaling pathways. In this regard, S6K1 is phosphorylated by multiple upstream kinases including PDK1 and protein kinase C [20].

The mechanism through which exercise-induced AMPK activation acts to repress translation of mRNAs encoding ribosomal proteins is unclear. Under a variety of conditions, translation of TOP mRNAs has been shown to be regulated by S6K1 [reviewed in 16]. Despite these observations, S6K1 is not the only pathway through which TOP mRNA translation is regulated. In two studies [21;22], translation of TOP mRNAs was shown to increase with no detectable change in phosphorylation of either S6K1 or rpS6. Instead, TOP mRNA translation was enhanced in cells expressing constitutively active forms of either PI 3-kinase or Akt [22], two kinases thought to be upstream of mTORC1 in the insulin signal transduction pathway. A more recent study [23] showed that in mouse embryo fibroblasts (MEF) in which the gene encoding S6 was mutated to change the five sites phosphorylated by S6K1 to Ala, translation of TOP mRNAs was enhanced by addition of serum to the medium of serum-deprived cells. In addition, in mice bearing a non-phosphorylatable S6 gene, the effect of partial hepatectomy to enhance TOP mRNA translation in the liver was unaffected [23]. Combined, the findings in MEFs and in mice suggest that S6K1 is not required for regulation of TOP mRNA translation. In the present study, the finding that translation of mRNAs encoding ribosomal proteins was repressed immediately after exercise-induced AMPK activation with no detectable change in S6K1 or rpS6 phosphorylation, provides further evidence that mTORC1 regulates TOP mRNA translation through a mechanism distinct from its effects on the phosphorylation state of those two proteins.

Activation of AMPK serves as a protective mechanism for maintaining adenylate energy charge during exercise [24;25;26]. Thus, AMPK activation represses global rates of protein synthesis in skeletal muscle while simultaneously enhancing glucose uptake. In liver, the downregulation of TOP mRNA translation observed in response to exercise-induced AMPK activation may allow ATP that would normally be used for ribosome biogenesis to instead be used for other metabolic processes. For example, AMPK targets several metabolic pathways in liver to ensure glucose homeostasis and fat oxidation during exercise [13;27]. Because of the relatively long half-life of ribosomes, a short-term reduction in ribosome biogenesis, an expected result of reduced TOP mRNA translation, would be unlikely to affect global rates of protein synthesis. However, because TOP mRNAs typically encode proteins that are involved in mRNA translation, continuous activation of AMPK could lead to a reduction in the capacity of the liver to synthesize protein. In this regard, a previous study [28] reported a decrease in global rates of liver protein synthesis following consecutive days of subjecting rats to endurance exercise.

In conclusion, the results of the present study demonstrate a reduction in TOP mRNA translation in liver in response to exercise-induced AMPK phosphorylation. The reduction is associated with decreased signaling through mTORC1, as assessed by reduced 4E-BP1 phosphorylation as well as decreased binding of eIF4G and increased binding of 4E-BP1 to eIF4E. However, no change in phosphorylation of either S6K1 or rpS6 was observed, suggesting that the change in TOP mRNA translation was mediated by a mechanism distinct from activation of the S6K1 arm of the mTORC1 signaling pathway.

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Fig. 1.

Effect of endurance exercise on AMPK phosphorylation in rat liver. (A) Hyperphosphorylation of AMPK was measured by Western blot analysis and quantitated as the fraction of the protein present in the hyperphosphorylated form. Results are presented as a fraction of the control value and expressed as mean $\pm$ SEM. Control rats, light grey bars (n=12); exercised rats, dark grey bars (n=11). A representative blot is shown as an inset. C, control; E, exercised rats. * $p < 0.001$ vs. control. (B) Liver homogenates were incubated with (+) or without (-) λ phosphatase as described under "Materials and Methods" prior to Western blot analysis for AMPK. A representative blot is shown (n=6 per condition).

Fig. 2.

Effect of exercise on mTORC1 signaling in rat liver. (A) Hyperphosphorylation of 4E-BP1 was measured by Western blot analysis and quantitated as the fraction of the protein present in the hyperphosphorylated β - and γ -forms. Results are presented as a fraction of the control value and expressed as mean $\pm$ SEM. Control rats, light grey bars; exercised rats, dark grey bars. Representative blots are shown as insets to each panel. In panel A, the α -, β -, and γ -forms of the protein are denoted to the left of the inset. C, control rats; E, exercised rats. * p<0.005 vs. control (n=12 per condition). (B) The association of 4E-BP1 with eIF4E was measured by Western blot analysis of eIF4E immunoprecipitates. Results are presented as a fraction of the control value and expressed as mean $\pm$ SEM. * p<0.05 vs. control (n= 5-6 per condition). (C) The association of eIF4G with eIF4E was measured by Western blot analysis using the same eIF4E immunoprecipitates as in panel B. * p<0.0001 vs. control (n= 5-6 per condition). (D) Hyperphosphorylation of S6K1 was assessed by Western blot analysis as described under

“Materials and Methods”. rpS6 phosphorylation on (E) Ser 235/236 and (F) Ser240/244 was assessed by Western blot analysis. Results were normalized to control values and expressed as mean $\pm$ SEM. (n=6 per condition).

Fig. 3. Effect of exercise on global rates of protein synthesis. The rate of protein synthesis was measured in livers in vivo as described under “Materials and Methods” immediately after cessation of treadmill running. Results are presented as a fraction of the control value and expressed as mean $\pm$ SEM (n=5 per condition).

Fig. 4.

Effect of exercise on the translation of TOP mRNAs. The distribution of mRNAs encoding rpS6, rpS8, eEF1 α , β -actin, and GAPDH between polysomal and nonpolysomal fractions was assessed by sucrose density gradient centrifugation. A representative polysome profile is shown for (A) control liver and (B) liver from exercised rats, $n=6$ per group. The non-shaded area represents the subpolysomal fraction, and the shaded area represents the polysomal fraction. Quantitative RT-PCR was used to measure the amount of mRNAs encoding specific proteins. Results were normalized to the total amount of RNA recovered in the individual fractions and are expressed as a ratio of mRNA present in polysomal vs. nonpolysomal fractions. (C) Distribution of mRNAs encoding β -actin and GAPDH as a ratio of polysomal:subpolysomal. Control rats, light grey bars; exercised rats, dark grey bars. (D) Distribution of mRNAs encoding eEF1 α , rpS6, and rpS8 as a ratio of polysomal: subpolysomal. Control rats, light grey bars; exercised rats, dark grey bars ($n=5-6$ per condition). * $p<0.05$ vs. control rats by one-tailed t-test.