

Hepatic amino acid-dependent signaling is under the control of AMP-dependent protein kinase

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Abstract It has become increasingly clear in recent years that amino acids can stimulate a signal transduction pathway resulting in the phosphorylation of mammalian target of rapamycin downstream targets. We have now found that amino acid-dependent phosphorylation of p70S6 kinase and of S6 in hepatocytes is prevented when AMP-dependent protein kinase (AMPK) is activated by either the purine ribonucleoside analogue AICARiboside, fructose or glycerol. Insulin-dependent phosphorylation of protein kinase B is not affected by AMPK activation. Protein synthesis is strongly inhibited when AMPK is activated. It is concluded that amino acid-dependent signaling, a protein-anabolic signal, can be effectively antagonized by activation of AMPK. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Signal transduction; AICAR; AMP-dependent protein kinase; Amino acid; Mammalian target of rapamycin; p70S6 kinase

1. Introduction

Recent research, initiated by our laboratory [1], has demonstrated that amino acids, in addition to their role as intermediary metabolites, can stimulate mammalian target of rapamycin (mTOR)-dependent phosphorylation of signal transduction components which also participate in insulin signaling [2–4]. Although in various cell types insulin alone can stimulate PI 3-kinase-dependent phosphorylation of protein kinase B (PKB), the hormone cannot activate mTOR downstream targets unless low concentrations of certain amino acids, leucine in particular, are also present. By contrast, high concentrations of amino acids stimulate phosphorylation of mTOR downstream targets in the absence of insulin but do not effect PKB and, presumably, also do not affect PI 3-kinase although the latter issue is controversial [4]. The mechanism by which amino acids stimulate mTOR-mediated signaling is not known but a possibility is that they either directly, or indirectly, stimulate mTOR activity or that they inhibit a pro-

tein phosphatase acting on mTOR and/or mTOR downstream targets ([4], for review).

It is now generally accepted that mTOR functions as an amino acid sensor, not only in mammalian cells but also in yeast, controlling protein synthesis and degradation in a reciprocal fashion [3,5]. In an elegant study with HEK293 cells, Dennis et al. [6] proposed that mTOR may also act as a sensor of intracellular ATP concentration. It was noted that among the various protein kinases, the K_m of mTOR for ATP in vitro was exceptionally high and within the physiological (mM) range of ATP concentrations. Moreover, by inhibition of either mitochondrial or glycolytic ATP production in intact cells, a correlation was found between intracellular ATP levels and the degree of phosphorylation of p70S6 kinase or 4E-BP1, as indicators of in situ mTOR activity. Because inhibition of ATP production may also affect AMP levels via the adenylate kinase equilibrium [7–9], we investigated whether activation of AMP-dependent protein kinase (AMPK), which shuts off ATP-dependent pathways [10], may also contribute to mTOR inhibition. Our data, obtained with isolated hepatocytes, indicate that this is, indeed, the case.

2. Materials and methods

2.1. Materials

Insulin, rapamycin, AICAR (AICARiboside, imidazole-4-carboxamide 1- β -D-ribofuranoside) and the chemicals for enhanced chemiluminescence (ECL) were from Sigma (St. Louis, MO, USA). LY294002 (2-(4-morpholinyl)-8-phenylchromone) was obtained from Biomol (Plymouth Meeting, PA, USA). Phosphospecific anti-PKB (Thr³⁰⁸) was from New England Biolabs. Phosphospecific anti-AMPK (Thr¹⁷²) was from Cell Signaling Technology Inc. (Leusden, The Netherlands). Rabbit anti-p70S6 kinase was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit horseradish peroxidase was from Bio-Rad (Hercules, CA, USA). Silicone oil AR200 was from Wacker-Chemie GmbH (Burghausen, Germany). [³²P]Phosphate and [³H]valine were from Amersham Corp. ('s Hertogenbosch, The Netherlands). All other chemicals and enzymes were obtained from either Boehringer (Mannheim, Germany) or Sigma (St. Louis, MO, USA).

Rapamycin and LY294002 were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration did not exceed 0.25% (v/v). This concentration of DMSO did not affect the processes that were studied.

2.2. Preparation of hepatocytes

Hepatocytes were isolated from 16 to 20 h starved male Wistar rats (200–250 g) by collagenase perfusion [11].

2.3. Hepatocyte incubation

Hepatocytes (5–10 mg dry weight/ml) were pre-incubated for the indicated time periods at 37°C in minimal medium (Krebs–Henseleit bicarbonate buffer plus 10 mM Na–HEPES, pH 7.4, and 20 mM

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Abbreviations: AICAR, AICARiboside, imidazole-4-carboxamide 1- β -D-ribofuranoside; AMPK, AMP-dependent protein kinase; mTOR, mammalian target of rapamycin; PKB, protein kinase B

glucose). In all experiments, hepatocytes were first incubated in this medium for 20 min to allow for temperature adjustment. This was then followed by addition of the components as indicated in the legends. Final incubation volume was 2–3 ml. The gas atmosphere was O₂/CO₂ (19:1, v/v).

For measurement of S6 phosphorylation, hepatocytes were first loaded with [³²P]phosphate (50 µCi/ml), exactly as described in [1].

Protein synthesis was measured with [³H]valine (0.5 µCi/ml), as in [1]. In order to ensure uniformity of the labeling of the intracellular valine pool, a concentration of 5 mM valine was used.

The composition of the complete mixture of all amino acids (4xAA) used in the experiments was such that the concentration of each of the amino acids was present at a concentration four times that found in the portal vein of a fasted rat. The composition of the 1xAA mixture was exactly as described in [11] except that the leucine concentration was 250 µM.

At the end of the incubations, hepatocytes were collected for gel analysis by centrifugation in 5 volumes of an ice-cold solution of 150 mM NaCl plus 10 mM Na-HEPES (pH 7.4) for 5 s in an Eppendorf centrifuge. For the SDS-PAGE procedures, the pellet was lysed by addition of Laemmli sample buffer and subsequently incubated at 95°C for 5 min. Cells for determination of ATP were centrifuged in a microcentrifuge through a layer of silicone oil into a layer of 14% (m/v) HClO₄. The acidified samples underneath the oil were re-extracted by stirring, centrifuged again and neutralized to pH 7 with a small volume of a mixture of 2 M KOH plus 0.3 M MOPS.

2.4. Gel retardation assay

An equivalent of 40–60 µg of cell protein per sample was separated by SDS-PAGE. For separation of the different phosphorylated forms of p70S6 kinase a 7.5% (w/v) polyacrylamide gel was used. After separation, a standard Western blotting procedure was performed and the blot was incubated with an antibody against p70S6 kinase. The proteins were visualized by ECL.

2.5. Phosphorylation of PKB and of AMPK

An equivalent of 30–40 µg of cell protein per sample was separated by 10% (w/v) SDS-PAGE. After separation, a standard Western blotting procedure was performed. The blot was subsequently incubated with an antibody against the phosphorylated form of PKB (Thr³⁰⁸) or against the phosphorylated form of AMPK (Thr¹⁷²). ECL was performed to visualize the proteins.

2.6. Phosphorylation of S6

An equivalent of 100 µg of cell protein per sample was separated by 10% (w/v) SDS-PAGE. Gel slabs were dried and subjected to autoradiography. Protein phosphorylation was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

2.7. Determination of ATP

ATP was determined fluorimetrically with NADP⁺, glucose, hexokinase and glucose 6-phosphate dehydrogenase [12].

2.8. Statistics

The data are summarized as means ± S.E.M., with the number of different hepatocyte preparations in parentheses (Fig. 2). Statistical significance was determined using Student's *t*-test (*P* < 0.05).

3. Results

3.1. Effect of AMPK activation on p70S6 kinase and S6 phosphorylation

The effect of AMPK activation on amino acid-dependent signaling was studied by titrating AICAR, a specific activator of AMPK [13], in the presence of amino acids. Phosphorylation of AMPK at Thr¹⁷², which has been shown to parallel its activity [14,15], was maximal at 250 µM AICAR (Fig. 1A). Neither the absence of amino acids nor the presence of rapamycin, an inhibitor of mTOR activation, affected basal AMPK phosphorylation. Phosphorylation of p70S6 kinase greatly increased upon addition of amino acids (in agreement

Table 1
Intracellular ATP

Additions	ATP (µmol/g dry weight)
None	12.8 ± 0.4 (5)
4AA	12.7 ± 0.3 (5)
4AA+AICAR 250 µM	12.1 ± 0.4 (5)
4AA+fructose 15 mM	2.6 ± 0.7 (4) ^a
4AA+glycerol 10 mM	6.6 ± 0.4 (4) ^a
4AA+rapamycin 100 nM	11.9 ± 0.6 (3)

Hepatocytes were incubated as described in the legend to Fig. 1.

^aSignificantly different from the control in the presence of amino acids alone (*P* < 0.05).

with existing literature; [4], for review); the effect was completely prevented by rapamycin and also counteracted by AICAR at concentrations similar to those required for activation of AMPK, AICAR being maximally effective at a concentration of 250 µM (Fig. 1B). In order to demonstrate that activity of p70S6 kinase was also diminished by AICAR, its effect on amino acid-stimulated phosphorylation of ribosomal protein S6, a measure of the *in situ* activity of p70S6 kinase [16], was also studied. Similar to p70S6 kinase, phosphorylation of S6 was stimulated by amino acid addition in a rapamycin-sensitive manner (cf. [1]), and this was prevented by AICAR (Fig. 1C).

AMPK could also be activated by addition of either fructose (cf. [17]) or glycerol. In hepatocytes, these two compounds are rapidly phosphorylated and decrease intracellular ATP because of intracellular phosphate depletion [18,19] (Table 1). Like AICAR, both fructose and glycerol increased AMPK phosphorylation and antagonized amino acid-dependent stimulation of p70S6 kinase and S6 phosphorylation (Fig. 1A–C).

It is important to stress that intracellular ATP was not affected by AICAR (in agreement with [13]) nor was it influenced by amino acid depletion or rapamycin addition (Table 1).

3.2. Effect of AMPK activation on PKB phosphorylation

In another series of experiments we tested the effect of AICAR on PKB phosphorylation. In these experiments, insulin had to be used to stimulate PKB phosphorylation because amino acids do not affect PKB ([4], for review).

In contrast to amino acid-dependent stimulation of p70S6 kinase and S6 phosphorylation, insulin-dependent stimulation of PKB phosphorylation was not affected by AICAR (Fig. 1D). As a control, the effect of the PI 3-kinase inhibitor LY294002 was also tested. As expected, this compound completely prevented insulin-dependent phosphorylation of PKB (Fig. 1D). The lack of inhibition of insulin-stimulated PKB phosphorylation by activation of AMPK is in agreement with similar observations in other cell types [20,21].

3.3. Inhibition of protein synthesis by AMPK activation

Because mTOR downstream targets are involved in the regulation of protein synthesis [2,3,5,6], the effect of AMPK activation on protein synthesis was investigated and compared with the effect of rapamycin. Protein synthesis was two-fold stimulated by the presence of amino acids, was completely inhibited by cycloheximide and strongly inhibited by AMPK activation, whether induced by AICAR or fructose (Fig. 2). The strong inhibition of hepatic protein synthesis by fructose confirms existing literature [18]. Rapamycin inhibited protein synthesis by 20% only (cf. [1]).

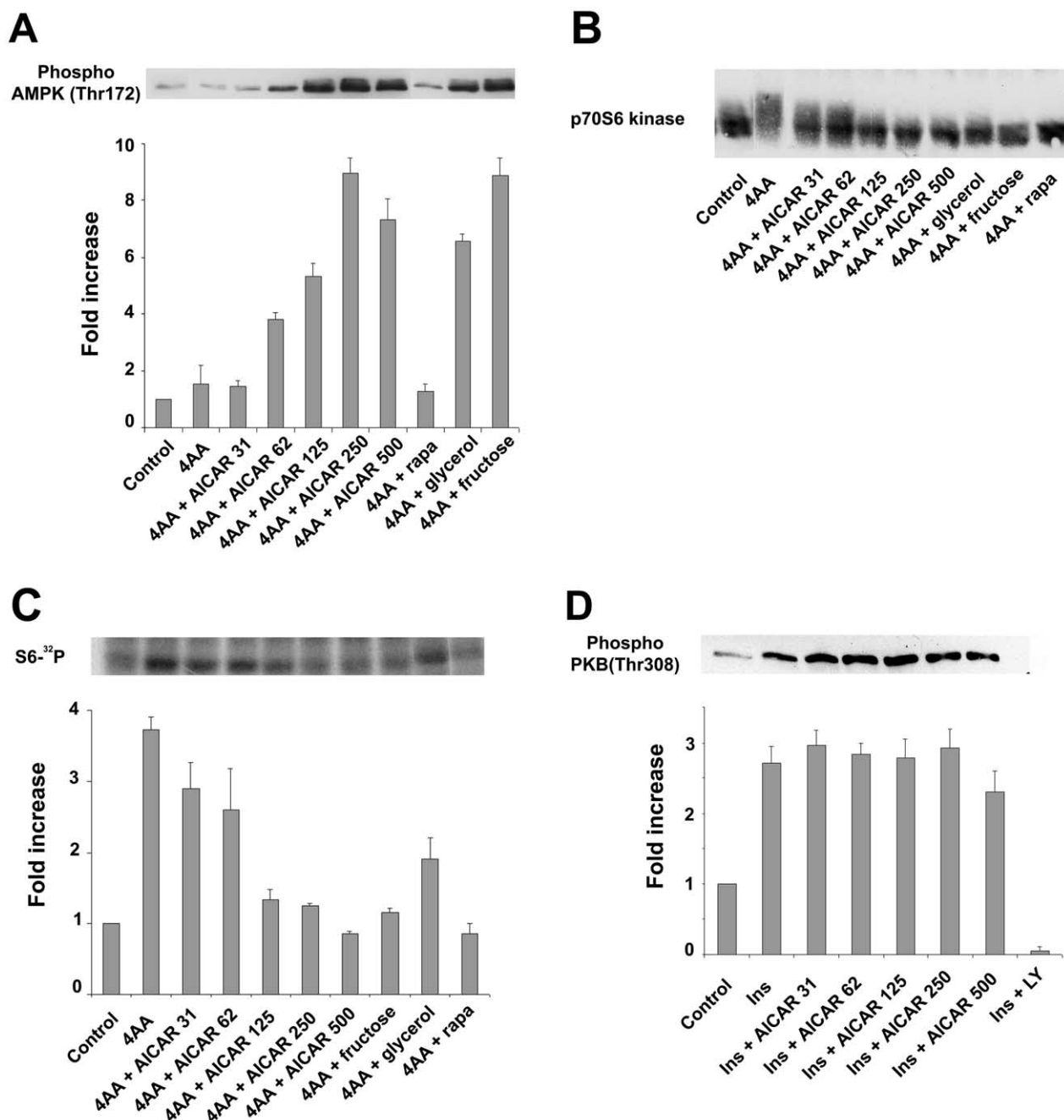


Fig. 1. Phosphorylation of AMPK (A), p70S6 kinase (B), S6 (C) and PKB (D). Hepatocytes were preincubated for 15 min in the absence or presence of AICAR (at the concentrations indicated in μM), 100 nM rapamycin, 15 mM fructose, or 10 mM glycerol; amino acids (4xAA; dissolved in the minimal incubation medium) were then added and incubation was continued for another 30 min. When amino acids were not present, the same protocol was followed except that an equivalent volume of minimal medium without amino acids was added to the cell suspension. In D, insulin (10^{-7} M) was added 4 min before the end of the incubation. The data are from experiments carried out with four different hepatocyte preparations. Fig. 1B is a representative experiment. Data (A,C,D) are expressed as fold increase over the control value. Abbreviations: rapa, rapamycin; ins, insulin; LY, LY294002.

4. Discussion

In hepatocytes, mTOR-mediated signaling can be stimulated by either a combination of insulin and low concentrations of amino acids acting in synergy, or by high concentrations of amino acids alone [1,4]. The interference of AMPK with signaling appears to be confined to amino acid-dependent, mTOR-mediated signaling because insulin-stimulated PKB phosphorylation was not affected.

It has recently been demonstrated that mTOR activity is kinetically controlled by the cytosolic ATP concentration, and it was proposed that mTOR, in addition to its function as a sensor of amino acids, is also a sensor of ATP [6]. Our data strongly suggest that AMPK is an additional element in the control of mTOR-dependent signaling. In this context, the recent observation that mTOR is associated with the mitochondrial outer membrane [22] is highly relevant, because adenylate kinase, which controls AMP concentration, is predom-

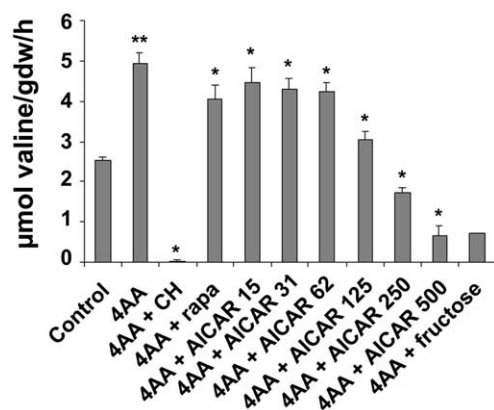


Fig. 2. Protein synthesis. Hepatocytes were incubated for 1 h in the absence or presence of the amino acid mixture (4xAA). The concentration of valine was 5 mM under all conditions. Where present, 10 μ M cycloheximide, 100 nM rapamycin, 15 mM fructose or AICAR (at the indicated concentrations in μ M) were added. Data are the means \pm S.E.M. from experiments carried out with four to six hepatocyte preparations. Values represent the amount of [3 H]valine incorporated into protein. With fructose, only two experiments were carried out. *, Significantly different from the corresponding control in the presence of amino acids alone; **, significantly different from the control in the absence of amino acids. Abbreviations: rapa, rapamycin; CH, cycloheximide.

inantly located in the mitochondrial intermembrane space and is not present in the mitochondrial matrix [23]. mTOR is thus in a perfect position to be controlled by changes in ATP/AMP ratio.

Clearly, the strong inhibitory effect of AMPK activation on protein synthesis cannot solely be ascribed to an effect on mTOR-dependent signaling, because inhibition of protein synthesis by rapamycin was much less effective (Fig. 2). Apparently, AMPK inhibits protein synthesis not only through inhibition of mTOR-mediated signaling but also at other steps controlling the protein synthesizing machinery.

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References

- [1] Blommaart, E.F.C., Luiken, J.J.F.P., Blommaart, P.J.E., van Woerkom, G.M. and Meijer, A.J. (1995) *J. Biol. Chem.* 270, 2320–2326.
- [2] Shah, O.J., Anthony, J.C., Kimball, S.R. and Jefferson, L.S. (2000) *Am. J. Physiol.* 279, E715–E729.
- [3] Raught, B., Gingras, A.C. and Sonenberg, N. (2001) *Proc. Natl. Acad. Sci. USA* 98, 7037–7044.
- [4] van Sluijters, D.A., Dubbelhuis, P.F., Blommaart, E.F.C. and Meijer, A.J. (2000) *Biochem. J.* 351, 545–550.
- [5] Dennis, P.B., Fumagalli, S. and Thomas, G. (1999) *Curr. Opin. Genet. Dev.* 9, 49–54.
- [6] Dennis, P.B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S.C. and Thomas, G. (2001) *Science* 294, 1102–1105.
- [7] Veech, R.L., Lawson, J.W., Cornell, N.W. and Krebs, H.A. (1979) *J. Biol. Chem.* 254, 6538–6547.
- [8] Moore, F., Weekes, J. and Hardie, D.G. (1991) *Eur. J. Biochem.* 199, 691–697.
- [9] Bontemps, F., Vincent, M.F. and Van den Berghe, G. (1993) *Biochem. J.* 290, 671–677.
- [10] Hardie, D.G., Carling, D. and Carlson, M. (1998) *Annu. Rev. Biochem.* 67, 821–855.
- [11] Meijer, A.J., Lof, C., Ramos, I.C. and Verhoeven, A.J. (1985) *Eur. J. Biochem.* 148, 189–196.
- [12] Williamson, J.R. and Corkey, B.E. (1969) *Methods Enzymol.* 13, 434–513.
- [13] Corton, J.M., Gillespie, J.G., Hawley, S.A. and Hardie, D.G. (1995) *Eur. J. Biochem.* 229, 558–565.
- [14] Hawley, S.A., Davison, M., Woods, A., Davies, S.P., Beri, R.K., Carling, D. and Hardie, D.G. (1996) *J. Biol. Chem.* 271, 27879–27887.
- [15] Stein, S.C., Woods, A., Jones, N.A., Davison, M.D. and Carling, D. (2000) *Biochem. J.* 345, 437–443.
- [16] Pullen, N. and Thomas, G. (1997) *FEBS Lett.* 410, 78–82.
- [17] Gillespie, J.G. and Hardie, D.G. (1992) *FEBS Lett.* 306, 59–62.
- [18] Mäenpää, P.H., Raivio, K.O. and Kekomäki, M.P. (1968) *Science* 161, 1253–1254.
- [19] Woods, H.F. and Krebs, H.A. (1973) *Biochem. J.* 132, 55–60.
- [20] Jakobsen, S.N., Hardie, D.G., Morrice, N. and Tornqvist, H.E. (2001) *J. Biol. Chem.* 276, 46912–46916.
- [21] Fisher, J.S., Gao, J., Han, D.-H., Holloszy, J.O. and Nolte, L.A. (2002) *Am. J. Physiol. Endocrinol. Metab.* 282, E18–E23.
- [22] Desai, B.N., Myers, B.R. and Schreiber, S.L. (2002) *Proc. Natl. Acad. Sci. USA* 99, 4319–4324.
- [23] Schnaitman, C. and Greenawalt, W. (1968) *J. Cell Biol.* 38, 158–175.