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Amino acid availability regulates S6K1 and protein synthesis in avian insulin-insensitive QM7 myoblasts

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Abstract The regulation of S6K1 by nutritional status and insulin has been recently reported in vivo in chicken muscle despite the relative insulin resistance of this tissue as estimated by phosphatidylinositol 3-kinase (PI3-kinase) activity. The present work aimed to study the impact of amino acids on S6K1 activity in quail muscle (QM7) myoblasts. Firstly, we characterized S6K1 in QM7 cells and demonstrated the absence of insulin receptors in these cells. Secondly, we showed that amino acids in the absence of insulin induced S6K1 phosphorylation on Thr389 and concomitantly increased its enzymatic activity. Amino acid-induced S6K1 activation was inhibited by LY294002 (PI3-kinase inhibitor) and rapamycin (inhibitor of the mammalian target of rapamycin, mTOR), suggesting the involvement of an avian homolog of mTOR. The availability of individual amino acids (methionine or leucine) regulated S6K1 phosphorylation on Thr389 and QM7 protein synthesis. In conclusion, amino acids regulate S6K1 phosphorylation and activity in QM7 cells through the mTOR/PI3-kinase pathway in an insulin-independent manner.

Key words: Quail muscle (QM7) cell line; Amino acid; S6K1; Protein synthesis

1. Introduction

The cytoplasmic serine/threonine kinase S6K1 plays a critical role in the regulation of protein translation in mammals. S6K1 phosphorylates 40S ribosomal protein S6 and selectively stimulates the translation of elongation factors and ribosomal proteins [1]. This enzyme is activated through multisite phosphorylation in response to insulin via a signal transduction pathway involving phosphatidylinositol 3-kinase (PI3-kinase) and the mammalian target of rapamycin (mTOR) [2,3]. There is also strong evidence of amino acid control of S6K1 through a mechanism involving the mTOR signaling pathway. This issue has been the subject of recent studies [4,5] but the underlying mechanisms remain to be elucidated. We have recently shown that S6K1 is expressed in chicken muscle and demonstrated its activation following refeeding and insulin treatment in vivo [6]. S6K1 stimulation was observed despite the relative resistance of chicken muscle to exogenous insulin [7] and the lack of a refeeding-related response of chicken muscle PI3-kinase [8].

The availability of an avian muscle cell line would represent a useful tool to clarify the signal transduction pathway involved in S6K1 activation in bird muscle. A stable and permanent myogenic cell line (QM7 cells) has been developed by Antin and Ordahl [9]. QM7 cells were derived from a preexisting quail fibrosarcoma cell line (QT6) cultured from methylcholanthrene-induced pectoralis fibrosarcomas of Japanese quail *Coturnix coturnix japonica*. To our knowledge this is the only avian muscle cell line available thus far. In the present study we showed the absence of insulin receptors on the cell surface of QM7 cells leading us to use this cellular model to investigate the regulation of S6K1 activity in response to amino acids independently of insulin action.

2. Materials and methods

2.1. Materials

All culture media and additives were obtained from Gibco-BRL (Life Technologies). Rabbit polyclonal anti-S6K1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-phospho-Thr389 S6K1 was from Cell Signaling technology (Beverly, MA, USA). The S6K1 assay kit was obtained from Upstate Cell Signaling (Lake Placid, NY, USA). Monocomponent porcine insulin was purchased from Novo Industrie Pharmaceutique (Paris, France). Monoiodinated A-14 human ^{125}I-labeled insulin and L-[U-^{14}C]phenylalanine (450 mCi/mmol) were from Amersham (Les Ulis, France). [γ-^{33}P]ATP was from NEN Life sciences (Zaventem, Belgium).

2.2. Cell culture and treatments

QM7 quail myoblast cells were grown in McCoy medium supplemented with 10% fetal calf serum and 1% chicken serum to 90–100% confluence, fasted 24 h in serum-free medium, washed once with amino acid-deprived medium (Earle's balanced salt solution containing MEM vitamins and 2 g/l glucose), and incubated in the same medium for 2 h. Cells were then incubated in fresh medium containing various concentrations of amino acids as indicated in each experiment. The 1×concentration of amino acids (MEM as reference) was defined as the following (in mM): L-arginine HCl 1.1, L-cysteine 0.2, L-histidine HCl H_2O 0.2, L-isoleucine 0.4, L-leucine 0.4, L-lysine HCl 0.4, L-methionine 0.1, L-phenylalanine 0.2, L-threonine 0.4, L-tryptophan 0.05, L-tyrosine 0.2, L-valine 0.4, L-alanine 0.2, L-asparagine 0.2, L-aspartic acid 0.2, L-glutamic acid 0.2, glycine 0.2, L-proline 0.2, L-serine 0.2, L-glutamine 2.0. NaCl was added as needed to maintain equal osmolarity. In some studies, complete RPMI medium or RPMI media

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Abbreviations: mTOR, mammalian target of rapamycin; PI3-kinase, phosphatidylinositol 3-kinase; QM7, quail muscle cell line; S6K1, p70 S6 kinase

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without methionine or leucine were used instead of the above-mentioned media.

2.3. Western blotting

At the end of incubation, QM7 myoblasts were prepared as previously described [10]. S6K1 activation was determined by gel mobility shift assays. The phosphorylated active form of the kinase is determined as a band with lower mobility on SDS–PAGE and the unphosphorylated form, which corresponds to the inactive form, as a band with higher mobility. Aliquots of supernatant (10 μg protein, assessed using a Bio-Rad kit) were separated on 7.5% SDS–PAGE and Western blotting as previously described [6]. For S6K1 phosphorylation on Thr389, 20 μg of QM7 proteins were separated on 10% SDS–PAGE and Western blotting using an anti-phospho-Thr389 S6K1 polyclonal antibody according to the manufacturer's procedure. The antibody also detects a band of 85 kDa corresponding to the nuclear S6K1 isoform (p85S6K1). Bands were visualized by enhanced chemiluminescence and quantified by Scion Image software. The S6K1 phosphorylation intensity was normalized for S6K1 content determined using a rabbit anti-S6K1 polyclonal antibody.

2.4. S6K1 assay

S6K1 activity was measured by immune kinase assay according to the procedure in the S6K1 assay kit. Briefly, QM7 lysates were immunoprecipitated with anti-S6K1 antibody and the specific enzyme activity of the protein was measured by estimating the phosphorylation of an artificial substrate (AKRRRLSSLRA) corresponding to an 11 amino acid sequence of the ribosomal protein S6 in the presence of labeled ATP.

2.5. Insulin binding to chicken liver and QM7 cell membranes

A liver sample taken from 3-week-old chickens was used as reference. After the chicken was killed, the liver sample was immediately frozen, ground in liquid nitrogen and stored at −80°C. The crude liver membranes were prepared by differential centrifugation as previously described [11]. For QM7 crude membrane preparation, confluent cells were extensively washed in ice-cold phosphate-buffered saline (PBS) and frozen for 24 h at −80°C; cells were then scraped from the culture dishes in the presence of cold membrane buffer containing protease and phosphatase inhibitors. The resulting mixture was treated as previously described for liver membrane preparation. Insulin binding was determined as previously described [11].

2.6. Protein synthesis

In order to measure protein synthesis in QM7 myoblasts, L-[U-^{14}C]-phenylalanine (0.25 μCi/ml) was added to the medium during the stimulation period. At the end of incubation (60 min), cells were washed once in ice-cold PBS, and then in ice-cold 10% trichloroacetic acid (TCA). TCA-insoluble material was washed three times with 10% TCA and solubilized in 0.5 M NaOH at 37°C for determination of protein (BCA, Pierce) and radioactivity incorporated into QM7 protein. Protein synthesis was expressed as nmol phenylalanine incorporated per mg protein/h [12].

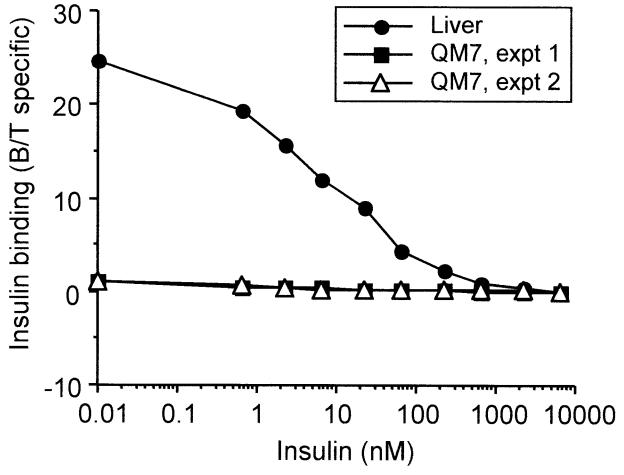

Fig. 1. ^{125}I-labeled insulin binding to chicken liver and QM7 cell membranes. Specific ^{125}I-labeled insulin binding to liver and QM7 cell membranes was inhibited by increasing concentrations of unlabeled insulin, using the same membrane protein concentration (0.4 mg/ml, final concentration). The specific binding of labeled insulin (B) is expressed as the percentage of total (T). Data were obtained with QM7 cells originating from two independent experiments and triplicate determinations were performed per curve point.

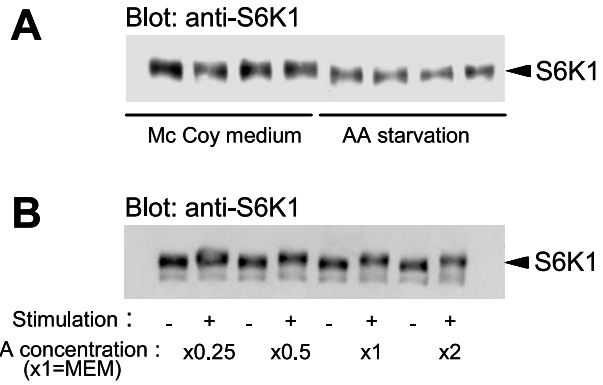

Fig. 2. Effects of amino acid deprivation and addition on S6K1 phosphorylation. A: Representative S6K1 gel mobility shift assay. QM7 cells were cultured in McCoy medium, fasted for 7 h, and subjected to amino acid deprivation for 1 h. B: Representative S6K1 gel mobility shift assay (of three independent experiments). QM7 cells were cultured in McCoy medium, fasted for 24 h, and subjected to amino acid deprivation for 2 h. The culture medium was replaced with amino acid-free medium or amino acid mixtures ranging in concentration from 0.25 to 2× (MEM as reference) for 30 min.

2.7. Statistical analysis

Statistical analysis was performed using analysis of variance (Statview Software program, version 5) to detect significant intergroup differences. Values are expressed as means ± S.E.M., and $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Insulin binding to QM7 membranes

The presence of insulin receptors in QM7 cells was investigated using iodinated insulin. Specific ^{125}I-labeled insulin binding to QM7 cell membranes and to liver membranes (control) was measured following 20 h incubation at 4°C. Fig. 1 indicates the absence of specific insulin binding to QM7 cell membranes. Similar results were obtained when insulin binding to QM7 cells was estimated on intact monolayer cells (data not shown). These findings reveal that the QM7 cell line is lacking insulin receptors.

3.2. Effects of amino acids on S6K1 phosphorylation

To study the impact of amino acid availability, QM7 cells were transferred to McCoy medium without serum for 7 h, then subjected to amino acid deprivation for 1 h. Amino acid deprivation reduced the phosphorylation of S6K1 as shown by the higher mobility of the protein on SDS–PAGE as compared to cells cultured in medium containing amino acids (Fig. 2A). This effect was reversed by re-supplying amino acids for 30 min, with a clear decrease in the electrophoretic mobility of S6K1 indicative of phosphorylation of the protein (Fig. 2B). In addition, we found that the S6K1 band was shifted for the lower amino acid concentrations (0.25 and 0.5×) corresponding to activation of the enzyme at physiological concentrations.

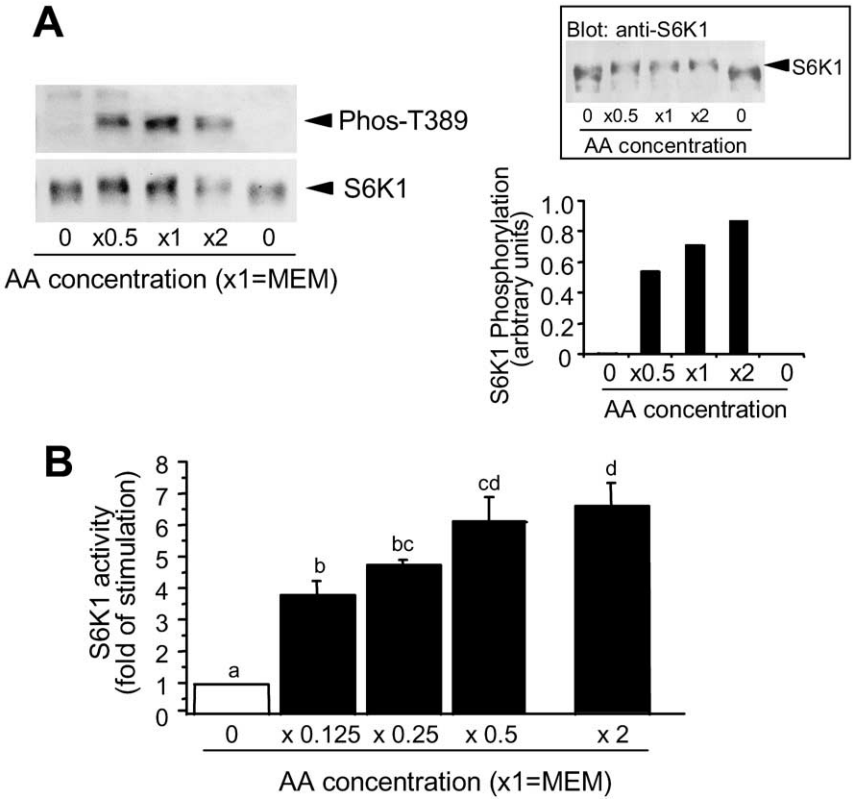

Fig. 3. Effects of amino acid concentration on S6K1 phosphorylation and activity. QM7 cells were cultured in McCoy medium, fasted for 24 h, and subjected to amino acid deprivation for 2 h. The culture medium was replaced with amino acid-free medium or amino acid mixtures ranging in concentration from 0.125 to 2× (MEM as reference) for 30 min. A: S6K1 phosphorylation on Thr389. Results of a representative S6K1 gel mobility shift assay are shown in the inset. B: S6K1 immune complex assay. Data are means ± S.E.M. ($n = 6$–7) from four independent experiments and are expressed as fold stimulation above basal level (amino acid-free medium). $^{a-d}$Means not sharing the same letter are significantly different ($P < 0.05$).

3.3. *Effects of amino acid concentration on Thr389 phosphorylation and S6K1 activity*

We next investigated the effects of amino acid concentration on S6K1 phosphorylation on residue Thr389. As shown in Fig. 3A, the mobility shift induced by amino acids was clearly associated with an increase in Thr389 phosphorylation level. Amino acids also increased S6K1 activity, in a dose-dependent manner (Fig. 3B). A significant increase was already observed with the lower amino acid concentrations (0.125×). The phosphorylation and activation of S6K1 induced by amino acids is in good agreement with data obtained in mammals [13–17]. The positive correlation between S6K1 activity and its phosphorylation on residue Thr389 is also consistent with the assumption that the phosphorylation of Thr389 is necessary for S6K1 activation [18,19].

3.4. *Effects of LY294002 and rapamycin on amino acid-induced S6K1 activation*

To explore the signaling pathway involved in amino acid-induced S6K1 activation, QM7 cells were incubated with or without treatment with 50 μM LY294002 (PI3-kinase inhibitor) or 100 nM rapamycin (specific mTOR inhibitor) for 1 h. Gel mobility shift assays showed that the increased phosphorylation of S6K1 by amino acids was blocked by both LY294002 and rapamycin (Fig. 4A). Similarly, the inhibitors completely abolished the amino acid-stimulated S6K1 activity (Fig. 4B). Our results therefore indicate that the rapamycin-sensitive pathway involving an avian homolog of mTOR is required. The present study also demonstrates an inhibitory effect of LY294002 in QM7 cells. It is possible that, at the concentration used in this study, LY294002 inhibits mTOR [20]. However, numerous papers have reported that both mTOR and other members of the PI3-kinase family are involved in amino acid-induced activation of S6K1 in mammalian cells [13,15,16,21,22]. Therefore, the activation of S6K1 implies mTOR and/or PI3-kinase pathways in QM7 cells.

3.5. *Effects of methionine and leucine availability on S6K1 phosphorylation and on protein synthesis assessed by phenylalanine incorporation*

The impact of amino acid composition on S6K1 activation was further investigated by incubating QM7 cells for 2 h in RPMI medium lacking methionine or leucine followed by the addition of the deprived amino acids for 1 h. Methionine or leucine deprivation caused a decrease in S6K1 phosphorylation on Thr389 compared to control cells (−35 and −50% for methionine and leucine deprivation, respectively: $P < 0.05$; Fig. 5A). The supply of the lacking amino acid restored phosphorylation status to the level observed in control cells. In cells deprived of leucine to which the deprived amino acid was returned at half concentration, phosphorylation status was also significantly increased and reached a level similar to controls. Thus, S6K1 was activated by leucine and methionine in avian species as in mammals [22–25]. In addition,

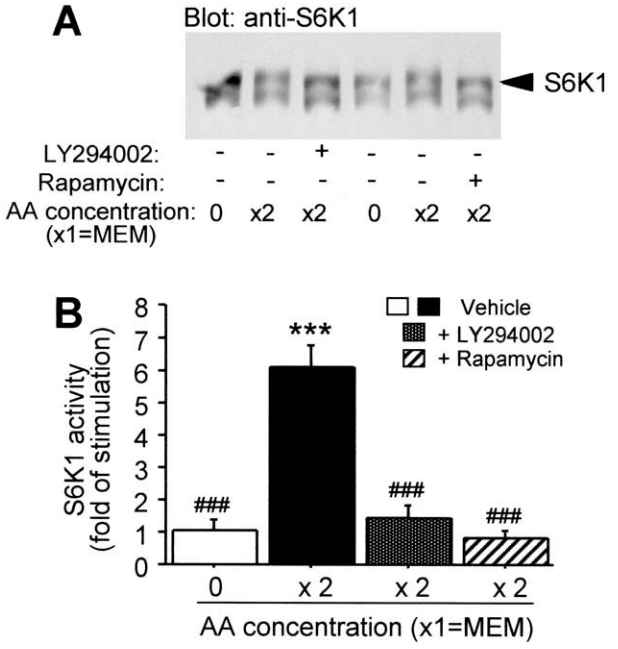

Fig. 4. Effects of LY294002 and rapamycin on amino acid-induced S6K1 phosphorylation and activity. QM7 cells were cultured in McCoy medium, fasted for 24 h, and subjected to amino acid deprivation for 2 h. During the last 30 min of preincubation, LY294002 (50 μM) or rapamycin (100 nM) was added to cells. The culture medium was replaced with amino acid-free medium or an amino acid mixture (2×, MEM as reference) with or without inhibitors as indicated for 30 min. Controls with the diluent of the inhibitors (dimethyl sulfoxide, final concentration 0.01–0.1%) were performed for these experiments. A: Representative S6K1 gel mobility shift assay (of three independent experiments). B: S6K1 immune complex assay. Data are means ± S.E.M. ($n = 4$–6) from three independent experiments and are expressed as fold stimulation above basal level (amino acid-free medium). $^{***}P < 0.001$ vs. basal (amino acid-free medium); $^{\#\#\#}P < 0.001$ vs. maximum stimulation (2× amino acid mixture).

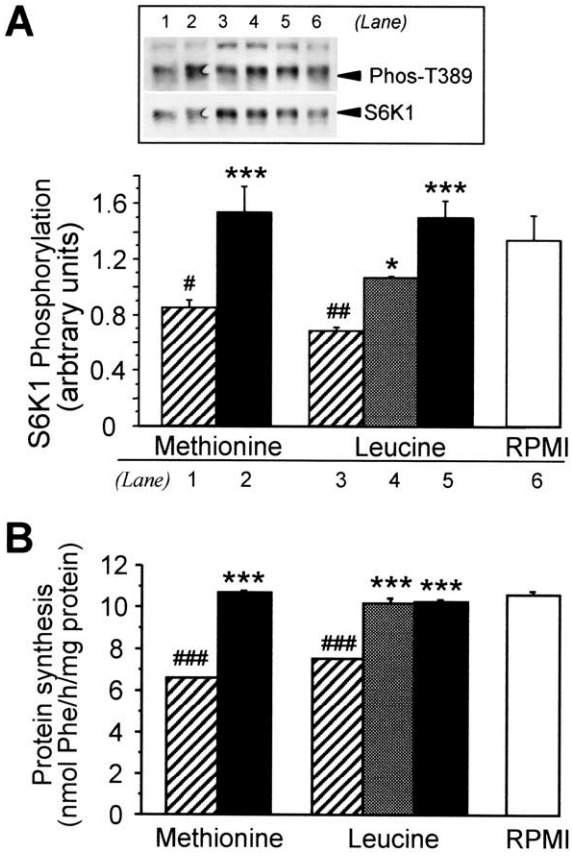

Fig. 5. Effects of methionine and leucine availability on S6K1 phosphorylation and phenylalanine (Phe) incorporation. QM7 cells were cultured in McCoy medium, fasted for 24 h, and subjected to single amino acid deprivation for 2 h (RPMI media without methionine or leucine). A: S6K1 phosphorylation on Thr389. The culture medium was replaced with RPMI media without methionine or leucine, or with complete RPMI medium for 60 min. Results of a representative blot are shown in the inset. In each panel, hatched bars represent cells deprived of methionine or leucine as indicated in the figure; black bars, cells deprived of methionine or leucine to which the deprived amino acid was returned; gray bar, cells deprived of leucine to which the deprived amino acid was returned at half concentration; white bar, cells maintained in serum-free RPMI as control (complete mixture of amino acids). Values represent means ± S.E.M. ($n = 3$). $^{*}P < 0.05$, $^{***}P < 0.001$ vs. basal level (RPMI media without methionine or leucine); $^{\#}P < 0.01$, $^{\#\#}P < 0.01$ vs. control cells maintained in serum-free RPMI. B: Phenylalanine incorporation. The culture medium was replaced with RPMI media without methionine or leucine, or with complete RPMI medium, and supplemented with L-[^{14}C]phenylalanine. Protein synthesis was determined as described in Section 2. Means ± S.E.M. ($n = 3$). $^{***}P < 0.001$ vs. basal level (RPMI media without methionine or leucine); $^{\#\#\#}P < 0.001$ vs. control cells maintained in serum-free RPMI.

methionine and leucine availability concomitantly regulated QM7 protein synthesis (Fig. 5B). Deprivation of methionine or leucine reduced protein synthesis compared to control cells (approximately −40 and −30% for methionine and leucine deprivation, respectively, $P < 0.001$) whereas their incorporation into culture media returned protein synthesis to control values. Taken together, our results demonstrate the role of leucine and methionine in S6K1 activation and in mediating stimulation in protein synthesis in QM7 myoblasts. Interestingly, the QM7 cell line is highly sensitive to amino acid availability and changes in culture media.

In conclusion, S6K1 is expressed in the quail muscle (QM7) cell line. This kinase is regulated by amino acid availability through signal transduction pathways involving mTOR and/or PI3-kinase independently of insulin. Additional studies on mTOR/PI3-kinase signaling pathways in QM7 cells are needed and may contribute to the understanding of the potential insulin resistance observed in chicken muscle.

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