L-Leucine availability regulates phosphatidylinositol 3-kinase, p70 S6 kinase and glycogen synthase kinase-3 activity in L6 muscle cells: evidence for the involvement of the mammalian target of rapamycin (mTOR) pathway in the L-leucine-induced up-regulation of System A amino acid transport

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Amino acid availability is known to regulate diverse cell processes including the activation of p70 S6 kinase, initiation factors involved in mRNA translation, gene expression and cellular amino acid uptake. Essential amino acids, in particular the branched-chain amino acids (e.g. leucine), have been shown to be the dominant players in mediating these effects, although the precise nature by which they regulate these processes remain poorly understood. In this study we have investigated the mechanisms involved in the leucine-induced modulation of p70 S6 kinase and addressed whether this kinase participates in the up-regulation of the System A amino acid transporter in L6 muscle cells. Incubation of muscle cells that had been amino acid-deprived for 1 h with L-leucine (2 mM) led to a rapid (> 2-fold) activation of p70 S6 kinase, which was suppressed by both wortmannin and rapamycin. Consistent with this finding, addition of leucine caused a rapid ($\approx$ 5-fold) but transient stimulation of phosphatidylinositol 3-kinase (PI3K). PI3K activation was inhibited by wortmannin and was not dependent upon insulin receptor substrate-1 activation. Unlike stimulation by insulin, activation of neither protein kinase B nor p42/p44 mitogen-activated protein kinase accompanied the leucine-induced stimulation of PI3K. However, the leucine-induced activation of PI3K and p70 S6 kinase did result in the concomitant inactivation of glycogen synthase kinase-3 (GSK-3). Leucine enhanced System A transport by $\approx$ 50%. We have shown previously that this stimulation is protein-synthesis-dependent and in the current study we show that it was blocked by both wortmannin and rapamycin. Our findings indicate that PI3K and the mammalian target of rapamycin are components of a nutrient signalling pathway that regulates the activation of p70 S6 kinase and induction of System A in L6 cells. The activation of this pathway by leucine is also responsible for the inactivation of GSK-3, and this is likely to have important regulatory implications for translation initiation.

Key words: branched-chain amino acid, 4E-BP1, insulin, PKB/Akt, protein synthesis.

INTRODUCTION

It is well established that amino acids, in particular the branched-chain amino acids (BCAAs; leucine, isoleucine and valine), can exert powerful regulatory influences upon carbohydrate metabolism [1], amino acid transport [2], protein turnover [3,4] and gene expression [5]. How these diverse cell processes are regulated by amino acid availability remains poorly understood. However, there is now growing evidence that amino acids *per se* can act as initiators of cellular signalling, and that they may be important in the control of key cellular functions. A number of recent studies [6–11] have shown, for example, that resupply of amino acids following a period of amino acid deprivation can stimulate the activity of p70 S6 kinase, a serine/threonine kinase whose ability to phosphorylate ribosomal protein S6 is considered important in the control of mRNA translation [12]. The amino acid-dependent regulation of p70 S6 kinase is also sensitive to the macrolide rapamycin [8–10]. This immunosuppressant drug acts by forming an inhibitory complex with a 12-kDa cytosolic binding protein (FK506-binding protein, or FKBP), which targets a large-molecular-mass protein kinase called mammalian target of rapamycin (mTOR, also known as FRAP), which is

therefore likely to be upstream of p70 S6 kinase and also subject to regulation by amino acids.

A central issue that remains unresolved concerns the mechanism by which amino acids, particularly those belonging to the BCAA family, stimulate the mTOR/p70 S6 kinase pathway. However, like insulin, activation of p70 S6 kinase by amino acids can be suppressed by the phosphatidylinositol 3-kinase (PI3K) inhibitor, wortmannin, in some cell types [8,9]. This finding implies that molecules participating in proximal insulin signalling may also be activated by amino acids. One of the consequences of PI3K activation by hormonal and growth-factor stimuli is the downstream activation of protein kinase B (PKB)/Akt. PKB activation results in the phosphorylation and inactivation of glycogen synthase kinase-3 (GSK-3). The inactivation of GSK-3 is considered crucial for the hormonal activation of glycogen synthase [13] and dephosphorylation of the guanine nucleotide-exchange factor, eukaryotic initiation factor 2B (eIF2B) [14]. The hormonal activation of PKB has also been implicated in the insulin-induced phosphorylation of mTOR. This suggestion is based on the finding that mTOR possesses consensus PKB phosphorylation sites in its C-terminal region, and that expression of a conditionally active PKB results in mTOR activation [15].

Indeed, very recent work has shown that PKB can phosphorylate mTOR directly on Ser[2448] *in vitro* and that this site becomes phosphorylated *in vivo* in response to insulin, in a wortmannin-sensitive fashion [16]. Interestingly, cellular amino acid deprivation reduces insulin-mediated phosphorylation of Ser[2448], suggesting that amino acids may play a regulatory role in the activation of mTOR by PKB [16].

We have shown previously that BCAAs (and leucine in particular) can stimulate the activity of the short-chain neutral amino acid transporter, System A, in L6 muscle cells [2]. The mechanism by which this stimulation takes place remains poorly understood, but it is dependent upon protein synthesis. We hypothesized that since amino acids, including those belonging to the BCAA family, have been implicated in the regulation of protein synthesis, one mechanism by which leucine may enhance System A activity is via PKB and/or the mTOR/p70 S6 kinase pathway. In this study we show that leucine induces a rapid and transient activation of PI3K and p70 S6 kinase, but not of PKB, and that this activation underpins the ability of the amino acid to induce an increase in System A activity. We also provide novel evidence that activation of the mTOR pathway results in the associated inactivation of GSK-3.

EXPERIMENTAL PROCEDURES

Materials

Culture media (α-minimal essential medium, α-MEM), foetal calf serum and antimycotic/antibiotic solution were obtained from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). Wortmannin, insulin, rapamycin and Kodak X-Omat film were purchased from Sigma-Aldrich (Poole, Dorset, U.K.). Phospho-specific antibodies to p42/p44 mitogen-activated protein (MAP) kinases were obtained from New England Biolabs (Hitchin, Herts., UK). Horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Scottish Antibody Production Unit (Carluke, Lanarkshire, Scotland, U.K.). Reagents for enhanced chemiluminesence (ECL) were purchased from Pierce & Warriner (Chester, Cheshire, U.K.). Hybond nitrocellulose membranes, and Protein A– and Protein G–Sepharose were obtained from Amersham Life Sciences (Little Chalfont, Bucks., UK). [γ-^{32}P]ATP was purchased from ICN (Costa Mensa, CA, U.S.A.). Antibodies against GSK-3α, PKBα and p70 S6 kinase (for immunoprecipitation) and peptide substrates for these kinases were generously provided by Professor Sir Philip Cohen (MRC Protein Phosphorylation Unit, University of Dundee, Dundee, Scotland, U.K.) and Professor Chris Proud (Department of Anatomy and Physiology, University of Dundee). Antibodies against insulin receptor substrate-1 (IRS-1), anti-phosphotyrosine (anti-PY) and the p85 subunit of PI3K were purchased from UBI (Lake Placid, NY, U.S.A.).

Cell culture

Monolayers of L6 muscle cells were cultured to the stage of myotubes as described previously [17–19] in α-MEM containing 2% (v/v) foetal calf serum and antimycotic/antibiotic solution (100 unit/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B) at 37 °C in an atmosphere of 5% CO_2/95% air. Cells were cultured in 10-cm-diameter dishes for analysis of kinase activities. Upon formation of myotubes, cells were deprived of serum by incubation in serum-free α-MEM for 4 h followed by a 1-h amino acid-deprivation period in Hepes-buffered saline (HBS, 20 mM Hepes/Na, pH 7.4, 140 mM NaCl, 2.5 mM $MgSO_4$, 5 mM KCl and 1 mM $CaCl_2$). Subsequent additions (e.g. insulin, amino acids or inhibitors) to the cells were

made at the times and at the concentrations indicated in the Figure legends.

Analysis of p70 S6 kinase, PKB and GSK-3 activity in L6 lysates

L6 cells were extracted from 10 cm dishes using ice-cold lysis buffer [50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X-100, 1 mM Na_3VO_4, 10 mM sodium β-glycerophosphate, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 μM micro-cystin-LR, 0.27 M sucrose, 0.2 mM PMSF, 1 mM benzamidine, 10 μg/ml leupeptin and 0.1% (v/v) 2-mercaptoethanol]. p70 S6 kinase was immunoprecipitated from 50 μg of cell lysate and assayed by monitoring phosphorylation of a 32-amino acid-long S6 peptide (KEAKEKRQEQIAKKRRLSSLRASTSKSE-SSQK). PKBα was immunoprecipitated from 100 μg of L6 lysate using an isoform-specific antibody and its activity assayed using 'crosstide', a synthetic peptide (GRPRTSSFAEG) corresponding to the GSK-3 sequence surrounding the Ser residue phosphorylated by MAP kinase-activated protein kinase 1 (MAPKAP-K1) [13]. GSK-3α was immunoprecipitated from 100 μg of cell lysate and incubated with or without 25 m-unit/ml protein phosphatase 2A$_1$ (Promega, Southampton, Hants., U.K.), prior to assay using phospho-GS peptide-1 (where GS is glycogen synthase) [20]. In the present study 1 unit of p70 S6 kinase and PKB activity was defined as the amount that catalysed the phosphorylation of 1 nmol of substrate in 1 min. Protein concentrations were determined using the method of Bradford [21].

Analysis of PI3K activity in L6 myotubes

Following treatment with insulin or leucine, L6 myotubes were extracted from culture dishes using ice-cold lysis buffer (see above). Lysates (200 μg of protein) were incubated with either anti-p85 or anti-PY antibody complexed to Protein G–Sepharose beads for 2 h at 4 °C. Immunoprecipitates were washed and incubated with phosphatidylinositol (0.1 mg/ml) for 20 min at 30 °C, in a buffer containing 50 mM [γ-^{32}P]ATP, 1.2 mM Na_3VO_4, 5 mM $MgCl_2$ and 25 mM Hepes, pH 7.4. The reaction was terminated by adding 20 μl of 8 M HCl and 160 μl of methanol/chloroform (1:1, v/v). After centrifugation at 13 000 g for 5 min at 4 °C, the lower phase was freeze-dried and dissolved in 15 ml of methanol/chloroform (1:1, v/v). The products were separated by TLC on silica gel G-60 plates in chloroform/methanol/4.3 M ammonia (9:7:2). [^{32}P]-Labelled phosphatidyl-inositol 3-phosphate spots were visualized and associated radioactivity was quantified using an Instantimager (Canberra Packard) for electronic autoradiography

IRS-1 immunoprecipitation from L6 lysates

L6 cells were extracted on 10-cm-diameter culture plates in ice-cold lysis buffer. IRS-1 was immunoprecipitated from cell lysates (500 μg of protein) using a C-terminal IRS-1 antibody. Immuno-complexes were captured by incubation with Protein A–Sepharose beads and solubilized in Laemmli buffer prior to SDS/PAGE and immunoblotting using anti-PY or anti-p85 antibodies as described below.

SDS/PAGE and immunoblotting

Cell lysates (30 μg of protein) or immune complexes were subjected to SDS/PAGE and immunoblotting as described previously [19]. Separated proteins were transferred on to nitrocellulose membranes and blocked with Tris-buffered saline (pH 7.0) containing 5% BSA and 0.05% (v/v) Tween-20. Membranes

were probed with anti-phospho p42/p44 MAP kinase (used at a final dilution of 1:1000), anti-p85 (1:100) or anti-PY (1:3000) antibodies. Following primary antibody incubation, membranes were washed then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:1000) or anti-mouse IgG (1:500) as appropriate. Immunoreactive protein bands were visualized by ECL on Kodak X-Omat film.

Measurement of System A amino acid transport

System A activity was assayed by measuring the uptake of methyl-aminoisobutyrate (Me-AIB, a paradigm System A substrate) as described previously [17]. Briefly, L6 myotubes were incubated with 10 μM [^{14}C]Me-AIB (1 μCi/ml) for 10 min. Non-specific tracer binding was determined using [^{3}H]mannitol as an extracellular marker and also by determining cell-associated radioactivity in the presence of a saturating dose of unlabelled Me-AIB (10 mM). Me-AIB uptake was determined by aspirating the radioactive medium rapidly, followed by three cell washes in ice-cold isotonic saline solution (0.9% NaCl, w/v). Muscle cells were lysed in 0.05 M NaOH, and cell-associated radioactivity was determined by liquid scintillation counting. Total cell protein was determined by the Bradford method [21].

Statistical analysis

Statistical analysis for multiple comparisons was performed using one-way analysis of variance (ANOVA) followed by the Newman–Keuls post test. Data analysis was performed using GraphPad Prism software and considered statistically significant at P values of < 0.05.

RESULTS

Leucine stimulates p70 S6 kinase in L6 myotubes

A number of studies have reported that leucine and other members of the BCAA family are the dominant players in the amino acid-induced regulation of p70 S6 kinase [8,9,22,23]. To determine whether p70 S6 kinase participates in the leucine-induced activation of System A, it was necessary to first establish that this amino acid was capable of activating the kinase in L6 myotubes. Muscle cells that had been maintained in serum-free α-MEM containing a full complement of amino acids for 5 h were subsequently incubated for 10 min with 2 mM L-leucine, a concentration that we have shown previously to exert a maximal stimulatory effect on System A transport [2]. This treatment resulted in a modest, but significant, stimulation (40%) of p70 S6 kinase activity (basal activity was 18.8 ± 0.8 m-unit/mg of protein, mean $\pm$ S.E.M.). However, since L6 cells had been incubated in media containing a complete amino acid mixture we postulated that this may have contributed to a rise in the basal activity of the kinase and reduced the stimulation seen in response to leucine. To test this possibility we assayed p70 S6 kinase in muscle cells that were subjected to a brief (1 h) amino acid-deprivation period prior to incubation with 2 mM L-leucine for periods up to 20 min. Under these conditions the basal p70 S6 kinase activity was significantly lower (7.1 ± 0.3 m-unit/mg of protein) and the kinase was activated by $\approx$ 2-fold upon addition of leucine to the incubation medium (Figure 1a). This stimulation was maximal within 5–10 min of incubating the cells with leucine and was comparable in magnitude with that seen in response to a 10-min insulin challenge, but fell rapidly to basal values within 20 min.

Recent work has suggested that amino acid availability influences the insulin-induced phosphorylation of mTOR [16]. It
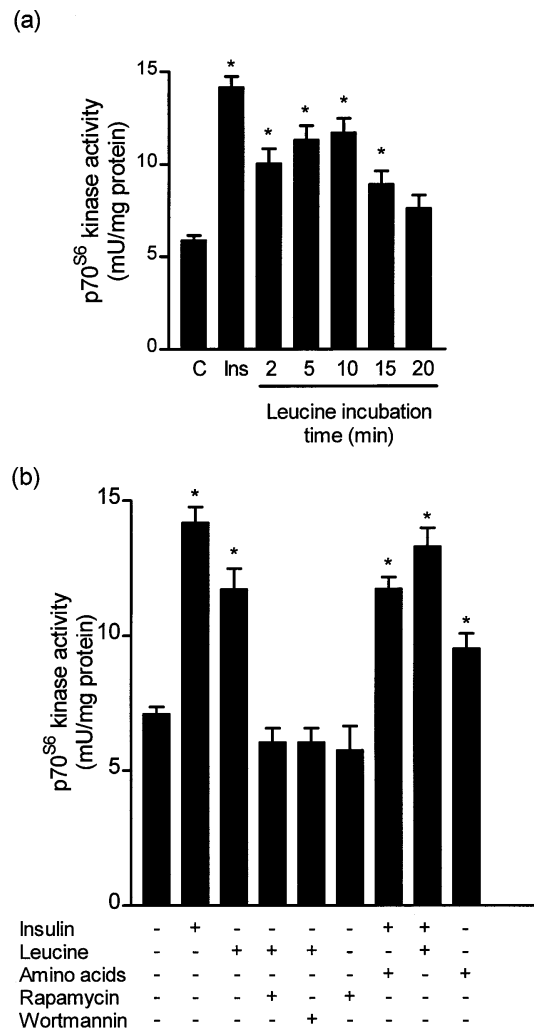
Figure 1   Effect of insulin and L-leucine on p70 S6 kinase activity in L6 cells

(**a**) Cells were amino acid-deprived for 1 h in HBS as described and incubated in the absence or presence of insulin (Ins, 100 nM, 10 min) or L-leucine (2 mM) for the times indicated prior to assaying p70 S6 kinase. (**b**) L6 cells were amino acid-deprived for 1 h in HBS prior to incubation in absence or presence of insulin (100 nM), 2 mM L-leucine or a complete amino acid mixture for 10 min. Cells were exposed to rapamycin (100 nM) and wortmannin (100 nM) during the amino acid-deprivation period for 15 min prior to incubation with insulin, L-leucine or a complete amino acid mixture (composition as in α-MEM), and analysis of p70 S6 kinase activity. Results represent means $\pm$ S.E.M. of 3–5 experiments, each performed in triplicate. Asterisks represent a statistically significant change ($P < 0.05$) from the appropriate untreated control (C).

is thus conceivable that the observed hormonal activation of p70 S6 kinase in our study may have been compromised by the absence of amino acids in the media. We therefore investigated whether treatment of cells with L-leucine or a complete amino acid mixture prior to incubating cells with insulin could further enhance the hormonal activation of p70 S6 kinase. This experimental manipulation did not result in any detectable enhancement in the insulin-mediated stimulation of p70 S6 kinase, suggesting that, in L6 cells, short periods of amino acid deprivation do not limit the hormonal activation of p70 S6 kinase (Figure 1b). It is also noteworthy that these experiments revealed that leucine was just as potent, if not more so, than a complete amino acid mixture in stimulating p70 S6 kinase (Figure 1b). Moreover, consistent with a number of recent studies, the leucine-
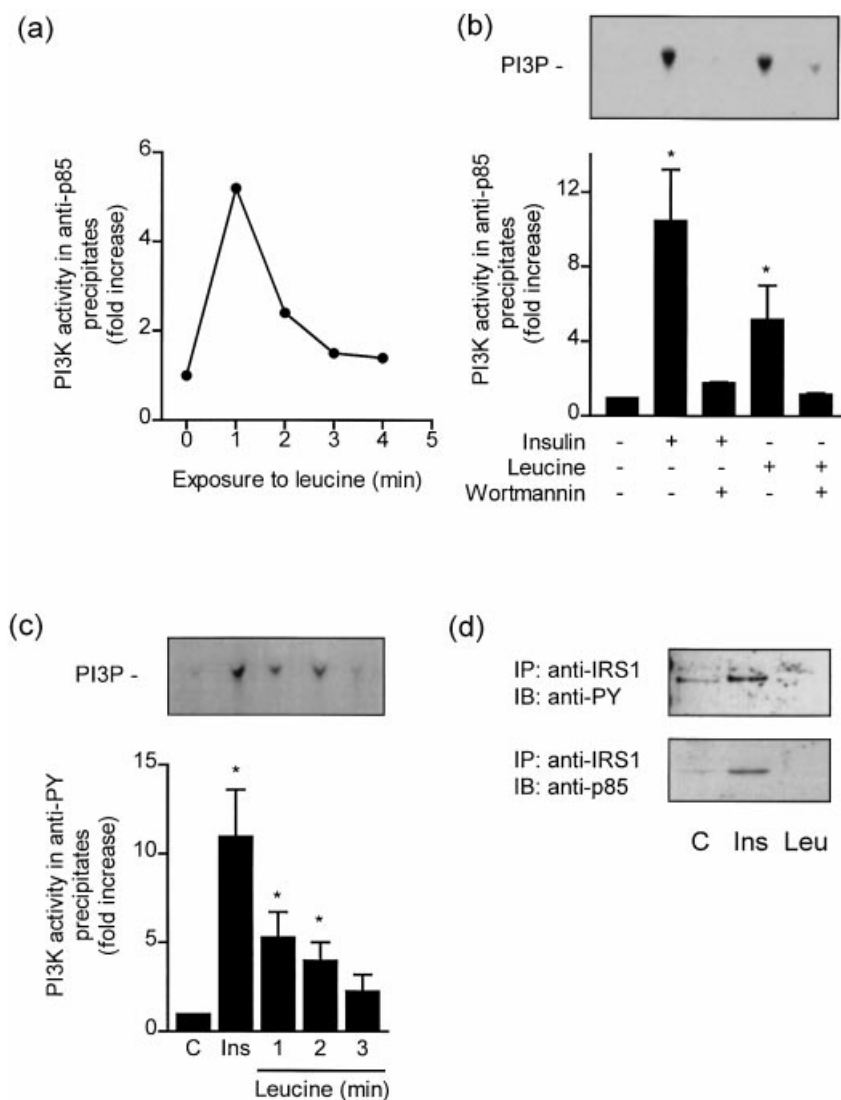
Figure 2   Effects of L-leucine and insulin on PI3K activity, IRS-1 tyrosine phosphorylation and IRS-1/p85 PI3K association

(**a**) L6 cells were amino acid-deprived for 1 h in HBS prior to incubation with L-leucine (2 mM) for the times indicated or (**b**) incubated in the absence or presence of insulin (100 nM, 10 min), L-leucine (2 mM, 1 min) or pre-treated with wortmannin (100 nM, 15 min during the amino acid-deprivation period), prior to assaying PI3K activity in anti-p85 immunoprecipitates assayed as described in the text. (**c**) PI3K activity was also assayed in anti-PY immunoprecipitates following incubation of L6 cells in amino acid-free HBS for 1 h and treatment with either insulin (Ins, 100 nM, 10 min) or L-leucine (2 mM) for the times indicated. Results in (**b**) and (**c**) represent means $\pm$ S.E.M. of three experiments. Asterisks represent a statistically significant change ($P < 0.05$) from the appropriate untreated control. (**d**) L6 cells were incubated in the absence or presence of insulin or L-leucine (Leu; as described for **b**), prior to cell lysis and IRS-1 immunoprecipitation. IRS-1 immunoprecipitates (IP) were immunoblotted (IB) subsequently with anti-p85 or anti-PY antibodies. C, control; PI3P, phosphatidylinositol 3-phosphate.

induced activation of p70 S6 kinase was suppressed by both rapamycin and wortmannin [6–9] (Figure 1b). The above findings implicate both mTOR and PI3K as upstream players in the nutrient-induced regulation of p70 S6 kinase and suggest that the activity of upstream signalling proteins may also be transiently modulated by leucine availability.

Leucine availability regulates PI3-kinase in L6 myotubes

To address whether increased leucine availability was able to activate PI3K in L6 myotubes, we assayed kinase activity in anti-p85 and anti-PY immunoprecipitates. Figure 2 shows that leucine induced a very rapid transitory activation in PI3K activity ($\approx$ 5-fold, as monitored by the generation of phosphatidylinositol 3-phosphate) in both anti-p85 and anti-PY precipitates within

2 min, which fell rapidly thereafter to baseline values (Figures 2a and 2c). The increase in PI3K activity was greater ($\approx$ 10–12-fold) in muscle cells exposed to 100 nM insulin for 10 min (Figures 2b and 2c). The activation seen in response to both insulin and leucine was prevented when muscle cells were pre-incubated with 100 nM wortmannin (Figure 2b). The hormonal activation of PI3K requires the interaction of its p85 subunit with phospho-tyrosine residues on insulin receptor substrates such as IRS-1. We therefore immunoprecipitated IRS-1 from L6 myotubes following incubation with either insulin or leucine, and probed the immune pellet with anti-PY antibodies or anstisera against the p85 subunit. This approach revealed that, whereas insulin promoted tyrosine phosphorylation of IRS-1 and its association with the p85 subunit of PI3K, leucine did not elicit these responses (Figure 2d). This latter finding signified that the
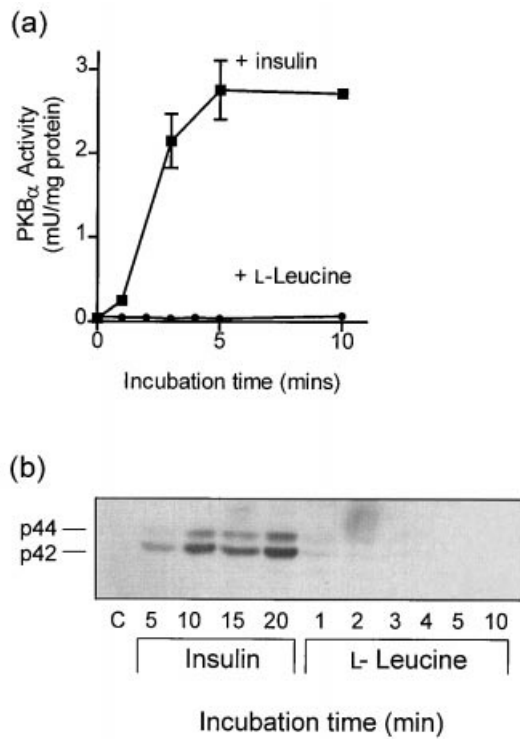
Figure 3 Insulin, but not leucine, stimulates PKB and p42/p44 MAP kinases in L6 cells

L6 myotubes were amino acid-deprived for 1 h in HBS as described in the text and cell lysates were prepared following cell incubation with insulin (100 nM for up 20 min) or with L-leucine (2 mM for up to 10 min) and used subsequently for analysis of (**a**) *in vitro* PKBα activity and (**b**) immunoblotting with phospho-specific antibodies to p42/44 MAP kinases. The blot is representative of data from three experiments. C, control.

Figure 4 Effects of wortmannin and rapamycin on leucine-induced inactivation of GSK-3 in L6 muscle cells

(**a**) L6 myotubes were amino acid-deprived for 1 h in HBS as described in the text and incubated in the absence or presence of insulin (Ins, 100 nM, 10 min), D- or L-leucine (Leu, 2 mM, for the times indicated) with or without wortmannin (WM, 100 nM, 15 min during the amino acid-deprivation period before incubation with insulin or leucine), prior to cell lysis and analysis of GSK-3 activity. (**b**) L6 cells were incubated in the absence or presence of insulin (100 nM, 10 min), L-leucine (2 mM, 3 min) with or without rapamycin (100 nM, 15 min before stimulation with insulin or leucine), prior to cell lysis and analysis of GSK-3 activity. Data are the means ± S.E.M. from 3–5 experiments. Asterisks represent a statistically significant change ($P < 0.05$) from the appropriate untreated control. PP2A1, protein phosphatase 2A₁.

leucine-induced activation of PI3K in anti-PY precipitates was not due to its association with IRS-1, but most probably involves recruitment of the kinase to some other tyrosine-phosphorylated signalling complex.

Leucine inactivates GSK-3 independently of PKB

Based on our finding that increased leucine availability caused a transient activation of PI3K, we next examined whether there was a synchronous activation of PKB in L6 cells incubated with the amino acid. PKBα was immunoprecipitated from cell lysates following treatment of amino acid-deprived muscle cells with either 100 nM insulin or 2 mM leucine for periods up to 10 min, and its activity assayed using crosstide. Figure 3(a) shows that insulin promoted a rapid activation of PKBα, which was maximal within 5 min. However, despite the transient activation in PI3K that was elicited by leucine (Figure 2a), the amino acid caused no detectable activation of PKBα (Figure 3a). Leucine also failed to activate PKBβ and PKBγ (results not shown) and, unlike insulin, did not induce the phosphorylation of p42 and p44 MAP kinases (Figure 3b). In separate experiments we were able to establish that amino acid deprivation for 1 h, or cell incubation with leucine for times that stimulated p70 S6 kinase, did not result in the activation of stress-activated protein kinase (SAPK)2/p38 MAP kinase or c-Jun N-terminal kinase (JNK; results not shown). This latter finding indicates that a short period of amino acid deprivation does not induce a cellular stress response, and
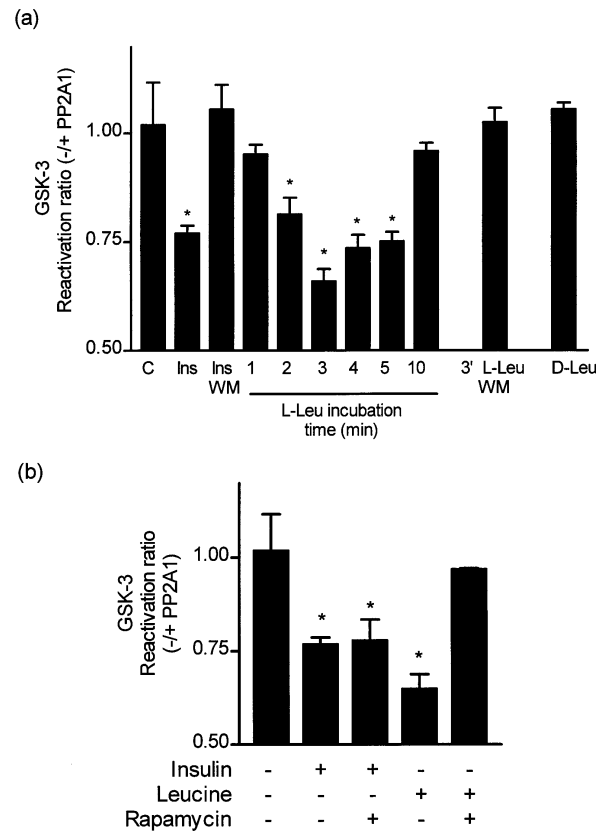
that these stress kinases do not participate in the activation of p70 S6 kinase.

GSK-3, in addition to phosphorylating and inhibiting glycogen synthase, also mediates the phosphorylation and inactivation of eIF2B, a guanine nucleotide-exchange factor that plays an important role in the initiation of mRNA translation [14]. Since leucine activated p70 S6 kinase and has been shown to promote protein synthesis in L6 cells [2], we assessed whether incubation of muscle cells with leucine modulated GSK-3 activity. Figure 4(a) shows that when GSK-3α was immunoprecipitated from cell lysates following incubation of amino acid-deprived cells with leucine for periods of up to 10 min, we observed a significant inactivation of GSK-3, which was maximal within the first 5 min. The inhibition of GSK-3 was comparable with that elicited by a 10-min incubation with insulin (Figure 4a). GSK-3 inactivation was not apparent in muscle cells that had been incubated with leucine for periods in excess of 10 min. Importantly, both the hormonal and leucine-induced inactivation of GSK-3α was lost when muscle cells were preincubated with 100 nM wortmannin, and the inactivation did not take place if D-leucine was substituted for L-leucine (Figure 4a). Since supply of L-leucine induces a
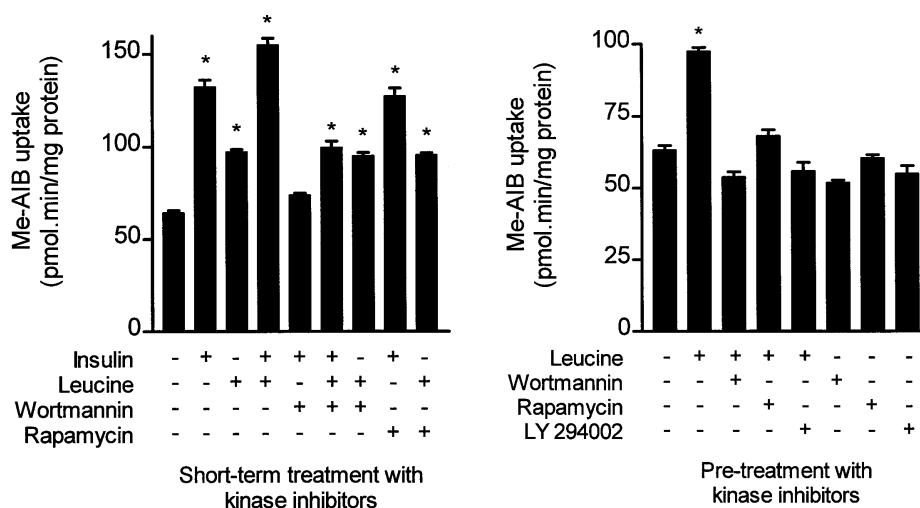
Figure 5   Effects of wortmannin and rapamycin on insulin- and L-leucine-stimulated System A amino acid transport in L6 cells

Left-hand panel: L6 myotubes were incubated in the absence or presence of insulin (100 nM, 30 min) and/or L-leucine (2 mM, 3 h). Cells were exposed to wortmannin (100 nM) or rapamycin (100 nM) for 45 min (i.e. 15 min prior to incubation with insulin, but 2 h 15 min following treatment with L-leucine). Right-hand panel: L6 cells were incubated in the absence or presence of 2 mM L-leucine for 3 h. Wortmannin (100 nM), LY 294002 (50 μM) or rapamycin (100 nM) were added 15 min prior to initiating the L-leucine incubation. Results represent the means $\pm$ S.E.M. of between 3 and 11 experiments, each performed in triplicate. Asterisks represent a statistically significant change ($P < 0.05$) from the appropriate untreated control.

rapid activation in p70 S6 kinase, we postulated that this kinase may participate in the leucine-induced inactivation of GSK-3. Figure 4(b) shows that when L6 cells were preincubated with rapamycin, the ability of leucine to inactivate GSK-3 was completely abolished. However, in line with previous work performed in L6 cells, the inhibitor failed to suppress the insulin-induced inactivation of GSK-3 [20].

Leucine induces System A in a PI3K- and p70 S6 kinase-dependent fashion

To establish whether the leucine-induced activation of PI3K and p70 S6 kinase were involved in the induction of System A transport, the effects of inhibitors of these kinases on the ability of leucine to stimulate System A activity were studied. When L6 cells were incubated with 100 nM insulin for 30 min, or with 2 mM leucine for 3 h, we observed the characteristic stimulation in System A transport reported previously [2,24]. When the two stimuli were combined there was a near additive stimulation in Me-AIB uptake (Figure 5, left-hand panel). Incubating L6 cells with wortmannin, but not with rapamycin, prior to an acute (30 min) insulin challenge suppressed completely that component of System A transport activated by the hormone (Figure 5, left-hand panel). Since it was necessary to incubate L6 cells with leucine for up to 3 h to observe the stimulation of System A transport, neither inhibitor exerted an inhibitory effect on this stimulation when presented to cells after incubation with leucine (Figure 5, left-hand panel). However, when muscle cells were incubated with either wortmannin or LY 294002 (two structurally unrelated PI3K inhibitors), or with rapamycin prior to a 3-h incubation with 2 mM leucine, the leucine-induced up-regulation of System A transport was abolished (Figure 5, right-hand panel).

DISCUSSION

In this study we show that leucine, a member of the BCAA family, can exert rapid stimulatory effects on PI3K and p70 S6

kinase, which culminate in the activation of the System A amino acid transporter. The finding that PI3K is activated in L6 cells by leucine is consistent with a number of recent studies, showing that the leucine-induced activation of p70 S6 kinase, or phosphorylation of the translational repressor molecule 4E-BP1, are blocked by the PI3K inhibitor wortmannin [7,9]. However, some investigators have failed to detect any amino acid-induced changes in PI3K activity, despite showing that the stimulatory effects of amino acids on p70 S6 kinase were sensitive to wortmannin [8]. This discrepancy may be explained partly by differences in experimental design. Our data indicate that leucine induces a rapid activation of PI3K that is very short-lived, lasting no longer than a few minutes. However, in some previous studies PI3K activity (in anti-PY and anti-p85 immunoprecipitates) was assayed following a 30-min cell incubation with amino acids [8]. It is possible, therefore, that activation of PI3K may have been overlooked in these studies, whereas any transient stimulation of the kinase would have been blocked by pre-treatment of cells with wortmannin, with a resulting loss in p70 S6 kinase activation.

The transient stimulation that we observe in PI3K appears insufficient to elicit the activation of PKB. This finding is in agreement with the work of Kimball et al. [23], who were also unable to detect any leucine-induced phosphorylation of the Ser[473] residue of PKB in L6 myoblasts. The suggestion that activation of PI3K is not necessarily accompanied by an increase in PKB activity is not unprecedented. One recent study has shown, for example, that insulin-like growth factor-1 (IGF-1), oxidative stress and osmotic stress all stimulate PI3K, but exert differential effects on PKB activation in Swiss 3T3 cells [25]. IGF-1 and osmotic stress both promote the near equivalent accumulation of phosphatidylinositol 3,4,5-trisphosphate, whereas oxidative stress specifically induces the generation of phosphatidylinositol 3,4-bisphosphate. However, of the three stimuli, only IGF-1 and oxidative stress activated PKB [25]. These findings suggest that, while generation of 3-phosphoinositides is necessary for PKB activation, their production alone may not be sufficient for activation of the kinase. It is plausible that the lack of PKB activation seen in response to leucine in our

studies may, in part, be due to the production of insufficient amounts of the appropriate phosphoinositides required for PKB activation [26], or that the lipids are produced in a cellular compartment that is inaccessible to PKB.

Recent work has suggested that PKB participates in the insulin-induced phosphorylation of Ser[2448] on mTOR, thus raising the possibility that PKB may participate in the activation or phosphorylation of proteins downstream of mTOR, such as p70 S6 kinase and 4E-BP1 [15,16]. Of interest has been the demonstration that the insulin-mediated phosphorylation of the Ser[2448] mTOR site is reduced substantially in cells incubated in an amino acid-free environment [16]. This finding implies that amino acids may play a permissive role in the hormonal activation of mTOR, and thereby that of its downstream targets, including p70 S6 kinase and 4E-BP1. However, our data indicate that insulin is capable of stimulating p70 S6 kinase in L6 myotubes that have been maintained in an amino acid-free environment for 1 h. Moreover, this activation appears not to be enhanced by re-supply of leucine or a complete amino acid mixture (Figure 1b). Indeed, incubating muscle cells with either leucine or an amino acid mixture following a 1-h amino acid-deprivation period stimulates p70 S6 kinase to a level comparable with that seen in response to insulin, and this activation is wortmannin- and rapamycin-sensitive. These observations imply that in L6 cells: (i) amino acid and insulin signalling are likely to converge at a point upstream of mTOR, possibly at the level of PI3K; (ii) amino acids do not act as permissive agents in the hormonal activation of p70 S6 kinase; and (iii) given that leucine does not activate PKB in our system, the phosphorylation of mTOR at Ser[2448] is unlikely to be a functional requirement for the amino acid-mediated activation of p70 S6 kinase (unless another amino acid-regulated kinase also phosphorylates this serine site). Clearly, addressing whether phosphorylation of Ser[2448] is important for mTOR signalling in response to insulin remains an issue requiring further investigation.

The insulin-induced inactivation of GSK-3 is mediated largely via the activation of PKB [26], a signalling event important for promoting an increase in the cellular synthesis of glycogen and protein. However, to our knowledge, the present study represents the first report showing that GSK-3 can also be inactivated in response to a nutrient (amino acid) stimulus. Incubation of muscle cells with leucine induced a transitory inhibition of GSK-3, which occurred over a time frame that was compatible with the upstream activation of PI3K and p70 S6 kinase. Indeed, the finding that wortmannin blocks the inactivation of GSK-3 is consistent with the notion that PI3K participates in suppressing GSK-3 activity in response to leucine. The sensitivity to rapamycin also implicates mTOR as a component of the nutrient-regulated pathway that inactivates GSK-3. mTOR is unlikely to feature in the insulin-induced inhibition of GSK-3, since rapamycin fails to block GSK-3 inactivation in response to the hormone. Given that insulin promotes phosphorylation of mTOR [16] and activates p70 S6 kinase (Figure 1), we believe that the ability of this pathway to inactivate GSK-3 in L6 cells must be regulated in a stimulus-dependent manner. The amino acid-induced activation of p70 S6 kinase and the concomitant inactivation of GSK-3 is likely to play an important role in promoting translation of mRNAs encoding proteins that help the cell to respond and/or adapt to changes in nutrient availability.

One cellular process that adapts in this manner is the System A amino acid transporter whose functional activity can be assayed using the non-metabolizable N-methylated amino acid derivative Me-AIB. Leucine is not a substrate for this amino acid transporter [27], yet it induces an increase in System A

activity (even when presented to cells in the presence of other amino acids) in a transcription- and translation-dependent manner [2]. The present study shows that leucine induces this up-regulation in System A transport via the PI3K/mTOR pathway. This proposition is based on the ability of both wortmannin and rapamycin to suppress the stimulatory effects of leucine when presented to L6 cells prior to incubation with the amino acid (Figure 5, right-hand panel). The finding that these inhibitors have no effect when presented to cells following incubation with leucine implies that the transient increases in PI3K and p70 S6 kinase activity elicited by the amino acid play a key role in enhancing System A activity. It is noteworthy that evidence exists showing that a transient increase in PI3K activity is sufficient to promote delayed responses such as changes in gene expression. Alberta et al. [28] have recently shown, for example, that in Balb/c 3T3 cells a transient (10-min) activation of PI3K can induce the expression (some 90 min later) of the intermediate-early gene, MCP-1. It is plausible, therefore, that in our cell system the transient activation of PI3K by leucine also serves to trigger the downstream cascade of events that participates in the delayed up-regulation of System A expression/activity in response to leucine.

In summary, this study has provided evidence for the involvement of PI3K and p70 S6 kinase in the leucine-induced up-regulation of System A transport. The ability of rapamycin to suppress the leucine-induced activation of p70 S6 kinase and System A transport implies that mTOR is also likely to be a component of this nutrient-regulated pathway. The activation of this pathway by amino acids is also responsible for the nutrient-induced inactivation of GSK-3, an event that is likely to have important regulatory implications for the initiation phase of mRNA translation. How supply of leucine triggers these cellular responses remains unknown, but attempting to elucidate the nature and identity of the sensing mechanism(s) represents an important investigative goal that will help advance our conceptual understanding of how nutrients modulate cell function.

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