THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 41, Issue of October 12, pp. 38052–38060, 2001

*Printed in U.S.A.*

# Amino Acid and Insulin Signaling via the mTOR/p70 S6 Kinase Pathway

## A NEGATIVE FEEDBACK MECHANISM LEADING TO INSULIN RESISTANCE IN SKELETAL MUSCLE CELLS*

Received for publication, July 17, 2001
Published, JBC Papers in Press, August 9, 2001, DOI 10.1074/jbc.M106703200

**Frédéric Tremblay‡ and André Marette§**

*From the Department of Physiology and Lipid Research Unit, Laval University Hospital Research Center, Ste-Foy, Québec G1V 4G2, Canada*

**Amino acids have emerged as potent modulators of the mTOR/p70 S6 kinase pathway. The involvement of this pathway in the regulation of insulin-stimulated glucose transport was investigated in the present study. Acute exposure (1 h) to a balanced mixture of amino acids reduced insulin-stimulated glucose transport by as much as 55% in L6 muscle cells. The effect of amino acids was fully prevented by the specific mTOR inhibitor rapamycin. Time course analysis of insulin receptor substrate 1 (IRS-1)-associated phosphatidylinositol (PI) 3-kinase activity revealed that incubation with amino acids speeds up its time-dependent deactivation, leading to a dramatic suppression (−70%) of its activity after 30 min of insulin stimulation as compared with its maximal activation (5 min of stimulation). This accelerated deactivation of PI 3-kinase activity in amino acid-treated cells was associated with a concomitant and sustained increase in the phosphorylation of p70 S6 kinase. In marked contrast, inhibition of mTOR by rapamycin maintained PI 3-kinase maximally activated for up to 30 min. The marked inhibition of insulin-mediated PI 3-kinase activity by amino acids was linked to a rapamycin-sensitive increase in serine/threonine phosphorylation of IRS-1 and a decreased binding of the p85 subunit of PI 3-kinase to IRS-1. Furthermore, amino acids were required for the degradation of IRS-1 during long term insulin treatment. These results identify the mTOR/p70 S6 kinase signaling pathway as a novel modulator of insulin-stimulated glucose transport in skeletal muscle cells.**

Translational control by amino acid-dependent signaling has received considerable attention in recent years (for review, see Refs. 1 and 2). This pathway participates in the phosphorylation of p70S6k[1] and 4E-BP1, two translational modulators located downstream of mTOR (3–8). First discovered as a target of the immunosuppressive drug rapamycin, mTOR (also known as FRAP (FK506-binding protein-rapamycin-associated protein) or RAFT1 (rapamycin and FKBP12 (FK506-binding protein 12) targets)) is thought to act as a sensor of ambient amino acid concentrations (3–8). Indeed, cells deprived of amino acids show a rapid decline in the phosphorylation of p70S6k and 4E-BP1, which is rapidly reversed after amino acid re-addition in a rapamycin-sensitive manner (4–6). Furthermore, amino acid starvation did not affect the activity of a mutant form of p70S6k that is resistant to the action of rapamycin (5). Reciprocally, amino acid supplementation was still able to promote p70S6k phosphorylation in the presence of rapamycin in cells expressing a rapamycin-resistant mutant form of mTOR (4). Taken together, these findings strongly suggest that amino acids signal to p70S6k via mTOR or an mTOR-controlled element.

The mTOR nutrient pathway also integrates signal arising from phosphatidylinositol (PI) 3-kinase triggered by insulin or mitogenic signaling (9–12). This is achieved through the phosphorylation of mTOR by the serine/threonine kinase Akt (also termed protein kinase B), which requires the lipid products of PI 3-kinase, PI 3,4-bisphosphate, and/or PI 3,4,5-triphosphate for its activation (13–17). Recent advances in our understanding of mTOR regulation have shown that both hormonal and nutrient signals are prerequisite inputs necessary to fully activate this pathway (5, 7). For instance, in Chinese hamster ovary cells expressing insulin receptors, insulin-induced phosphorylation of p70S6k and 4E-BP1 is greatly increased in the presence of amino acids (5). Interestingly, this occurred without any amino acid-dependent modulation of IR/IRS tyrosine phosphorylation or PI 3-kinase and Akt activation by insulin (5), suggesting that both stimuli signal to mTOR via different pathways. In marked contrast, it was found that amino acids impair the ability of insulin to stimulate the tyrosine phosphorylation of IRS proteins and PI 3-kinase activity in hepatoma and muscle cells (7). This was observed in the face of normal insulin-induced phosphorylation of Akt, p70S6k, and 4E-BP1 (7). Whether such discrepant findings are solely a matter of cellular context remains to be established.

The regulatory role of amino acids on cellular processes is not without precedent. For instance, amino acids have been shown to increase muscle protein synthesis (18, 19), inhibit autophagic proteolysis in liver (20–22), stimulate glucose-induced protein synthesis in pancreatic β-cells (3), and facilitate multicellular clustering of rat adipocytes (8). All of these processes are dependent upon activation of a rapamycin-sensitive pathway.

* This work was supported by grants from the Canadian Diabetes Association and the Canadian Institutes of Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by studentships from the Québec Hypertension Society and the Fonds FCAR-FRSQ-Santé.

§ Supported by scholarships from the Canadian Institutes of Health Research and the Fonds de la Recherche en Santé du Québec. To whom correspondence should be addressed: Dept. of Physiology and Lipid Research Unit, Laval University Hospital Research Center, 2705, Laurier Blvd., Ste-Foy, Québec G1V 4G2, Canada. Tel.: 418-656-4141 (ext. 7549); Fax: 418-654-2176; E-mail: andre.marette@crchul.ulaval.ca.

[1] The abbreviations used are: p70S6k, 70-kDa ribosomal S6 kinase; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; mTOR, mammalian target of rapamycin; PI, phosphatidylinositol; IR, insulin receptor; IRS, insulin receptor substrate; MEM, minimal essential medium; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

In addition, amino acids were shown to blunt insulin effect on whole-body and skeletal muscle glucose disposal in humans (23–26). Although there is accumulating evidence that the positive effect of amino acids on translational events occurred via an mTOR-dependent pathway, the mechanism whereby amino acids negatively modulate glucose transport is still obscure.

Insulin stimulates glucose transport in muscle and fat cells by activation of the insulin receptor tyrosine kinase and phosphorylation of intracellular substrates of the IRS family (mainly IRS-1) (27). Tyrosine-phosphorylated IRS proteins propagate the signal to Src homology 2 domain-containing proteins such as the p85 regulatory subunit of PI 3-kinase, which in turn activates its p110 catalytic subunit (27). It is believed that PI 3-kinase activation is essential for the stimulation of glucose transport by insulin (17). Downstream effectors of PI 3-kinase includes Akt, which is thought to participate in the stimulation of glucose transport by insulin in muscle cells (28, 29).

The goal of the present study was to test the hypothesis that amino acids impair insulin action on glucose transport by specifically activating the mTOR/p70S6k-signaling pathway in L6 muscle cells. Furthermore, we examined whether amino acids modulate glucose transport by altering component(s) of the insulin signal transduction pathway in an mTOR/p70S6k-dependent fashion.

## EXPERIMENTAL PROCEDURES

*Materials*—All cell culture solutions and supplements were purchased from Life Technologies, Inc. except for fetal bovine serum, which was purchased from Wisent (St-Bruno, QC, Canada). Reagents for SDS-polyacrylamide gel electrophoresis and immunoblotting were from Bio-Rad. ECL and [2-$^3$H]Deoxyglucose were from PerkinElmer Life Sciences. [$\gamma$-$^{32}$P]ATP, protein A- and G-Sepharose, and anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase were purchased from Amersham Pharmacia Biotech. Polyclonal antibodies against IRS-1 (raised against 20 C-terminal amino acids (C-20)) and Akt (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-specific antibodies against Akt (Ser-473) and p70 S6 kinase (Thr-421/Ser-424) were from New England Biolabs (Beverly, MA). Antibodies against phosphotyrosine (4G10 clone), IRS-1 and PI 3-kinase, and Akt substrate (Crosstide) were obtained from Upstate Biotechnology (Lake Placid, NY). Human insulin was obtained from Eli Lilly (Toronto, ON, Canada). Rapamycin was purchased from Biomol (Plymouth Meeting, PA). L-$\alpha$-Phosphatidylinositol was from Avanti Polar Lipids (Alabaster, AL). Oxalate-treated TLC silica gel H plates were obtained from Analtech (Newark, DE). All other chemicals were of the highest analytical grade.

*Cell Culture and Treatment*—A line of L6 skeletal muscle cells (kind gift of Dr. Amira Klip, Hospital for Sick Children, Toronto, ON, Canada) clonally selected for high fusion potential was used in the present study. The L6 cell line was derived from neonatal rat thigh skeletal muscle cells and retains many morphological, biochemical, and metabolic characteristics of skeletal muscle. Cells were grown and maintained in monolayer culture in $\alpha$-MEM containing 2% (v/v) fetal bovine serum and 1% (v/v) antibiotic/antimycotic solution (10000 units/ml penicillin, 10000 $\mu$g/ml streptomycin and 25 $\mu$g/ml amphotericin B) in an atmosphere of 5% $CO_2$ at 37 °C. Fully differentiated L6 myotubes were deprived of serum 4 h before experimental treatments. Then cells were incubated either in amino acid-free medium (Earle's balanced salt solution (EBSS)) or in EBSS containing 1× or 2× amino acids either individually or as a mixture, as found in MEM for 1 h. The concentrations (in $\mu$M) of amino acids (1×) in MEM were as follows: Arg, 126; Cys, 100; Gln, 2000; His, 200; Ile, 400; Leu, 400; Lys, 400; Met, 100; Phe, 200; Thr, 400; Trp, 50; Tyr, 200 and Val, 400. Vehicle (0.01% $Me_2SO$) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated with insulin for different times as indicated in figure legends.

*Measurement of 2-Deoxyglucose Uptake*—2-Deoxyglucose was determined as previously described (30). Briefly, after experimental treatments, cells were rinsed once with HEPES-buffered solution (20 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, and 1 mM $CaCl_2$) and were subsequently incubated for 8 min in HEPES-buffered solution containing 10 $\mu$M 2-deoxyglucose and 0.3 $\mu$Ci/ml 2-deoxy-[$^3$H]glucose. After the incubation in transport medium, cells were rinsed three times with ice-cold 0.9% NaCl solution and then disrupted by adding 50 mM NaOH. Cell-associated radioactivity was determined by scintillation counting. Protein concentrations were determined by the bicinchoninic acid method, and the results were expressed in pmol/min/mg. Glucose uptake values were corrected for non-carrier-mediated transport by measuring hexose uptake in the presence of 10 $\mu$M cytochalasin B (5–10% of total uptake).

*PI 3-Kinase Assay*—After experimental treatment, medium was removed, and cells were rinsed twice in ice-cold phosphate-buffered saline (PBS) and lysed in 20 mM Tris, pH 7.4, 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40, 2 mM $Na_3VO_4$, and 10 mM NaF. 500 $\mu$g of lysates were immunoprecipitated with 2 $\mu$g of anti-IRS-1 coupled to protein A-Sepharose overnight at 4 °C. Immune complexes were washed twice with wash buffer I (PBS, pH 7.4, 1% Nonidet P-40, and 2 mM $Na_3VO_4$), twice with wash buffer II (100 mM Tris, pH 7.5, 500 mM LiCl, and 2 mM $Na_3VO_4$), and twice with wash buffer III (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 2 mM $Na_3VO_4$). Beads were resuspended in 70 $\mu$l of kinase buffer (8 mM Tris, pH 7.5, 80 mM NaCl, 0.8 mM EDTA, 15 mM $MgCl_2$, 180 $\mu$M ATP, and 5 $\mu$Ci of [$\gamma$-$^{32}$P]ATP) and 10 $\mu$l of sonicated PI mixture (20 $\mu$g of L-$\alpha$-PI, 10 mM Tris, pH 7.5, and 1 mM EGTA) for 15 min at 30 °C. The reaction was stopped by the addition of 20 $\mu$l of 8 M HCl mixed with 160 $\mu$l of $CHCl_3$:$CH_3OH$ (1:1) and centrifuged. The lower organic phase was spotted on an oxalate-treated silica gel TLC plate and developed in $CHCl_3$:$CH_3OH$:$H_2O$:$NH_4OH$ (60:47:11.6:2). The plate was dried and visualized by autoradiography with intensifying screen at −80 °C.

*Akt Kinase Assay*—After experimental treatment, the medium was removed, cells were rinsed twice in ice-cold PBS and lysed in 50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1% Triton X-100, 2 mM $Na_3VO_4$, and 10 mM NaF. 200–300 $\mu$g of lysates were immunoprecipitated with 2 $\mu$g of anti-Akt1 coupled to protein G-Sepharose for 2 h at 4 °C. Immune complexes were washed 3 times in 25 mM HEPES, pH 7.4, 10% glycerol, 1% Triton X-100, 0.1% bovine serum albumin, 1 M NaCl, 1 mM dithiothreitol, and 200 $\mu$M $Na_3VO_4$ and 2 times in kinase buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, and 1 mM dithiothreitol). The reaction was started by adding 30 $\mu$l of kinase buffer (containing 8 $\mu$M ATP, 2 $\mu$Ci of [$\gamma$-$^{32}$P]ATP and 100 $\mu$M Crosstide) for 30 min at 30 °C. The reaction product was resolved on 40% acrylamide-urea gel and visualized by autoradiography with intensifying screen at −80 °C.

*Immunoprecipitation and Immunoblotting*—IRS-1 was immunoprecipitated as described for PI 3-kinase assay. Tyrosine-phosphorylated proteins were immunoprecipitated with 2 $\mu$g of anti-phosphotyrosine (clone 4G10) coupled to protein A-Sepharose from 500 $\mu$g of cell lysate. Immune complexes were washed 3 times in PBS, pH 7.4, 1% Nonidet P-40, and 2 mM $Na_3VO_4$, resuspended in SDS sample buffer, and boiled for 5 min. Immunoprecipitates or cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Polyvinylidene difluoride membranes were then blocked for 1 h at room temperature in TBS (50 mM Tris, pH 7.4, 150 mM NaCl) containing 0.04% Nonidet P-40, 0.02% Tween 20, and 5% nonfat milk followed by overnight incubation at 4 °C with primary antibodies. The polyvinylidene difluoride membranes were then washed for 30 min followed by a 1-h incubation with either anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase in TBS containing 2% nonfat milk. The polyvinylidene difluoride membranes were washed for 30 min in TBS, and the immunoreactive bands were detected by the enhanced chemiluminescence method.

*Stastistical Analysis*—The effects of amino acids, insulin, and rapamycin were compared by analysis of variance tests followed by least mean square determination. Differences were considered to be statistically significant at $p < 0.05$.

## RESULTS

*Amino Acids Inhibit Insulin-stimulated Glucose Transport in a Dose-dependent Manner*—The inhibitory action of amino acids on glucose transport in skeletal muscle has been observed *in vivo* (23–26). To test whether such effect can be reproduced *in vitro*, L6 myocytes were either incubated in an amino acid-free medium or in a 1× or 2× amino acids mixture for 1 h (a 1× amino acid mixture was defined as the concentration of amino acids found in MEM (See "Experimental Procedures")). After experimental treatments, glucose transport was measured in cells that were stimulated or not with insulin (100 nM). Increasing the concentrations of amino acids had no significant effect on basal glucose transport (Fig. 1*A*). However, the effect of a submaximal concentration of insulin (100 nM) on glucose trans-

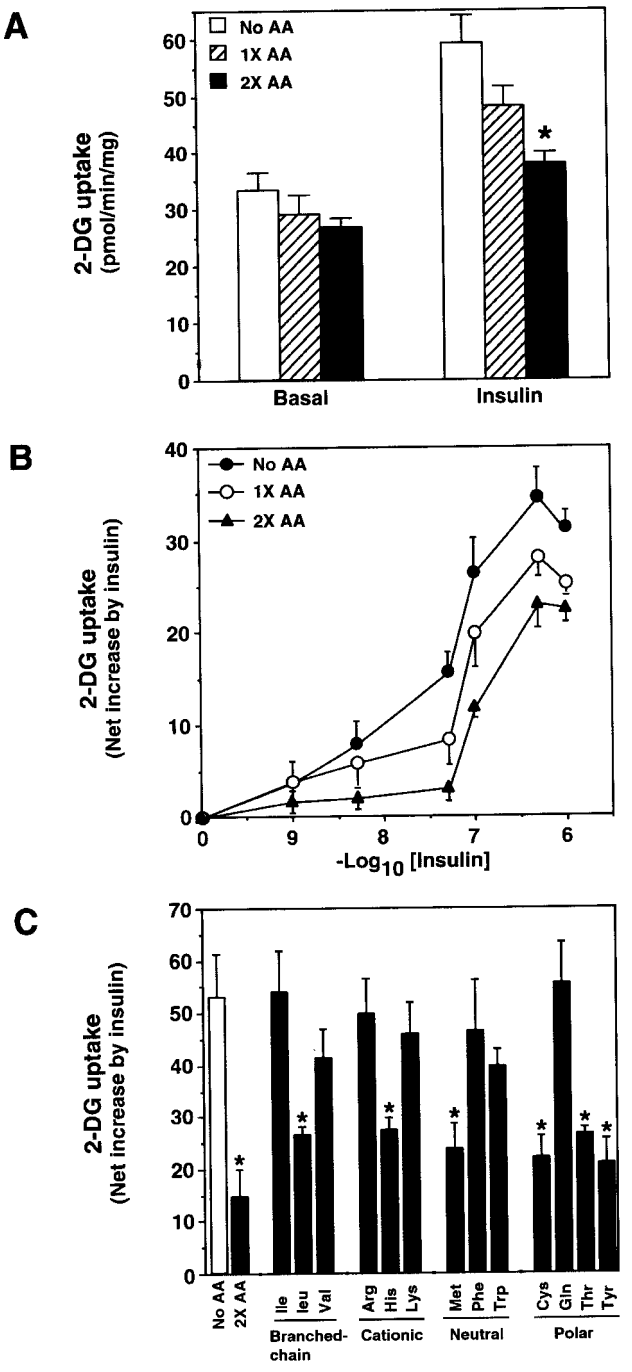


FIG. 1. **Amino acids inhibit insulin-stimulated glucose transport in a dose-dependent manner.** Serum-deprived L6 cells were incubated either in amino acid-free medium (*No AA*) or in medium containing a 1× or 2× amino acid mixture, as found in MEM for 1 h. *A*, cells were stimulated or not with 100 nM insulin for the last 45 min of incubation before glucose transport measurements, as described under "Experimental Procedures." *B*, cells were stimulated with various doses of insulin (1 nM to 1 $\mu$M) for the last 45 min of incubation before glucose transport measurements. *C*, cells were exposed to individual amino acid at the concentration found in 2× MEM for 1 h and stimulated with 100 nM insulin for the last 45 min of incubation before glucose transport measurements. For *B* and *C*, results were expressed as net increases in glucose transport mediated by insulin (insulin minus basal). The means ± S.E. from at least four individual experiments, each performed in triplicate, are shown. *, $p < 0.05$ *versus* cells deprived of amino acids (*No AA*). *2-DG*, 2-deoxyglucose.

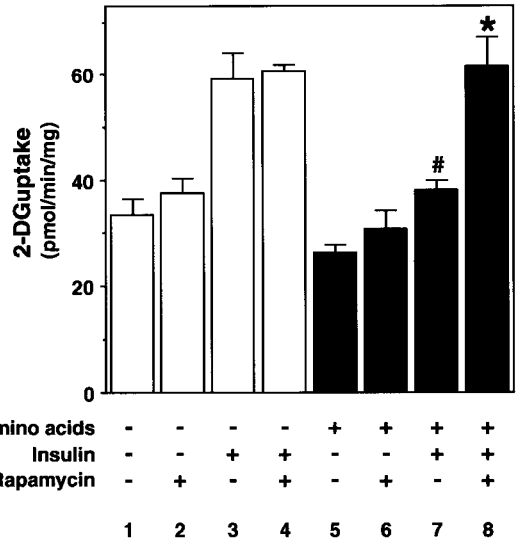


FIG. 2. **Amino acids impair insulin-stimulated glucose transport via the mTOR pathway.** Serum-deprived L6 cells were incubated either in amino acid-free medium (*open bars*) or in medium containing a 2× amino acid mixture, as found in MEM (*black bars*) for 1 h. Vehicle (0.01% $Me_2SO$) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated or not with 100 nM insulin for the last 45 min of incubation before 2-deoxy-D-glucose (*2-DG*) uptake measurements, as described under "Experimental Procedures." The means ± S.E. from at least 4 individual experiments, each performed in triplicate, are shown. #, $p < 0.05$ *versus* cells deprived of amino acids. *, $p < 0.05$ *versus* cells treated with 2× amino acids mixture + rapamycin.

port was inhibited by as much as 30 and 55% in myocytes treated with 1× or 2× amino acid mixtures as compared with cells deprived of amino acids, respectively (Fig. 1*A*). We next determined if the effect of amino acids could be observed over a wide range of insulin concentrations. Amino acid-treated cells were exposed to increasing doses of insulin, and glucose transport was determined. As depicted in Fig. 1*B*, the suppressive effect of amino acids was noticeable at 5 nM insulin and could still be observed at maximal doses of the hormone (0.5–1 $\mu$M). These results clearly indicate that acute exposure (1 h) to amino acids impairs the ability of insulin to stimulate glucose transport in muscle cells. To identify which amino acids were responsible for insulin resistance, we measured glucose transport in cells treated with individual amino acids at the concentration found in 2× amino acid mixtures. It was found that Cys, His, Leu, Met, Thr, and Tyr were inhibitory, whereas the remaining (Arg, Glu, Ile, Lys, Phe, Trp, and Val) amino acids failed to inhibit insulin-stimulated glucose uptake (Fig. 1*C*). It is noteworthy that the inhibitory influence of amino acids were neither limited to a particular chemical group (*e.g.* branched-chain, cationic, neutral, or polar) nor to a particular amino acid transport system (*e.g.* A, ASC, or L). Interestingly, glutamine, an amino acid known to activate the hexosamine biosynthetic pathway and to cause insulin resistance in rat adipocytes (31, 32), was without effect in L6 muscle cells. This indicates that build-up of hexosamines is unlikely to be involved in the acute inhibition of insulin-stimulated glucose transport by amino acids in muscle cells.

*Amino Acids Impair Insulin-stimulated Glucose Transport via the mTOR Pathway*—We next tested the hypothesis that amino acids impair insulin-stimulated glucose transport via the activation of mTOR. The involvement of the mTOR pathway in amino acid-induced insulin resistance was evaluated by using rapamycin, a highly specific inhibitor (33) that forms a complex with FKBP12 (FK506-binding protein 12), which binds and inactivates mTOR (34). In cells deprived of amino acids for 1 h, neither basal nor insulin-stimulated glucose transport was affected by the addition of rapamycin to the medium (Fig. 2, *lane 1 versus 2* and *lane 3 versus 4*). Strikingly, the inhibitory effects of amino acids on insulin-stimulated glu-

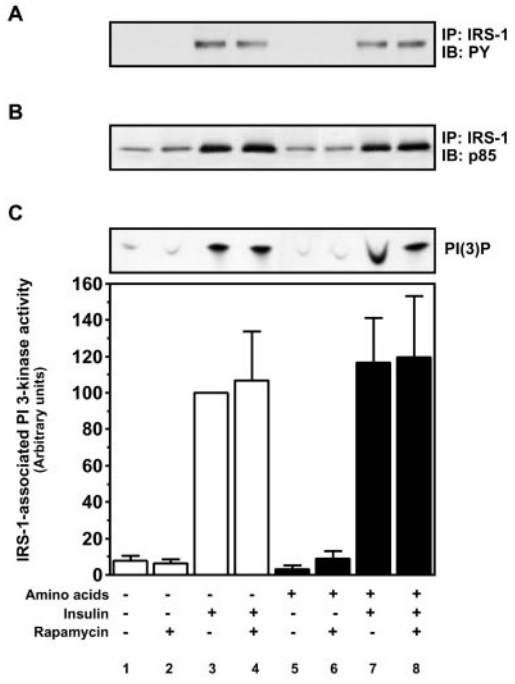


FIG. 3. **Amino acids fail to impair the acute effect of insulin on IRS-1 tyrosine phosphorylation and PI 3-kinase recruitment and activation.** Serum-deprived L6 cells were incubated either in amino acid-free medium (*open bars*) or in medium containing a 2× amino acids mixture as found in MEM (*black bars*) for 1 h. Vehicle (0.01% $Me_2SO$) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated or not with 100 nM insulin for the last 5 min of incubation, rinsed twice in ice-cold PBS, and lysed as described under "Experimental Procedures." Measurements of tyrosine phosphorylation (*PY*) of IRS-1 (*A*), association of the p85 subunit of PI 3-kinase with IRS-1 (*B*), and PI 3-phosphate (*PI(3)P*) produced by IRS-1-associated PI 3-kinase activity (*C*) were determined as described under "Experimental Procedures." For *A* and *B*, results shown are one representative experiment repeated at least three times. The means ± S.E. from at least five individual experiments are shown in *C*. *IB*, immunoblot.

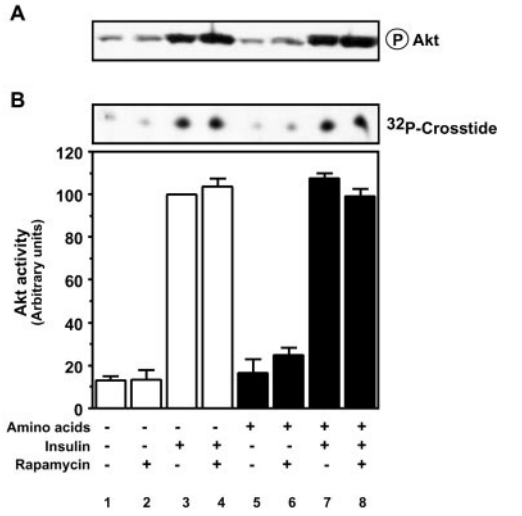


FIG. 4. **Amino acids fail to inhibit the acute effect of insulin on Akt phosphorylation and activity.** Serum-deprived L6 cells were incubated either in amino acid-free medium (*open bars*) or in medium containing a 2× amino acids mixture as found in MEM (*black bars*) for 1 h. Vehicle (0.01% $Me_2SO$) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated or not with 100 nM insulin for the last 10 min of incubation, rinsed twice in ice-cold PBS, and lysed as described under "Experimental Procedures." Measurements of Akt phosphorylation (*P*) using a phospho-specific antibody that detects Akt only when phosphorylated at Ser-473 (*A*) and Akt kinase activity assayed using Crosstide as substrate (*B*) were determined as described under "Experimental Procedures." For *A*, results are shown of one representative experiment repeated at least three times. The means ± S.E. from at least three individual experiments are shown in *B*.

cose transport (Fig. 2, compare *lanes 3 versus 7*) were completely prevented by treatment with rapamycin (Fig. 2, *lanes 7 versus 8*). These results indicate that the inhibitory amino acids (Fig. 1*C*) contained in the 2× amino acids mixture decrease insulin-stimulated glucose transport by activating the mTOR pathway. Furthermore, the observation that rapamycin regulates glucose transport only in medium enriched in amino acids strengthened the hypothesis that rapamycin specifically acts on mTOR to antagonize amino acid-dependent signaling.

*Amino Acids Fail to Impair the Acute Effect of Insulin on IRS-1 Tyrosine Phosphorylation and PI 3-Kinase Recruitment and Activation*—In an attempt to understand how amino acids induced insulin resistance, we next examined the activation of key signaling proteins involved in insulin action. Among them, IRS-1 and its associated PI 3-kinase activity play a prominent role in insulin-stimulated glucose transport in muscle (35, 36). L6 cells were incubated in media containing either no amino acids or a 2× amino acid mixture with or without rapamycin for 1 h and stimulated with insulin during the last 5 min. IRS-1 was immunoprecipitated from cell lysates, resolved by SDS-polyacrylamide gel electrophoresis, and incubated with anti-phosphotyrosine antibody. As shown in Fig. 3*A*, insulin-induced tyrosine phosphorylation of IRS-1 was not affected by amino acids or rapamycin treatments. Similarly, the amount of p85 regulatory subunit of PI 3-kinase recovered in IRS-1 immune complexes was strongly increased by insulin and was not affected by amino acids and/or rapamycin (Fig. 3*B*). Consistent with the lack of effect of amino acids on IRS-1 tyrosine phosphorylation and p85 recruitment, we observed no effects of amino acids or rapamycin on basal or insulin-induced PI 3-kinase activity in IRS-1 precipitates (Fig. 3*C*).

*Amino Acids Fail to Inhibit the Acute Effect of Insulin on Akt Phosphorylation and Activity*—It has been recently reported that dephosphorylation and/or relocalization of PI 3,4,5-triphosphate reduced Akt phosphorylation in response to insulin despite the fact that PI 3-kinase activity was intact (37, 38). Therefore, although incubation with amino acids failed to affect PI 3-kinase activity, it was of interest to evaluate the modulation of Akt phosphorylation and activity by amino acids and/or rapamycin. Using a phospho-specific antibody, we found that insulin robustly enhanced the phosphorylation state of Akt in cells incubated with or without amino acids and/or rapamycin (Fig. 4*A*). In accordance with Akt phosphorylation data, insulin-stimulated Akt kinase activity was unaffected by either amino acids or rapamycin (Fig. 4*B*).

*Different Temporal Activation of PI 3-Kinase and p70S6k by Insulin: Relationship with Insulin-induced Glucose Transport*—Since the acute burst (5 min) of PI 3-kinase activity in response to insulin was not impaired by amino acids (Fig. 3), we sought to determine whether there was any relationship between insulin-stimulated PI 3-kinase activity and mTOR/p70S6k activation over time. L6 cells were placed in 2× amino acid medium for 1 h and treated with insulin for the last 5, 15, or 30 min of incubation. It was found that IRS-1-associated PI 3-kinase is maximally activated after 5 min of insulin stimulation and progressively decrease, reaching ~30% of maximal activity after 30 min (Fig. 5*A*). In contrast to PI 3-kinase, the activation of p70S6k phosphorylation by insulin was barely detectable after 5 min but was much higher after 30 min of stimulation (Fig. 5*A*). Accordingly, the amino acid-induced impairment of glucose uptake was also dependent on insulin treatment duration and was not observed in cells treated for only 5 or 15 min with insulin (Fig. 5*B*).

*Amino Acids Speed up the Deactivation of Insulin-induced PI 3-Kinase Activity: Relationship with p70S6k Activation*—To

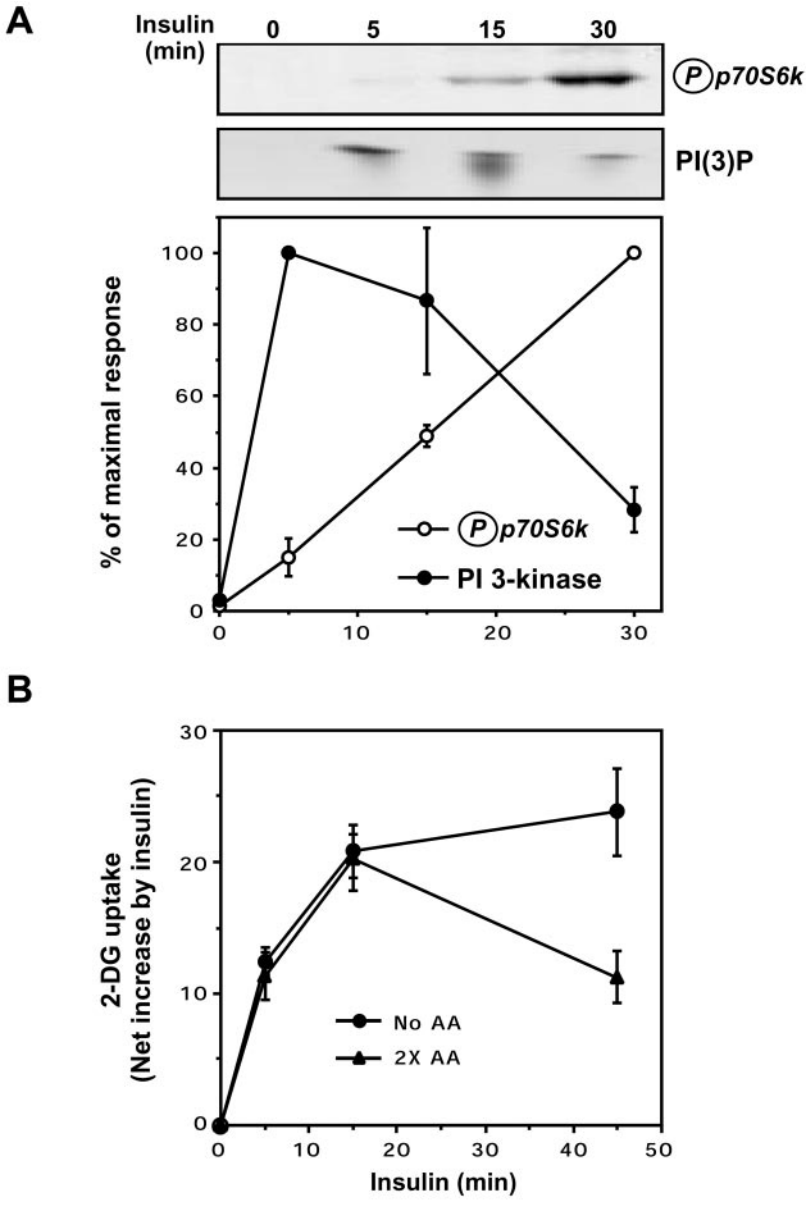


FIG. 5. **Different temporal activation of PI 3-kinase and p70S6k by insulin: relationship with insulin-induced glucose transport.** *A*, serum-deprived L6 cells were incubated in medium containing 2× amino acids mixture as found in MEM for 1 h. Cells were stimulated or not with 100 nM insulin for the last 30, 15, and 5 min of incubation, rinsed twice in ice-cold PBS, and lysed as described under "Experimental Procedures." Measurements of p70S6k phosphorylation using a phospho-specific antibody that detects p70S6k only when phosphorylated (*P*) at Thr-421/Ser-424 (*upper panel*) and PI 3-phosphate (*PI(3)P*) produced by IRS-1-associated PI 3-kinase activity (*lower panel*) were determined as described under "Experimental Procedures." Quantification of p70S6k phosphorylation (*open circle*) and IRS-1-associated PI 3-kinase activity (*filled circle*) are presented in the *line graph*. *B*, serum-deprived L6 cells were incubated either in amino acid-free medium (*filled circle*) or in medium containing 2× amino acids (*AA*) mixture as found in MEM (*filled triangle*) for 1 h. Cells were stimulated with 100 nM insulin for the last 5, 15, and 45 min of incubation before glucose transport measurement, as described under "Experimental Procedures." The means ± S.E. from at least four individual experiments are shown. *2-DG*, 2-deoxyglucose.

determine whether the apparent inverse relationship between the temporal activation of PI 3-kinase and p70S6k by insulin was of biochemical significance, we treated muscle cells with or without amino acids in the presence or absence of rapamycin. Although PI 3-kinase activity was marginally reduced after 30 min of insulin stimulation in amino acid-deprived cells (~30% reduction), the addition of amino acids dramatically accelerated the time-dependent deactivation of PI 3-kinase, leading to a marked reduction (~70%) of its activity after 30 min of insulin exposure (Fig. 6, *A*, *upper panel*, and *C*, *left panel*). The magnitude of inhibition of PI 3-kinase was closely correlated with the activation status of mTOR/p70S6k, as monitored by the increased phosphorylation and decreased electrophoretic mobility of p70S6k (Fig. 6*B*, *upper panel*). Indeed, the more p70S6k was phosphorylated, the more PI 3-kinase was inhibited (Fig. 6, *A* and *B*, *lane 4 versus lane 8*, and Fig. 6*C*). This time-dependent decrease in PI 3-kinase activity in both amino acid-treated and -deprived cells was completely prevented by rapamycin (Fig. 6*A*, *lower panel*) and was associated with a complete abolition of p70S6k phosphorylation (Fig. 6*B*, *lower panel*). Similar observations were made when a lower concentration (5 nM) of insulin was used in these experiments (data not shown). These results indicate that amino acids potentiate insulin-induced activation of mTOR/p70S6k pathway, leading to an accelerated deactivation of IRS-1-associated PI 3-kinase activity (Fig. 6*C*).

*Mechanism of Impaired Insulin-stimulated PI 3-Kinase Activity by Amino Acids*—The amino acid-induced deactivation of IRS-1-associated PI 3-kinase activity after 30 min of insulin stimulation (Fig. 6*C*) was associated with a decrease binding of the p85 regulatory subunit of PI 3-kinase to IRS-1 (Fig. 7*B*) despite normal tyrosine phosphorylation of the insulin receptor and IRS-1 (Fig. 7*A*). Coincubation with rapamycin fully restored PI 3-kinase recruitment and activation in these cells (Fig. 7, *B–C*). Interestingly, rapamycin treatment of amino acid-deprived cells also increased PI 3-kinase recruitment and activation (Fig. 7, *B–C*) to the levels measured in cells stimulated for 5 min with insulin.

The amino acid-mediated reduction in IRS-1-dependent PI 3-kinase activity was also associated with a significant mobility shift of IRS-1 after 30 min of insulin stimulation (Fig. 7*D*, compare *lane 5 versus 6*), which was prevented by rapamycin (compare *lane 7 versus 8*). This insulin-induced mobility shift of IRS-1 was virtually undetectable in amino acid-deprived cells (*lanes 1–4*). These data suggest that an increased IRS-1 Ser/Thr phosphorylation is responsible for the decrease binding of

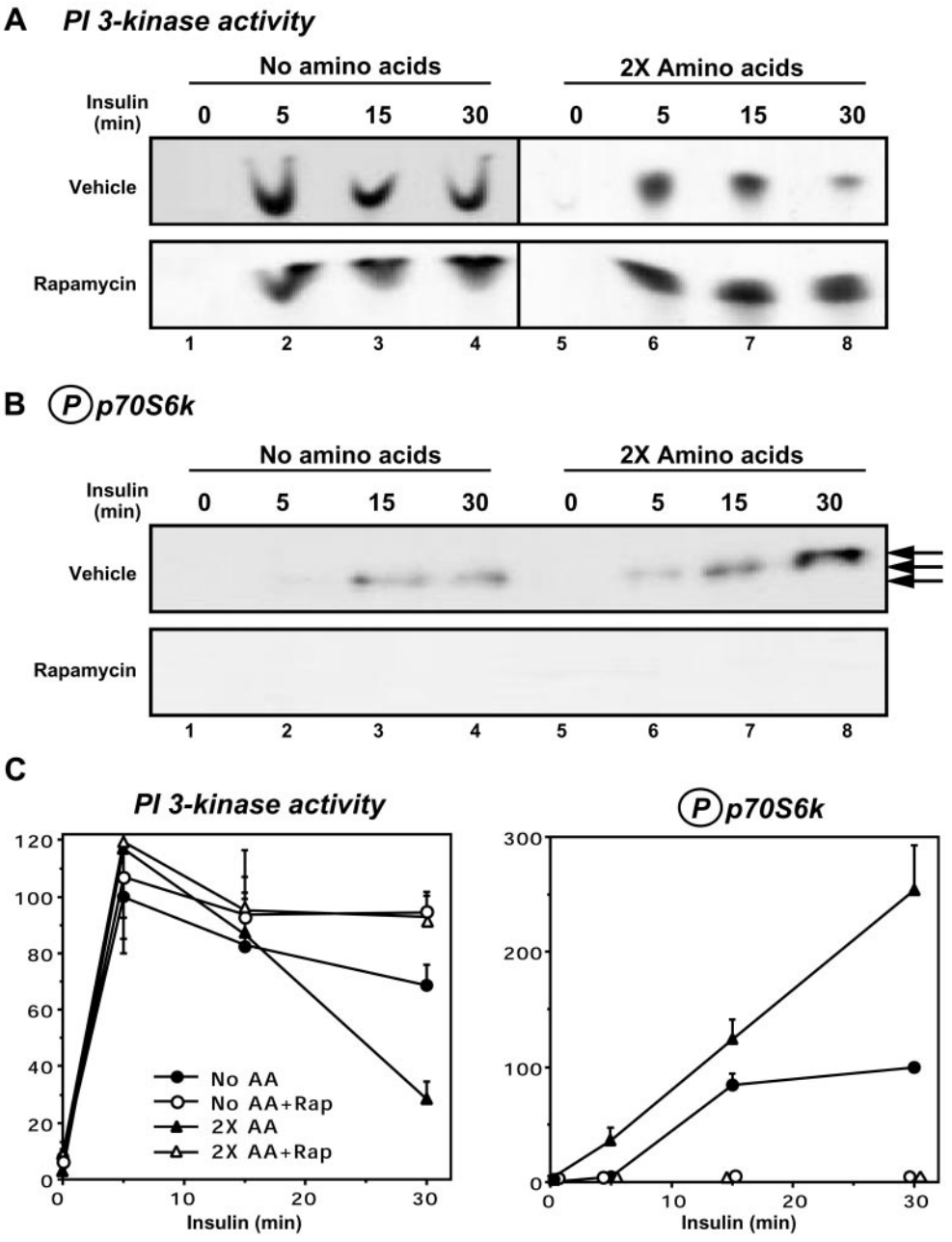


FIG. 6. **Amino acids speed up the deactivation of insulin-induced PI 3-kinase activity: relationship with p70S6k activation.** Serum-deprived L6 cells were incubated either in amino acid-free medium (*no AA*) or in medium containing 2× amino acids mixture as found in MEM for 1 h. Vehicle (0.01% $Me_2SO$) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated or not with 100 nM insulin for the last 30, 15, and 5 min of incubation, rinsed twice in ice-cold PBS, and lysed as described under "Experimental Procedures." Measurements of PI 3-phosphate produced by IRS-1-associated PI 3-kinase activity (*A*) and p70S6k phosphorylation (*P*) (*B*) using a phospho-specific antibody that detects p70S6k only when phosphorylated at Thr-421/Ser-424 were determined as described under "Experimental Procedures." The *arrows* indicate the different electrophoretic species of phosphorylated p70S6k, the slower migrating immunoreactive bands being a hyperphosphorylated form of p70S6k. *C*, quantification of IRS-1-associated PI 3-kinase activity (*left panel*) and p70S6k phosphorylation (*right panel*) of cells treated with no amino acids (*No AA*, *filled circle*), no amino acids + rapamycin (*Rap*, *open circle*), 2× amino acids (*filled triangle*), and 2× amino acids + rapamycin (*open triangle*) are presented in the *line graphs*. The means ± S.E. from at least four individual experiments are shown.

the p85 subunit to IRS-1 (Fig. 7*B*) and PI 3-kinase activation (Fig. 7*C*) in amino acid-treated cells. Importantly, this rapamycin-sensitive increase in Ser/Thr phosphorylation was not accompanied by a rapid IRS-1 degradation over the 30-min period.

*Dissociation between Insulin-induced PI 3-Kinase and Akt Activities in Amino Acid-treated Cells*—We next examined whether this amino acid-dependent deactivation of IRS-1-associated PI 3-kinase activity had any repercussion on downstream insulin signaling by measuring the effect of amino acids and/or rapamycin on Akt phosphorylation (Fig. 7*D*) and activity (Fig. 7*E*). Surprisingly, we found that Akt phosphorylation and kinase activity were still maximally stimulated after 30 min of insulin stimulation of amino acid-exposed cells, despite the marked suppression of PI 3-kinase activity in the same conditions (Fig. 7*C*). This indicates that the state of insulin resistance induced by amino acids is selective for PI 3-kinase but not for Akt.

*Amino Acids Are Necessary for Insulin-induced Ser/Thr Phosphorylation and Degradation of IRS-1 after More Prolonged Insulin Treatment*—Since the mTOR pathway was recently shown to control proteosomal degradation of IRS-1 in

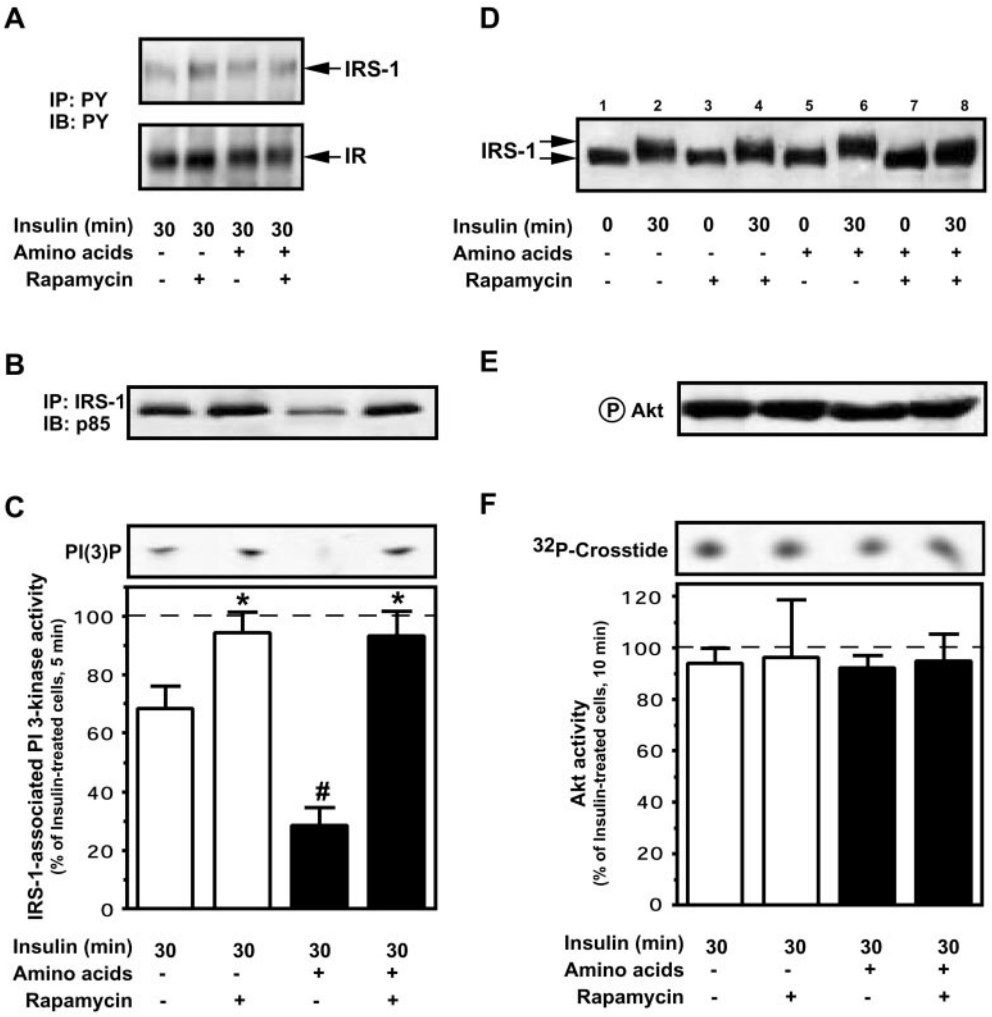


FIG. 7. **Mechanism of impaired insulin-stimulated PI 3-kinase activity by amino acids.** Serum-deprived L6 cells were incubated either in amino acid-free medium (*open bars*) or in medium containing 2× amino acids mixture as found in MEM (*black bars*) for 1 h. Vehicle (0.01% $Me_2SO$) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated or not with 100 nM insulin for the last 30 min of incubation as indicated, rinsed twice in ice-cold PBS, and lysed as described under "Experimental Procedures." Measurements of tyrosine phosphorylation of the IR and IRS-1 (*A*), association of the p85 subunit of PI 3-kinase with IRS-1 (*B*), and IRS-1-associated PI 3-kinase-mediated production of PI 3-phosphate (*PI(3)P*) (*C*) were determined as described under "Experimental Procedures." *D*, IRS-1 electrophoretic mobility on 6% SDS-polyacrylamide gel electrophoresis was determined as described under "Experimental Procedures." The *arrows* indicate the different electrophoretic species of IRS-1, the slower migrating band being a more heavily Ser/Thr phosphorylated form of IRS-1. Measurement of Akt phosphorylation (*P*) using a phospho-specific antibody that detects Akt only when phosphorylated at Ser-473 (*E*) and Akt kinase activity assayed using Crosstide as substrate (*F*) were determined as described under "Experimental Procedures." For *A*, *B*, *D*, and *E*, results are shown of one representative experiment repeated at least three times. The means ± S.E. from at least five individual experiments are shown for *C* and *F*. *, $p < 0.05$ *versus* cells treated in the absence of rapamycin. *IB*, immunoblot.

3T3-L1 adipocytes incubated in amino acid-containing medium (39), we thus examined the role of amino acids in a more prolonged insulin treatment on IRS-1 Ser/Thr phosphorylation and degradation in L6 cells. It was found that insulin increases Ser/Thr phosphorylation and promotes degradation of IRS-1 after 1–4 h of insulin stimulation but only in the presence of amino acids (Fig. 8*A*). IRS-1 degradation was associated with a strong activation of p70S6k phosphorylation in these cells (Fig. 8*B*). Moreover, a close relationship was noted between the time course of Akt and p70S6k phosphorylation, further suggesting that insulin-induced Akt activation is required for mTOR activation in amino acid-treated cells (Fig. 8, *B–C*).

## DISCUSSION

The observation that amino acids stimulate the phosphorylation of p70S6k and 4E-BP1 in a rapamycin-dependent manner (3–8) uncovered an entirely new pathway by which nutrients can serve as activators/intermediates in signal transduction. Furthermore, the fact that both insulin and amino acids induce the activation of the mTOR/p70S6k pathway (5, 7) raised the important question as to whether amino acid-dependent signaling "cross-talks" with components of the insulin signal transduction pathway. Although some studies report that amino acids had no effect on insulin-stimulated PI 3-kinase and Akt activities (5, 21), others found that amino acids decreased PI 3-kinase activation by insulin (7). However, none of these studies had carefully examined the temporal relationship between the mTOR/p70S6k and PI 3-kinase activation by insulin. Our results revealed that the time-dependent decrease in IRS-1-associated PI 3-kinase activity in amino acid-treated cells was associated with a concomitant activation of the mTOR/p70S6k pathway. We further demonstrated the cause-effect relationship between these two events by using the immunosuppressive drug rapamycin. Indeed, we found that inhibition of the mTOR/p70S6k pathway by rapamycin prevented the negative effect of amino acids on insulin signaling, resulting in sustained activation of PI 3-kinase throughout the stimulation period. Therefore, the results described herein may help to reconcile the apparent discrepancy from earlier studies

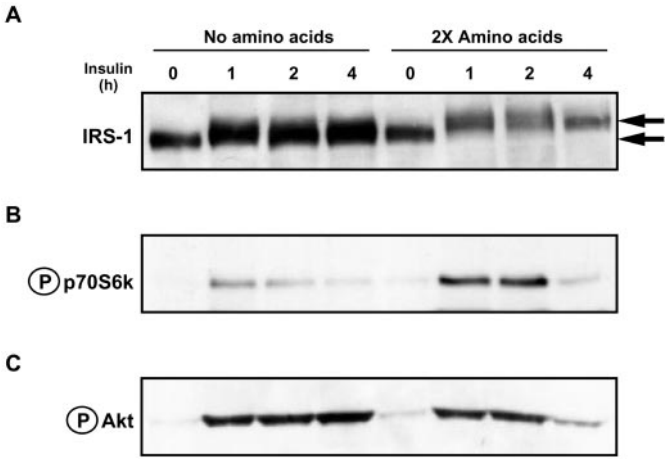


FIG. 8. **Amino acids are necessary for insulin-induced Ser/Thr phosphorylation and degradation of IRS-1 after more prolonged insulin treatment.** Serum-deprived L6 cells were incubated either in amino acid-free medium (*No amino acids*) or in medium containing a 2× amino acids mixture as found in MEM for 4 h. Cells were stimulated or not with 100 nM insulin for the last 1, 2, or 4 h of incubation as indicated, rinsed twice in ice-cold PBS, and lysed as described under "Experimental Procedures." Measurements of IRS-1 electrophoretic mobility (the *arrows* indicate the different electrophoretic species of IRS-1), the slower migrating band being a more heavily Ser/Thr-phosphorylated form of IRS-1 (*A*), p70S6k phosphorylation using a phospho-specific antibody that detects p70S6k only when phosphorylated (*P*) at Thr-421/Ser-424 (*B*), and Akt phosphorylation using a phospho-specific antibody that detects Akt only when phosphorylated at Ser-473 were determined as described under "Experimental Procedures" (*C*). Results are one representative experiment repeated three times.

(5, 7), where it was found that amino acids failed to affect PI 3-kinase activation when measured after 2 min of insulin stimulation (5) but severely impairs PI 3-kinase activation when measured after 30 min of insulin stimulation (7). Thus, the mTOR/p70S6k pathway is part of a feedback mechanism that negatively modulates the ability of insulin to transmit signal to PI 3-kinase via IRS-1.

Our data suggest that the uncoupling of IRS-1 and PI 3-kinase in amino acid-treated cells may be linked to an increased Ser/Thr phosphorylation of IRS-1, which subsequently reduces its affinity for the p85 regulatory subunit of PI 3-kinase. Increased Ser/Thr phosphorylation of IRS-1 has been proposed as a major cause of insulin resistance induced by a variety of agents such as tumor necrosis factor-$\alpha$, okadaic acid, platelet-derived growth factor, protein kinase C activators, and chronic hyperinsulinemia (39–43). Furthermore, insulin resistance induced by platelet-derived growth factor, tumor necrosis factor-$\alpha$, or chronic hyperinsulinemia has been shown to involve the activation of a rapamycin-sensitive pathway (39, 40, 44, 45). Therefore, the present work together with previous studies (39, 40, 44, 45) provides strong evidence that the mTOR/p70S6k pathway modulates insulin action by increasing the phosphorylation of IRS-1 on Ser/Thr residues. However, it is presently unknown whether mTOR and/or p70S6k directly phosphorylate IRS-1. On one hand, mTOR kinase activity has been shown to be directed toward sites containing a (Ser/Thr)-Pro motif (46) such as that encountered in the IRS-1 structure (47). On the other hand, inhibition of an mTOR-controlled Ser/Thr phosphatase by amino acids (48) may also be involved in the increase Ser/Thr phosphorylation of IRS-1. Furthermore, the observation that amino acids are required for IRS-1 degradation during prolonged insulin treatment indicates that mTOR activation is an essential pathway involved in this process. This mTOR-dependent degradation of IRS-1 may proceed via a lactacystin-sensitive proteosomal pathway, as recently reported in 3T3-L1 adipocytes (39).

It will be important to identify the Ser/Thr residue(s) in IRS-1 that is (are) targeted after amino acid and insulin treatments. Preliminary experiments revealed that Ser-612, the residue that is phosphorylated by MAP kinase after treatment with protein kinase C activators (43, 49), is not involved in mTOR-mediated insulin resistance. Indeed, we found that a phospho-Ser-616-IRS-1 antibody (Ser-612 in rats) had no immunoreactivity on IRS-1 precipitates isolated from amino acid-treated cells after 30 min of insulin stimulation.[2] Furthermore, *in vitro* phosphorylation of a peptide containing the Ser-612-IRS-1 residue (43, 49) was not affected by amino acids.[2] This is in agreement with the finding that Ser-612 is not involved in the rapamycin-sensitive decrease of IRS-1-associated PI 3-kinase activity after platelet-derived growth factor treatment but, rather, involved phosphorylation on Ser-632, -662 , and/or -731 (40). Also, tumor necrosis factor-$\alpha$ was recently shown to induce phosphorylation of IRS-1 on Ser-636 and -639 via a PI 3-kinase/Akt/mTOR pathway (45). Furthermore, insulin and tumor necrosis factor-$\alpha$ induce IRS-1 phosphorylation on Ser-307 (50, 51), which may also represent a relevant target of the mTOR pathway. Clearly, a more detailed analysis will be needed to identify the serine and/or threonine residues that are phosphorylated after mTOR activation in myocytes.

An intriguing finding of the present study is that insulin-stimulated Akt activity was unaltered by amino acid treatment even though such conditions led to a marked inhibition of IRS-1-associated PI 3-kinase activity. This suggests that Akt only requires partial stimulation of PI 3-kinase by insulin to achieve a full activation as previously observed (52, 53). Furthermore, it appears that a maximal Akt kinase activity is able to drive insulin signal to promote p70S6k phosphorylation activity regardless of the PI 3-kinase activation status, at least for 2 h. A similar observation was recently made using a mutant form of IRS-1 that is constitutively bound to membrane. This mutant IRS-1 normally transduced insulin signal to both Akt and p70S6k despite its poor ability (~20% of control) to activate PI 3-kinase (54). In contrast, overstimulation of a rapamycin-sensitive pathway by prolonged hyperinsulinemia leads to a reduction in Akt phosphorylation in 3T3-L1 adipocytes (39), suggesting that a more chronic activation of this pathway is needed for impairing Akt activation by insulin. In agreement with the latter proposition, we have shown that long term (4 h) insulin stimulation reduces insulin-induced Akt phosphorylation in amino acid-treated, but not in amino acid-deprived, cells. Since Akt activation is required to activate the mTOR pathway (13–17), this might represent an adaptive mechanism to restrain the insulin-resistant effect of amino acids.

The functional significance of amino acid-induced activation of the mTOR/p70S6k pathway in the regulation of insulin action was confirmed by the finding that acute exposure to amino acids (1 h) caused a dose-dependent inhibition of insulin-stimulated glucose transport in L6 muscle cells in a rapamycin-sensitive manner. The mTOR/p70S6k pathway may therefore play an important role in the blunting effect of amino acids on whole-body and skeletal muscle glucose disposal in humans (23–26). These findings are also relevant to our recent observations that feeding rats with cod protein as compared with casein prevented the development of insulin resistance caused by high fat feeding (55). The plasma amino acid profiles of the casein- and cod protein-fed animals were quite different in that study. Of particular interest is that the concentration of several amino acids causing inhibition of insulin action (*e.g.* leucine, threonine, and tyrosine, see Fig. 1*C*) were found to be more

[2] F. Tremblay and A. Marette, unpublished observations.

elevated in the plasma of rats fed casein (60 ± 12%) as compared with those fed with cod protein (55). It is therefore tempting to speculate that dietary casein promotes muscle insulin resistance in high fat-fed rats because casein-derived amino acids induce greater mTOR activation as compared with cod protein-derived amino acids. The effect of feeding various dietary proteins on mTOR activation and insulin action in muscle *in vivo* as well as its reversal by rapamycin will be tested in the near future.

In conclusion, the present study provides a molecular basis for the observed decrease in insulin-mediated glucose transport (23–26) and PI 3-kinase activation (7) by amino acids in muscle. Our results demonstrate that amino acids potentiate the activation of the mTOR/p70S6k pathway by insulin and that this causes an accelerated temporal deactivation of PI 3-kinase activity. The data further show that the insulin-resistant effect of amino acids involves a rapamycin-sensitive increase in Ser/Thr phosphorylation of IRS-1 and impaired recruitment of PI 3-kinase to IRS-1. The mTOR/p70S6k pathway was also shown to participate in the degradation of IRS-1 during prolonged hyperinsulinemia. Further studies will be needed to test whether amino acids increase IRS-1 Ser/Thr phosphorylation by activation of mTOR itself or an mTOR-controlled kinase or if a rapamycin-sensitive inhibition of a Ser/Thr phosphatase is involved.

## REFERENCES


1. Van Sluijters, D. A., Dubbelhuis, P. F., Blommaart, E. F., and Meijer, A. J. (2000) *Biochem. J.* **351,** 545–550
2. Shah, O. J., Anthony, J. C., Kimball, S. R., and Jefferson, L. S. (2000) *Am. J. Physiol. Endocrinol. Metab.* **279,** 715–729
3. Xu, G., Marshall, C. A., Lin, T. A., Kwon, G., Munivenkatappa, R. B., Hill, J. R., Lawrence, J. C., Jr., and McDaniel, M. L. (1998) *J. Biol. Chem.* **273,** 4485–4491
4. Iiboshi, Y., Papst, P. J., Kawasome, H., Hosoi, H., Abraham, R. T., Houghton, P. J., and Terada, N. (1999) *J. Biol. Chem.* **274,** 1092–1099
5. Hara, K., Yonezawa, K., Weng, Q. P., Kozlowski, M. T., Belham, C., and Avruch, J. (1998) *J. Biol. Chem.* **273,** 14484–14494
6. Wang, X., Campbell, L. E., Miller, C. M., and Proud, C. G. (1998) *Biochem. J.* **334,** 261–267
7. Patti, M. E., Brambilla, E., Luzi, L., Landaker, E. J., and Kahn, C. R. (1998) *J. Clin. Invest.* **101,** 1519–1529
8. Fox, H. L., Kimball, S. R., Jefferson, L. S., and Lynch, C. J. (1998) *Am. J. Physiol.* **274,** C206–C213
9. Rhoads, R. E. (1999) *J. Biol. Chem.* **274,** 30337–30340
10. Cheatham, B., Vlahos, C. J., Cheatham, L., Wang, L., Blenis, J., and Kahn, C. R. (1994) *Mol. Cell. Biol.* **14,** 4902–4911
11. Mendez, R., Myers, M. G., Jr., White, M. F., and Rhoads, R. E. (1996) *Mol. Cell. Biol.* **16,** 2857–2864
12. Sharma, P. M., Egawa, K., Huang, Y., Martin, J. L., Huvar, I., Boss, G. R., and Olefsky, J. M. (1998) *J. Biol. Chem.* **273,** 18528–18537
13. Vanhaesebroeck, B., and Alessi, D. R. (2000) *Biochem. J.* **346,** 561–576
14. Nave, B. T., Ouwens, M., Withers, D. J., Alessi, D. R., and Shepherd, P. R. (1999) *Biochem. J.* **344,** 427–431
15. Sekulic, A., Hudson, C. C., Homme, J. L., Yin, P., Otterness, D. M., Karnitz, L. M., and Abraham, R. T. (2000) *Cancer Res.* **60,** 3504–3513
16. Scott, P. H., Brunn, G. J., Kohn, A. D., Roth, R. A., and Lawrence, J. C., Jr. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7772–7777
17. Shepherd, P. R., Withers, D. J., and Siddle, K. (1998) *Biochem. J.* **333,** 471–490
18. Anthony, J. C., Yoshizawa, F., Anthony, T. G., Vary, T. C., Jefferson, L. S., and Kimball, S. R. (2000) *J. Nutr.* **130,** 2413–2419
19. Svanberg, E., Jefferson, L. S., Lundholm, K., and Kimball, S. R. (1997) *Am. J. Physiol.* **272,** E841–E847
20. Shah, O. J., Antonetti, D. A., Kimball, S. R., and Jefferson, L. S. (1999) *J. Biol. Chem.* **274,** 36168–36175
21. Shigemitsu, K., Tsujishita, Y., Hara, K., Nanahoshi, M., Avruch, J., and Yonezawa, K. (1999) *J. Biol. Chem.* **274,** 1058–1065
22. Mortimore, G. E., Poso, A. R., and Lardeux, B. R. (1989) *Diabetes Metab. Rev.* **5,** 49–70
23. Tessari, P., Inchiostro, S., Biolo, G., Duner, E., Nosadini, R., Tiengo, A., and Crepaldi, G. (1985) *Diabetologia* **28,** 870–872
24. Pisters, P. W., Restifo, N. P., Cersosimo, E., and Brennan, M. F. (1991) *Metabolism* **40,** 59–65
25. Flakoll, P. J., Wentzel, L. S., Rice, D. E., Hill, J. O., and Abumrad, N. N. (1992) *Diabetologia* **35,** 357–366
26. Abumrad, N. N., Robinson, R. P., Gooch, B. R., and Lacy, W. W. (1982) *J. Surg. Res.* **32,** 453–463
27. Czech, M. P., and Corvera, S. (1999) *J. Biol. Chem.* **274,** 1865–1868
28. Wang, Q., Somwar, R., Bilan, P. J., Liu, Z., Jin, J., Woodgett, J. R., and Klip, A. (1999) *Mol. Cell. Biol.* **19,** 4008–4018
29. Hajduch, E., Alessi, D. R., Hemmings, B. A., and Hundal, H. S. (1998) *Diabetes* **47,** 1006–1013
30. Bedard, S., Marcotte, B., and Marette, A. (1997) *Biochem. J.* **325,** 487–493
31. Marshall, S., Garvey, W. T., and Traxinger, R. R. (1991) *FASEB J.* **5,** 3031–3036
32. Traxinger, R. R., and Marshall, S. (1989) *J. Biol. Chem.* **264,** 20910–20916
33. Davies, S. P., Reddy, H., Caivano, M., and Cohen, P. (2000) *Biochem. J.* **351,** 95–105
34. Abraham, R. T., and Wiederrecht, G. J. (1996) *Annu. Rev. Immunol.* **14,** 483–510
35. Higaki, Y., Wojtaszewski, J. F., Hirshman, M. F., Withers, D. J., Towery, H., White, M. F., and Goodyear, L. J. (1999) *J. Biol. Chem.* **274,** 20791–20795
36. Kido, Y., Burks, D. J., Withers, D., Bruning, J. C., Kahn, C. R., White, M. F., and Accili, D. (2000) *J. Clin. Invest.* **105,** 199–205
37. Yang, C., Watson, R. T., Elmendorf, J. S., Sacks, D. B., and Pessin, J. E. (2000) *Mol. Endocrinol.* **14,** 317–326
38. Nakashima, N., Sharma, P. M., Imamura, T., Bookstein, R., and Olefsky, J. M. (2000) *J. Biol. Chem.* **275,** 12889–12895
39. Haruta, T., Uno, T., Kawahara, J., Takano, A., Egawa, K., Sharma, P. M., Olefsky, J. M., and Kobayashi, M. (2000) *Mol. Endocrinol.* **14,** 783–794
40. Li, J., DeFea, K., and Roth, R. A. (1999) *J. Biol. Chem.* **274,** 9351–9356
41. Tanti, J. F., Gremeaux, T., van Obberghen, E., and Le Marchand-Brustel, Y. (1994) *J. Biol. Chem.* **269,** 6051–6057
42. Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M. F., and Spiegelman, B. M. (1996) *Science* **271,** 665–668
43. De Fea, K., and Roth, R. A. (1997) *Biochemistry* **36,** 12939–12947
44. Pederson, T. M., Kramer, D. L., and Rondinone, C. M. (2001) *Diabetes* **50,** 24–31
45. Ozes, O. N., Akca, H., Mayo, L. D., Gustin, J. A., Maehama, T., Dixon, J. E., and Donner, D. B. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4640–4645
46. Brunn, G. J., Fadden, P., Haystead, T. A. J., and Lawrence, J. C., Jr. (1997) *J. Biol. Chem.* **272,** 32547–32550
47. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) *Nature* **352,** 73–77
48. Peterson, R. T., Desai, B. N., Hardwick, J. S., and Schreiber, S. L. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4438–4442
49. De Fea, K., and Roth, R. A. (1997) *J. Biol. Chem.* **272,** 31400–31406
50. Aguirre, V., Uchida, T., Yenush, L., Davis, R., and White, M. F. (2000) *J. Biol. Chem.* **275,** 9047–9054
51. Rui, L., Aguirre, V., Kim, J. K., Shulman, G. I., Lee, A., Corbould, A., Dunaif, A., and White, M. F. (2001) *J. Clin. Invest.* **107,** 181–189
52. Kim, Y. B., Nikoulina, S. E., Ciaraldi, T. P., Henry, R. R., and Kahn, B. B. (1999) *J. Clin. Invest.* **104,** 733–741
53. Kim, Y. B., Zhu, J. S., Zierath, J. R., Shen, H. Q., Baron, A. D., and Kahn, B. B. (1999) *Diabetes* **48,** 310–320
54. Kriauciunas, K. M., Myers, M. G., Jr., and Kahn, C. R. (2000) *Mol. Cell. Biol.* **20,** 6849–6859
55. Lavigne, C., Tremblay, F., Asselin, G., Jacques, H., and Marette, A. (2001) *Am. J. Physiol. Endocrinol. Metab.* **281,** 62–71

# Amino Acid and Insulin Signaling via the mTOR/p70 S6 Kinase Pathway: A NEGATIVE FEEDBACK MECHANISM LEADING TO INSULIN RESISTANCE IN SKELETAL MUSCLE CELLS

Frédéric Tremblay and André Marette

*J. Biol. Chem. 2001, 276:38052-38060.*
*originally published online October 5, 2001*

---