

Activation of the Mammalian Target of Rapamycin Pathway Acutely Inhibits Insulin Signaling to Akt and Glucose Transport in 3T3-L1 and Human Adipocytes

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The mammalian target of rapamycin (mTOR) pathway has recently emerged as a chronic modulator of insulin-mediated glucose metabolism. In this study, we evaluated the involvement of this pathway in the acute regulation of insulin action in both 3T3-L1 and human adipocytes. Insulin rapidly ($t_{1/2} = 5$ min) stimulated the mTOR pathway, as reflected by a 10-fold stimulation of 70-kDa ribosomal S6 kinase 1 (S6K1) activity in 3T3-L1 adipocytes. Inhibition of mTOR/S6K1 by rapamycin increased insulin-stimulated glucose transport by as much as 45% in 3T3-L1 adipocytes. Activation of mTOR/S6K1 by insulin was associated with a rapamycin-sensitive increase in Ser636/639 phosphorylation of insulin receptor substrate (IRS)-1 but, surprisingly, did not result in impaired IRS-1-associated phosphatidylinositol (PI) 3-kinase activity. However, insulin-induced activation of Akt was increased by rapamycin. Insulin also activated S6K1 and increased phosphorylation of IRS-1 on Ser636/639 in human adipocytes. As in murine cells,

rapamycin treatment of human adipocytes inhibited S6K1, blunted Ser636/639 phosphorylation of IRS-1, leading to increased Akt activation and glucose uptake by insulin. Further studies in 3T3-L1 adipocytes revealed that rapamycin prevented the relocalization of IRS-1 from the low-density membranes to the cytosol in response to insulin. Furthermore, inhibition of mTOR markedly potentiated the ability of insulin to increase PI 3,4,5-triphosphate levels concomitantly with an increased phosphorylation of Akt at the plasma membrane, low-density membranes, and cytosol. However, neither GLUT4 nor GLUT1 translocation induced by insulin were increased by rapamycin treatment. Taken together, these results indicate that the mTOR pathway is an important modulator of the signals involved in the acute regulation of insulin-stimulated glucose transport in 3T3-L1 and human adipocytes. (*Endocrinology* 146: 1328–1337, 2005)

INSULIN IS A PLEIOTROPIC hormone mediating a wide range of biological processes, among which stimulating glucose transport in skeletal muscle and adipose tissue in the postprandial state is the most prominent (1, 2). It exerts its effect by binding and activating its receptor, leading to the tyrosyl-phosphorylation of various intracellular targets, such as insulin receptor substrate (IRS)-1 and IRS-2. Once phosphorylated, IRSs serve as docking proteins for Src homology 2 (SH2)-domain containing molecules, including the regulatory subunit of phosphatidylinositol (PI)3-kinase, which then enables the activation of the p110 catalytic subunit and production of PI 3,4-bisphosphate [PI(3,4)P₂] and PI 3,4,5-triphosphate [PI(3,4,5)P₃] (3). It is believed that PI(3,4,5)P₃ is the major lipid product of PI 3-kinase that mediates activation of downstream effectors, Akt, and atypical protein kinase C (PKC), via the phosphoinositide-dependent protein

kinase (PKC) enzymes (4–6). The exact contribution of PI 3-kinase effectors in the stimulation of GLUT4 translocation remains unresolved, but evidence exists implicating both Akt (7, 8) and atypical PKC (9) in this process.

An impaired ability of insulin to lower blood glucose is an important feature of the insulin resistance syndrome encountered in type 2 diabetes (10, 11). Several mechanisms that may account for the reduction of insulin-stimulated glucose transport in muscle and adipose cells have been proposed. Of those, inhibitory phosphorylation of IRS-1 on Ser/Thr residues, which converts IRS-1 to an inhibitor of insulin signaling to PI 3-kinase/Akt, has been shown to be mediated by a plethora of kinases, such as c-jun N-terminal kinase (12), PKC- ζ (13, 14), ERK (15), and mammalian target of rapamycin (mTOR) (16, 17).

mTOR is a serine/threonine kinase whose activation is enhanced by both hormonal and nutritional inputs (18, 19). Although activation of mTOR by insulin is relayed through the insulin receptor/IRS/PI (3)k/Akt pathway, the mechanism by which amino acids activate this enzyme is still largely unknown. mTOR mediates translational events by modulating the phosphorylation/activation status of 70-kDa ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (20). This pathway has also been shown to participate in the desensitization of insulin action induced by chronic exposure to platelet-derived growth factor (21), TNF- α (17), insulin (22), and amino acids (23, 24) via an increased Ser/Thr phosphorylation of IRS-1.

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Abbreviations: DMSO, Dimethylsulfoxide; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; EBSS, Earle's balanced salt solution; FBS, fetal bovine serum; IRS, insulin receptor substrate; LDM, low-density membrane(s); mTOR, mammalian target of rapamycin; PDK, phosphoinositide-dependent protein kinase; PI, phosphatidylinositol; PI(3,4)P₂, PI 3,4-bisphosphate; PI(3,4,5)P₃, PI 3,4,5-triphosphate; PKC, protein kinase C; PM, plasma membrane; RDU, relative densitometric units; S6K1, 70-kDa ribosomal S6 kinase 1; SH2, Src homology 2.

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We have recently observed that acute exposure of skeletal muscle cells to insulin and amino acids activates mTOR, leading to a rapid impairment in IRS-1-associated PI 3-kinase, while having no effect on its downstream effector, Akt (24). In 3T3-L1 adipocytes, chronic activation of mTOR by insulin is known to induce Ser/Thr phosphorylation of IRS-1 followed by its proteosomal degradation (22), a process that has been shown to be enhanced by addition of amino acids in the incubation medium (23). Although there is a growing number of studies indicating that chronic activation of mTOR is a mediator of insulin resistance in both muscle and adipose cells, very little is known regarding its possible role in the acute regulation of insulin action and signaling. In the present study, we evaluated the involvement of the mTOR pathway in the acute regulation of insulin-stimulated glucose transport in 3T3-L1 adipocytes. We found that the mTOR pathway rapidly modulates insulin-induced glucose uptake in fat cells and that this effect is linked to an acute regulation of PI(3,4,5)P₃ levels and Akt activation.

Materials and Methods

Materials

All cell culture solutions and supplements were purchased from Life Technologies, Inc. (Burlington, Ontario, Canada) except for fetal bovine serum (FBS), which was purchased from Wisent (St-Bruno, Québec, Canada). Reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Mississauga, Ontario, Canada). ECL and 2-[³H]deoxyglucose were from NEN Life Science Products (Boston, MA). [γ -³²P]ATP, myo[2-³H]inositol, protein A- and G-Sepharose, and antimouse or antirabbit IgG conjugated to horseradish peroxidase were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, Québec, Canada). Polyclonal antibodies against IRS-1 (raised against 20 C-terminal amino acids), S6K1 (raised against 18 C-terminal amino acids) and Akt (raised against 20 C-terminal amino acids), S6K1 substrate, protein kinase A, and PKC inhibitors were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-specific antibodies against Akt (Ser473 and Thr308), IRS-1 (Ser636/639), and S6K1 (Thr421/Ser424) were from New England Biolabs (Beverly, MA). Antibodies against IRS-1 and p85/PI 3-kinase, and Akt substrate (Crosstide) were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-GLUT4 polyclonal antibody was purchased from Santa Cruz Biotechnology, and anti-GLUT1 polyclonal antibody was a generous gift from Dr. Amira Klip (Hospital for Sick Children, Toronto, Ontario, Canada). Human insulin was obtained from Eli Lilly (Toronto, Ontario, Canada). Rapamycin was purchased from Biomol (Plymouth Meeting, PA). L- α -PI was from Avanti Polar Lipids (Alabaster, AL). Oxalate-treated thin-layer chromatography silica gel H plates were obtained from Analtech (Newark, DE). All other chemicals were of the highest analytical grade.

Cell culture and treatment

3T3-L1 cells (kind gift of Dr. A. Klip, Hospital for Sick Children) were grown in monolayer culture in DMEM supplemented with 20% (vol/vol) calf serum and 1% (vol/vol) antibiotic solution in an atmosphere of 10% CO₂ at 37 C. Two days post confluence, 3T3-L1 fibroblasts were differentiated into adipocytes in DMEM containing 10% FBS, 115 μ g/ml 3-isobutyl-1-methylxanthine, 390 ng/ml dexamethasone, and 10 μ g/ml insulin for 4 d, then the medium was replaced with DMEM supplemented with 10% FBS and 5 μ g/ml insulin for an additional 4–6 d. Medium was then replaced by DMEM containing 10% FBS until more than 85% of the cells exhibited an adipocyte morphology, typically between 8–12 d post differentiation. 3T3-L1 adipocytes were deprived of serum 2 h before experimental treatments. Then, adipocytes were incubated either in an amino acid-free medium [Earle's balanced salt solution (EBSS)] or in EBSS containing 2 \times amino acids mixture as found in MEM, for 1 h, as previously described (24). Vehicle [0.01% dimethylsulfoxide (DMSO)] or rapamycin (25 nM) was then added during the

1-h incubation and stimulated with or without insulin as indicated in figure legends.

Isolation and differentiation of human preadipocytes

The sc adipose tissue was obtained from four female patients undergoing abdominoplasty (approved by the Research Ethics Committee of the Ottawa Health Research Institute). The age of the subjects was 44 \pm 3 yr (mean \pm SE), and mean body mass index was 26 \pm 3. Preadipocytes were isolated as previously described, with minor modifications (25). Tissue was separated from connective tissue and capillaries by dissection, and then digested with collagenase CLS type 1 (200 U/g tissue; Worthington, Lakewood, NJ). The digested tissue was subjected to progressive size filtration and centrifugation, followed by incubation in erythrocyte lysis buffer (155 mM NH₄Cl, 5.7 mM K₂HPO₄, 0.1 mM EDTA, pH 7.3). Preadipocytes were seeded at a density of 3 \times 10⁴ cells/cm² and grown to confluence in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 U/ml nystatin. To induce differentiation, cultures of preadipocytes were maintained in the same media supplemented with 5 μ g/ml insulin, 100 μ M indomethacin, 0.5 μ M dexamethasone, and 0.25 mM 3-isobutyl-1-methylxanthine. After approximately 2 wk, differentiated adipocytes were deprived of serum 2 h before experimental treatments. Then, adipocytes were incubated in EBSS containing 2 \times amino acids mixture as found in MEM, for 1 h, and then stimulated with insulin (10 or 500 nM) for 30 min and processed for immunoblotting or 2-deoxyglucose uptake. Vehicle (0.01% DMSO) or rapamycin (25 nM) was also added during the 1-h incubation with amino acids.

Measurement of 2-deoxyglucose and 3-O-methylglucose uptake

Uptake of 2-deoxyglucose in adipocytes was determined as previously described (26). In brief, cells were incubated for 8 min (3T3-L1) or 15 min (human adipocytes) in HEPES-buffered saline containing 10 μ M unlabeled 2-deoxyglucose and 10 μ M D-2-deoxy-[³H]glucose (0.5 μ Ci/ml). The reaction was terminated by washing three times with ice-cold 0.9% NaCl (wt/vol). Nonspecific uptake was determined in the presence of 1 mM HgCl₂. Cell-associated radioactivity was determined by lysing the cells with 0.05 N NaOH, followed by liquid scintillation counting. 3-O-Methylglucose uptake was measured in a similar fashion with the following differences: 50 μ M 3-O-methylglucose (4 μ Ci/ml) was added to HEPES-buffered saline and uptake allowed to occur for 30 sec, a period over which 3-O-methylglucose uptake is known to be linear. Cell monolayers were then washed three times with 1 mM HgCl₂ in saline solution before lysis with 0.05 N NaOH.

S6K1 assay

S6K1 activity was measured using a method described by Somwar *et al.* (27). After experimental treatment, medium was removed, and cells were rinsed twice in ice-cold PBS and lysed in 50 mM HEPES, pH 7.5; 150 mM NaCl; 1 mM EDTA; 20 mM β -glycerophosphate; 1% NP-40; 2 mM Na₃VO₄; and 10 mM NaF. Lysates (200–300 μ g) were immunoprecipitated with 2 μ g anti-S6K1 coupled to protein A-Sepharose for at least 2 h at 4 C. Immune complexes were washed three times in 50 mM Tris-acetate, pH 8.0; 5 mM β -glycerophosphate; 1 mM EDTA; 1 mM EGTA; 0.1% 2-mercaptoethanol; 2 mM Na₃VO₄; and 10 mM NaF and twice in kinase buffer (50 mM 3-[N-morpholino]propanesulfonic acid, pH 7.5; 25 mM β -glycerophosphate; 5 mM EGTA; 2 mM EDTA; 20 mM MgCl₂; 1 mM dithiothreitol). The reaction was started by adding 50 μ l kinase buffer (containing 100 μ M ATP, 1 μ M protein kinase A and PKC inhibitors, 2 μ Ci [γ -³²P]ATP, and 100 μ M S6K1 substrate) for 10 min at 30 C. Reaction product was spotted on p81 filter paper (Whatman, Clifton, NJ) and washed 3 \times 15 min with 1% phosphoric acid. Incorporated radioactivity was determined by liquid scintillation counting.

Immunoprecipitation and immunoblotting

IRS-1 was immunoprecipitated from 500 μ g cell lysates or 150 μ g membrane fractions and subjected to SDS-PAGE analysis as previously described (24, 28).

PI 3-kinase assay

PI 3-kinase activity was measured in IRS-1 immunoprecipitates as previously described (24, 28).

Akt kinase assay

After experimental treatment, medium was removed, cells were rinsed twice in ice-cold PBS and lysed in 50 mM Tris, pH 7.4; 1 mM EDTA; 1 mM EGTA; 10 mM β -glycerophosphate; 0.1% 2-mercaptoethanol; 0.1% Triton X-100; 2 mM Na_3VO_4 ; and 10 mM NaF. Lysates (200–300 μg) were immunoprecipitated with 2 μg anti-Akt1, which cross-reacts with Akt2, coupled to protein G-Sepharose for 2 h at 4 C. Immune complexes were washed three times in lysis buffer containing 500 mM NaCl and two 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 μl kinase buffer (containing 16 μM ATP, 2 μCi [γ - ^{32}P]ATP, and 60 μM Crosstide) for 30 min at 30 C. Reaction product was spotted on p81 filter paper (Whatman) and washed 3 \times 15 min with 1% phosphoric acid. Incorporated radioactivity was determined by liquid scintillation counting.

Subcellular fractionation and GLUT4/GLUT1 translocation

Membrane fractionation of 3T3-L1 adipocytes was performed essentially as described by Takano *et al.* (23). After experimental treatment, medium was removed and cells were rinsed twice in ice-cold PBS, gently scraped in HES buffer (255 mM sucrose; 20 mM HEPES, pH 7.5; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1 mM Na_3VO_4 ; and 1 mM NaF) and homogenized using a Teflon-glass potter (26 strokes). Homogenates were centrifuged at $19,000 \times g$ for 20 min. The remaining supernatant was centrifuged at $41,000 \times g$ for 20 min. The resulting supernatant was centrifuged at $250,000 \times g$ for 90 min to obtain the low-density membrane (LDM) and the cytosol in the pellet and the supernatant fractions, respectively. Plasma membrane (PM) fraction was obtained by resuspending the pellet obtained from the $19,000 \times g$ spin in HES buffer, layering it onto a sucrose cushion (1.12 M), and centrifuging it at $100,000 \times g$ in a SW-40 swinging bucket. All membrane fractions were resuspended in HES buffer supplemented with protease inhibitors cocktail and kept at -80 C until further processing.

Equal amounts of protein from both the LDM and PM fractions were analyzed by immunoblot with antibodies directed against IRS-1, pSer473-Akt, GLUT4, or GLUT1, as indicated in legends to figures. After overnight incubation (4 C) with primary antibodies, membranes were incubated with either antimouse or antirabbit IgG conjugated to horseradish peroxidase for 1 h. Immunoreactive bands were detected by the enhanced chemiluminescence method (Renaissance ECL kit, NEN Life Science Products Life Science). The relative abundance of GLUT1 and GLUT4 in the PM and LDM fractions was estimated from the density of the immunoreactive bands and the recovered protein amount in each fraction. The recovery of proteins in the PM and LDM fractions was not different among experimental groups (in μg ; PM, basal: 1037 ± 226 , rapamycin: 1041 ± 221 , insulin: 1091 ± 225 , insulin + rapamycin: 1146 ± 163 ; LDM, basal: 509 ± 128 , rapamycin: 539 ± 125 , insulin: 447 ± 183 , insulin + rapamycin: 547 ± 133).

Radioisotope labeling and inositol phospholipid analysis

Differentiated 3T3-L1 adipocytes were placed in inositol-free DMEM supplemented with 10% calf serum for 24 h, and then labeled with 100 $\mu\text{Ci/ml}$ ^3H -myo-inositol in the presence of inositol-free DMEM supplemented with 0.5% calf serum for another 16 h. The cells were then stimulated as indicated. Lipids were extracted and deacylated directly, as described (29, 30). The resulting glycerolphosphoinositols were separated on a Whatman Partisphere 5 SAX column. Peaks representing the deacylated products of ^3H -PI(3,4) P_2 and ^3H -PI(3,4,5) P_3 were identified by measuring the radioactivity contained in 1-ml fractions with a Beckman LS3801 scintillation counter, as previously described (29, 30).

Statistical analysis

The effects of amino acids, insulin, and rapamycin were compared by ANOVA test followed by Bonferonni/Dunn *post hoc* analysis. For clarity

reasons, only the effects of rapamycin and amino acids were statistically represented in the figures. Differences were considered to be statistically significant at $P < 0.05$.

Results

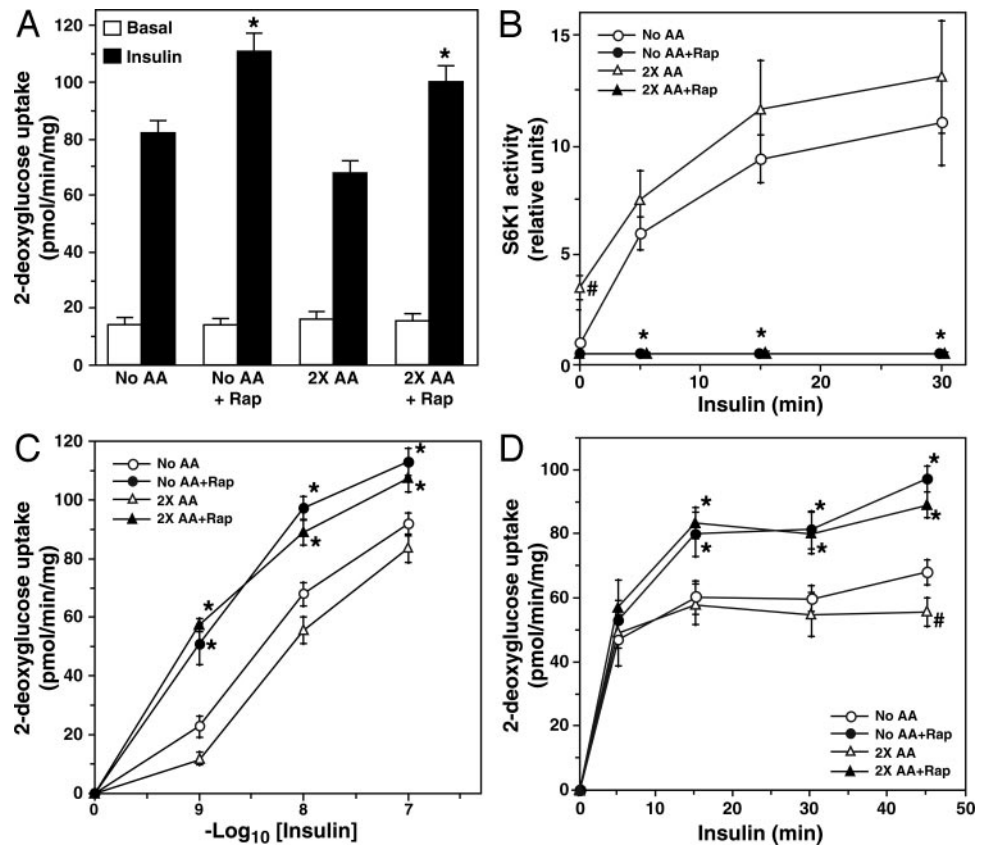
Effects of insulin and amino acids on insulin-stimulated glucose transport and its relationship with S6K1 activity

In our previous study, we have found that incubation of skeletal muscle cells with amino acids rapidly impairs the ability of insulin to stimulate glucose transport, compared with cells that were deprived of this nutrient (24). This insulin-resistant state was associated with enhanced activation of mTOR/S6K1 and could be entirely reversed by co-incubating cells with the mTOR inhibitor, rapamycin. We were therefore interested in determining whether this auto-regulation of glucose transport by amino acids could be re-produced in adipocytes, another important cellular target of insulin. 3T3-L1 adipocytes were incubated in medium that contained either no amino acids or a 2 \times amino acids mixture for 1 h, then glucose transport was measured. Amino acids slightly decreased insulin-stimulated glucose transport ($\sim 15\%$), but this failed to reach statistical significance (Fig. 1A). However, the stimulatory effect of insulin on glucose transport was markedly enhanced ($\sim 35\text{--}45\%$, $P < 0.05$) by treating cells with rapamycin (Fig. 1A). Surprisingly, the effect of rapamycin on insulin action was observed even in amino acid-deprived cells, which might indicate that the mTOR pathway is less dependent on availability of amino acids in adipocytes than it is in L6 myocytes. Indeed, whereas insulin alone (*i.e.* in the absence of amino acids for 1 h) was able to activate S6K1 in a time-dependent manner, reaching a near-maximal state of activation after 15 min of hormonal stimulation (~ 10 -fold over basal value, see Fig. 1B), supplementation with amino acids failed to potentiate S6K1 activation by insulin (Fig. 1B), as opposed to what we previously reported in L6 myocytes (24). As expected, blockade of mTOR by rapamycin completely abolished the effect of both insulin and amino acids on S6K1 activity ($P < 0.05$; Fig. 1B).

To further characterize the enhancement of insulin-stimulated glucose transport after mTOR inhibition, insulin dose-response and time-course analyses were performed (Fig. 1, C and D). We observed that rapamycin increases glucose uptake at all doses of insulin tested ($P < 0.05$ vs. insulin alone), which was reflected both by an increase in insulin sensitivity and maximal responsiveness (Fig. 1C). Furthermore, we confirmed the lack of importance of amino acids in this mTOR-dependent modulation of glucose transport in 3T3-L1 adipocytes, regardless of the insulin concentration used (Fig. 1C). Importantly, it was found that rapamycin-mediated increase in glucose uptake occurred rapidly, being readily observable after only 15 min of insulin stimulation ($P < 0.05$; Fig. 1D). This phenomenon was maintained for up to 45 min of insulin stimulation ($P < 0.05$) and, again, was minimally affected by amino acids (Fig. 1D).

To assess whether rapamycin enhanced 2-deoxyglucose transport or phosphorylation, we also measured the effect of the mTOR inhibitor on the uptake of 3-O-methylglucose, a glucose analog that is transported by glucose transporters but not phosphorylated by hexokinase. We found that rapamycin increased insulin-mediated 3-O-methylglucose (by

FIG. 1. Effect of insulin and amino acids on insulin-stimulated glucose transport and its relationship with S6K1 activity. Serum-deprived 3T3-L1 cells were treated with no amino acids, no amino acids + rapamycin, 2× amino acids, and 2× amino acids + rapamycin for 1 h. A, Cells were stimulated or not with 10 nM insulin for the last 45 min of incubation before glucose transport measurements as described under *Materials and Methods*. B, Cells were stimulated with 10 nM insulin for the last 5, 15, and 30 min of incubation before S6K1 activity measurements as described in *Materials and Methods*. C, Cells were stimulated with 1, 10, or 100 nM insulin for the last 45 min of incubation before glucose transport measurements as described in *Materials and Methods*. D, Cells were stimulated with 10 nM insulin for the last 5, 15, 30, and 45 min of incubation before glucose transport measurement as described under *Materials and Methods*. The means \pm SE from at least 12 (for A and C), 5 (for B), and 4 (for D) individual experiments, each performed in triplicate (for A, C, and D), are shown. For C and D, results are expressed as net increases in glucose transport mediated by insulin (insulin minus basal). *, $P < 0.05$ vs. corresponding cells treated without rapamycin; #, $P < 0.05$ vs. corresponding cells treated without amino acids.



43% \pm 18 and by 39% \pm 12 for amino acid-deprived and amino acid-treated cells, respectively; $P < 0.05$) to a similar extent as 2-deoxyglucose, suggesting that rapamycin mediates its effect by promoting hexose transport rather than hexokinase-dependent phosphorylation.

Effect of mTOR/S6K1 activation on IRS-1 Ser/Thr phosphorylation and PI 3-kinase recruitment and activity

One mechanism by which activation of mTOR leads to insulin resistance is through increased Ser/Thr phosphorylation of IRS-1 (22–24). We therefore next determined whether rapamycin could rapidly modulate IRS-1 Ser/Thr phosphorylation in adipocytes. Electrophoretic mobility of IRS-1 on SDS-PAGE was examined in cells that were treated or not with insulin (0–30 min), amino acids, and/or rapamycin. We found that insulin decreased the electrophoretic mobility of IRS-1 in a time-dependent manner in both amino acid-treated and -deprived cells (Fig. 2A). Such behavior of IRS-1 on SDS-PAGE has been shown to be mainly due to an increased phosphorylation of Ser/Thr residues, and this was largely prevented by pretreating cells with rapamycin (Fig. 2A). We further explored the possibility that Ser636/639, serine residues in IRS-1 that were shown to be phosphorylated by mTOR in TNF- α -treated myocytes (17), could be phosphorylated in cells where the mTOR/S6K1 pathway is acutely activated by insulin. We observed that insulin treatment for 5 or 30 min resulted in an increased pSer636/639-IRS-1 immunoreactivity in both amino acid-deprived [$+55 \pm 24$ relative densitometric units (RDU) and $+63 \pm 14$ RDU after 5 and 30 min, respectively, $P < 0.05$] and amino acid-

treated cells ($+93 \pm 38$ RDU and $+93 \pm 6$ RDU after 5 or 30 min, respectively, $P < 0.05$), an effect that was almost totally abolished by coincubation with rapamycin ($P < 0.05$). Whereas amino acids had little effect on insulin-mediated glucose transport (See Fig. 1, A, C, and D), they significantly increased the effect of insulin to promote phosphorylation of IRS-1 on Ser636/639 but only after 30 min of insulin stimulation ($+63 \pm 14$ RDU vs. $+93 \pm 6$ RDU for amino-deprived and -treated cells, respectively, $P < 0.05$; Fig. 2A).

To evaluate the functional consequences of Ser/Thr phosphorylation of IRS-1 in cells where mTOR is activated, PI 3-kinase recruitment and activity were measured in IRS-1 immunoprecipitates. Stimulating 3T3-L1 adipocytes for 5 or 30 min with insulin increased the amount of p85 regulatory subunit of PI 3-kinase recovered in IRS-1 immunoprecipitates (Fig. 2B). As expected from our glucose transport studies, PI 3-kinase recruitment by tyrosyl-phosphorylated IRS-1 was marginally influenced by amino acids. Surprisingly, however, rapamycin did not significantly alter binding of PI 3-kinase to IRS-1 (Fig. 2B). Consistent with the PI 3-kinase recruitment data, IRS-1-associated PI 3-kinase activity was strongly enhanced by insulin (more than 10-fold) but was affected neither by amino acids nor by rapamycin (Fig. 2C).

Effect of mTOR/S6K1 activation on Akt phosphorylation and activity

Activation of Akt, a downstream effector of PI 3-kinase, was next determined. Insulin-induced phosphorylation of Akt on both regulatory sites (Ser473 and Thr308) was sig-

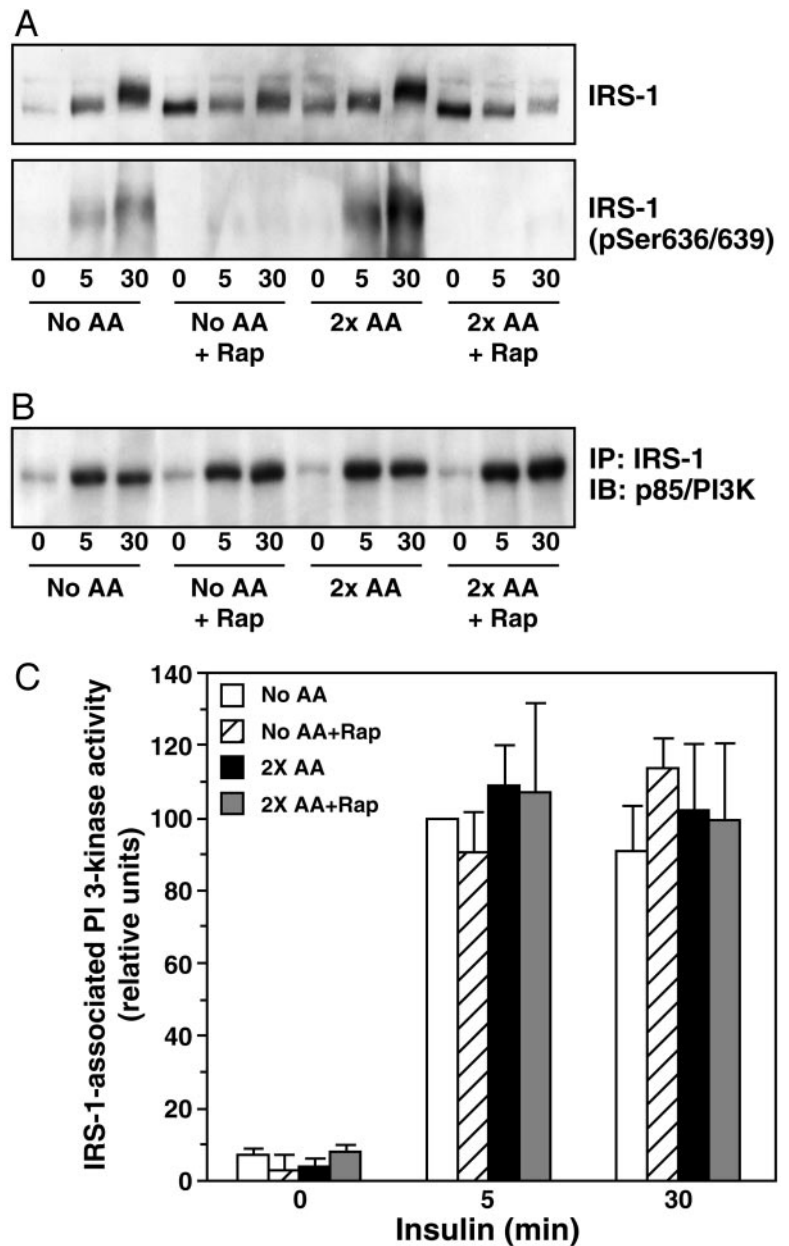


FIG. 2. Effect of mTOR/S6K1 activation on IRS-1 Ser/Thr phosphorylation and PI 3-kinase recruitment and activity. A, Serum-deprived 3T3-L1 cells were incubated either in amino acid-free medium (no AA) or in medium containing 2× amino acids mixture as found in MEM (2× AA) for 1 h. Vehicle (0.01% DMSO) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated or not with 10 nM insulin for the last 5 or 30 min of incubation before determining IRS-1 electrophoretic mobility on SDS-PAGE (*upper panel*) and pSer636/639-IRS-1 immunoreactivity (*lower panel*) as described in *Materials and Methods*. C and D, Cells were stimulated or not with 10 nM insulin for the last 5 or 30 min of incubation, rinsed twice in ice-cold PBS, and lysed as described in *Materials and Methods*. Measurement of B, association of the p85 subunit of PI 3-kinase with IRS-1, and C, PI(3)P produced by IRS-1-associated PI 3-kinase activity, were determined as described in *Materials and Methods*. For A and B, results are one representative experiment repeated at least three times. The means \pm SE from at least eight individual experiments are shown in C. IP, Immunoprecipitation; IB, immunoblotting.

nificantly enhanced by rapamycin (Fig. 3A). Quantification of immunoblots revealed that rapamycin increased phosphorylation of Akt on both Thr308 ($+61\% \pm 7$ and $+36\% \pm 19$ for amino acid-deprived and -treated cells, respectively; $P < 0.05$) and Ser473 ($+68\% \pm 14$ and $+72\% \pm 33$ for amino acid-deprived and -treated cells, respectively; $P < 0.05$) in adipocytes stimulated by insulin for 30 min. Inhibition of mTOR also potentiated the ability of insulin to increase Akt kinase activity, reaching statistical significance after 30 min of insulin stimulation ($P < 0.05$; Fig. 3B). This effect was not modulated by the presence of amino acids.

The mTOR pathway acutely controls insulin signaling to Akt and glucose transport in human adipocytes

To further confirm the biological significance of mTOR-dependent modulation of insulin signaling to glucose trans-

port, we next determined whether activation of the mTOR pathway also modulates insulin signaling to Akt and glucose transport in human adipocytes. Differentiated human adipocytes were treated with or without rapamycin and stimulated or not with insulin for 30 min, exactly as we did for 3T3-L1 cells. As we previously observed in murine adipocytes, inhibition of mTOR by rapamycin in human adipocytes prevented activation of S6K1 ($P < 0.05$, Fig. 4A), decreased phosphorylation of IRS-1 on Ser636/639 ($P < 0.05$, Fig. 4B), and enhanced phosphorylation of Akt ($P < 0.05$, Fig. 4C). Furthermore, we observed that rapamycin treatment increased insulin-stimulated 2-deoxyglucose uptake in human adipocytes, raising insulin action from 1.38-fold to a 1.63-fold stimulation of glucose uptake ($P < 0.05$, Fig. 4D). These experiments confirm that the mTOR pathway acutely controls insulin signaling to glucose transport through a very

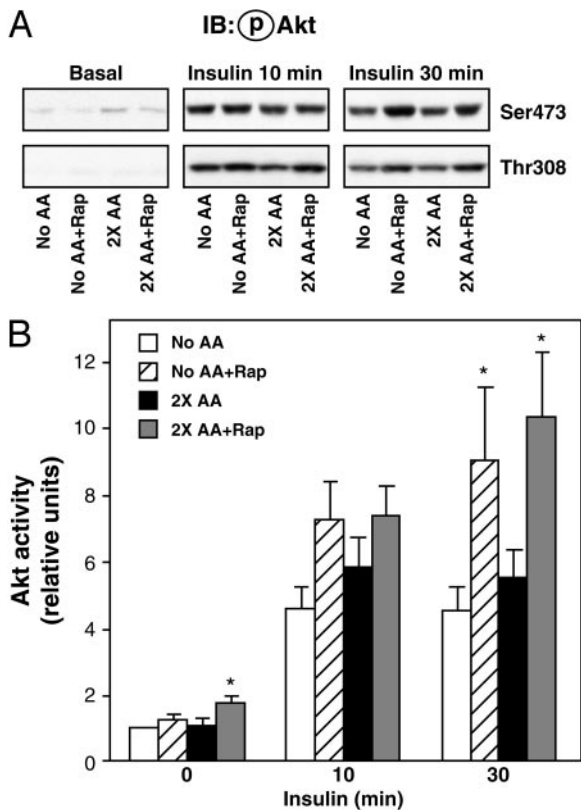


FIG. 3. Effect of mTOR/S6K1 activation on Akt phosphorylation and activity. Serum-deprived 3T3-L1 cells were incubated either in amino acid-free medium or in medium containing 2× amino acids mixture as found in MEM for 1 h. Vehicle (0.01% DMSO) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated or not with 10 nM insulin for the last 10 or 30 min of incubation, rinsed twice in ice-cold PBS, and lysed as described in *Materials and Methods*. Measurement of A, phosphorylation of Akt on Ser473 (upper panel) and Thr308 (lower panel), and B, Akt activity, were determined as described in *Materials and Methods*. For A, results are one representative experiment repeated at least three times. The means \pm SE from at least eight individual experiments are shown in B. *, $P < 0.05$ vs. corresponding cells treated without rapamycin.

similar, if not identical, mechanism in both murine and human adipocytes.

The mTOR pathway modulates IRS-1 cellular distribution and PI(3,4,5)P₃ levels in 3T3-L1 adipocytes

The observed dissociation between PI 3-kinase and Akt activities in rapamycin-treated 3T3-L1 adipocytes led us to test whether mTOR inhibition causes: 1) a retention of IRS-1/PI 3-kinase in a subcellular compartment where Akt activation is favored; and/or 2) an increased *in vivo* production of PI(3,4)P₂ or PI(3,4,5)P₃ resulting in a greater activation of Akt by PDK-1/2 enzymes. The former hypothesis is based on previous reports indicating that inhibitory Ser/Thr phosphorylation of IRS-1 is associated with a subcellular redistribution of IRS-1 (23, 31). Here we observed that, in the basal state, IRS-1 was mainly localized intracellularly in the LDM and, upon insulin stimulation, migrates to the cytosol (Fig. 5A). However, when cells were treated with rapamycin, IRS-1 was sequestered in the LDM in the insulin-stimulated state, and its appearance in the cytosol was largely prevented

compared with adipocytes in which the mTOR pathway was activated (Fig. 5A). Interestingly, the heavily Ser/Thr-phosphorylated form of IRS-1 (as evidenced by the gel mobility shift) in insulin-treated cells could be detected in both the LDM and cytosol fractions but not in rapamycin-treated adipocytes. Furthermore, retention of IRS-1 in the LDM isolated from rapamycin-treated cells was associated with a greater recruitment of the p85 subunit of PI 3-kinase to IRS-1 ($P < 0.05$ vs. cells treated with insulin alone; Fig. 5A).

To test our second hypothesis, PI(3,4)P₂ and PI(3,4,5)P₃ levels in both control and rapamycin-treated cells were measured. In the unstimulated state, PI(3,4)P₂ and PI(3,4,5)P₃ levels were found to be similar among both control and rapamycin-treated cells (Fig. 5B). Insulin alone raised the levels of PI(3,4)P₂ and PI(3,4,5)P₃, although it did not reach statistical significance. However, rapamycin treatment significantly raised PI(3,4,5)P₃, but not PI(3,4)P₂, levels in insulin-stimulated cells ($P < 0.05$ vs. corresponding basal value and vs. insulin alone; Fig. 5B). Enhanced PI(3,4,5)P₃ levels in rapamycin-treated cells was linked with a marked increase in Akt phosphorylation, not only in the LDM (2.3-fold vs. insulin alone, $P < 0.05$), where IRS-1 and p85 PI 3-kinase were sequestered, but also in the PM and in the cytosol (1.8- and 2.3-fold vs. insulin alone, respectively, $P < 0.05$; Fig. 5C).

Effect of mTOR/S6K1 activation on GLUT4 and GLUT1 translocation

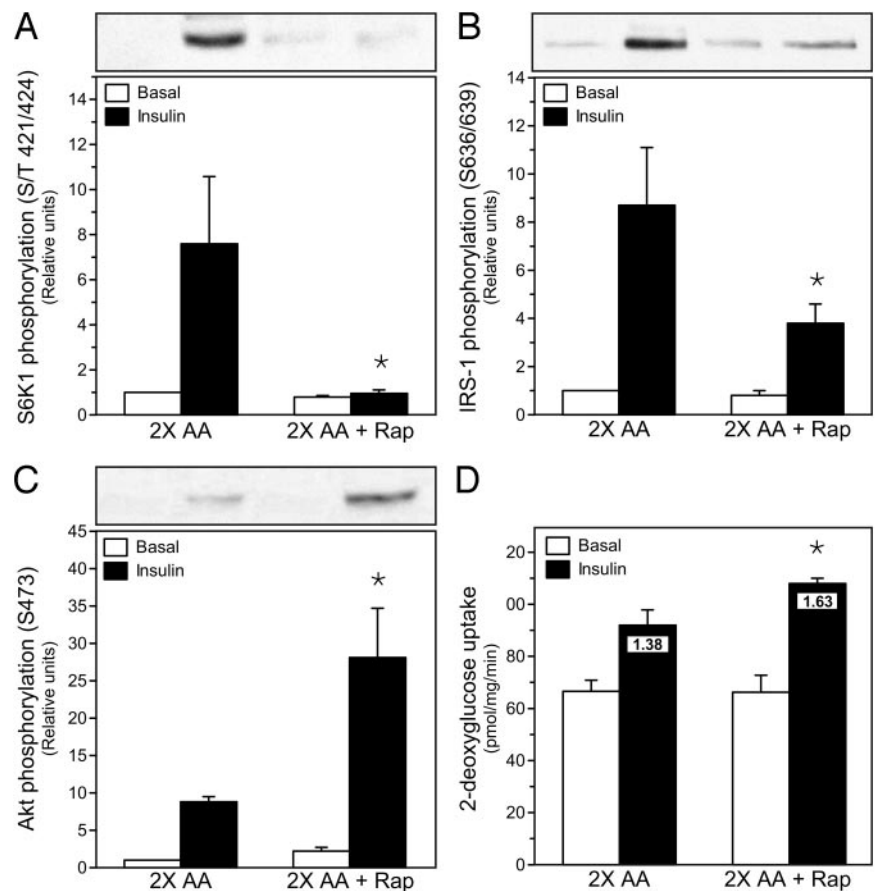
To further characterize the means by which glucose transport is elevated after mTOR inhibition, we evaluated the effect of rapamycin on insulin-induced translocation of GLUT4 and GLUT1 glucose transporters using subcellular fractionation. Although we found, as expected, that insulin stimulated the translocation of GLUT4 and, to a lesser extent, GLUT1 from the LDM to the PM, rapamycin failed to increase the abundance of either transporter in the PM fraction (Fig. 6).

Discussion

Integration of both hormonal and nutritional signals by mTOR has placed this checkpoint protein as a central regulator of cellular homeostasis (32). For instance, mTOR itself or its downstream effectors, S6K1 and 4E-BP1, were shown to play a role in glucose-induced protein synthesis in pancreatic β -cells (33), differentiation of muscle (34, 35) and adipose cells (36, 37), inhibition of autophagy in hepatocytes (38), and skeletal muscle protein synthesis (39). More recently, mTOR has been implicated in the development of insulin resistance in hyperinsulinemic and nutrient excess states through increased Ser/Thr phosphorylation of IRS-1 and disruption of PI 3-kinase signaling (22–24).

One key aspect of the present study is the observation that blockade of mTOR/S6K1 by rapamycin rapidly led to a substantial increase in insulin-stimulated glucose transport in both murine and human adipose cells. It is therefore believed that the mTOR pathway is not only involved in the chronic instauration of insulin resistance in 3T3-L1 adipocytes (22, 23) but also participates in the acute feed-back regulation of insulin action, thereby placing mTOR as an integral compo-

FIG. 4. The mTOR pathway acutely controls insulin action in human adipocytes. Serum-deprived human adipocytes were incubated in medium containing 2× amino acids mixture as found in MEM for 1 h. Vehicle (0.01% DMSO) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated or not with 10 nM (A–C) or 500 nM (D) insulin for the last 30 min of incubation before determining phosphorylation of S6K1 on Thr421/Ser424 (A), IRS-1 on Ser636/639 (B), and Akt on Ser473 (C) and glucose uptake (D) as described in *Materials and Methods*. The means ± SE from adipocytes obtained from four subjects are shown. *, $P < 0.05$ vs. corresponding cells treated without rapamycin.



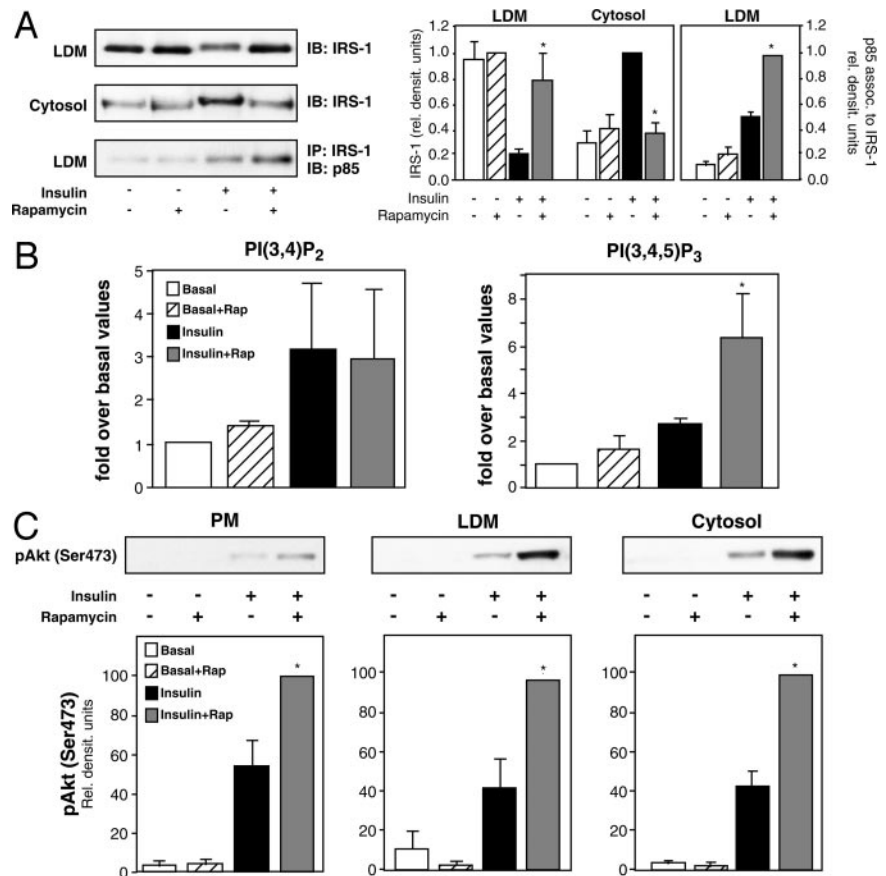
ment of the insulin signal transduction pathway modulating glucose transport. Indeed, we believe that our results underscore the physiological role of this pathway, because most of the studies in which glucose transport is measured used such short-term (15–45 min) stimulation with insulin where the mTOR/S6K1 is most likely activated and contributes to the fine-tuning of the IRS-1/PI(3)K/Akt pathway in fat cells.

The mechanism underlying mTOR-mediated inhibition of glucose uptake might result from the increased Ser/Thr phosphorylation of IRS-1. For instance, a rapamycin-sensitive pathway, activated by platelet-derived growth factor, was shown to cause insulin resistance in 3T3-L1 adipocytes through phosphorylation of IRS-1 on Ser 632, 662, and/or 731 residues (21). Another study has shown that both mTOR and S6K1 have the ability to phosphorylate IRS-1 *in vitro* and that mTOR-, but not S6K1-, mediated phosphorylation of IRS-1 impaired its tyrosyl-phosphorylation induced by JAK-1 (16). Furthermore, mTOR physically interacts with and catalyzes the phosphorylation (Ser636/639) of IRS-1 in TNF- α -treated myocytes (17). More recently, phosphorylation of IRS-1 on Ser632 (equivalent to Ser636 in human) was reported to be increased after exposure of adipocytes to insulin (40).

The negative impact of Ser/Thr phosphorylation of IRS-1 on insulin action has been previously recognized (see Ref. 41 for review). However we found, in this study, that whereas rapamycin blunted IRS-1 Ser/Thr phosphorylation, this neither affected IRS-1 association with the regulatory subunit of PI 3-kinase nor its associated PI 3-kinase activity. On the

other hand, rapamycin markedly potentiated the ability of insulin to enhance phosphorylation of Akt on both regulatory sites and a corresponding increase of its kinase activity. This was most likely contributed by Akt2, the major Akt isoform expressed in differentiated adipocytes and thought to mediate the bulk of insulin-stimulated glucose transport (42, 43). One possible mechanism that may link increased Akt in the face of unchanged PI 3-kinase activity is by segregation of the lipid kinase close to its cellular substrate via retention of IRS-1 (and its associated PI 3-kinase) in the LDM. There are precedents to suggest IRS-1 redistribution as a potential mechanism of uncoupling between PI 3-kinase and Akt. For instance, it has been shown that hydrogen peroxide or chronic GH treatment result in impaired insulin-induced activation of Akt without having any detrimental effect on PI 3-kinase activity in 3T3-L1 adipocytes (44, 45). These studies rationalized such an unexpected observation by suggesting that IRS-1/PI 3-kinase departure from the LDM to the cytosol was responsible for the defective activation of Akt (44, 45). Moreover, chronic activation of mTOR by insulin and amino acids was also shown to be associated with dysregulated IRS-1 subcellular compartmentalization (23). In agreement with the latter study, we observed a greater retention of IRS-1 (in association with the p85 subunit of PI 3-kinase) in the LDM after insulin stimulation in rapamycin-treated adipocytes. However, the functional consequence of IRS-1 segregation in the LDM on PI 3-kinase-mediated production of PI(3,4,5)P₃ is still unknown, but one could argue that PI

FIG. 5. Cellular mechanism(s) responsible for the dissociation between PI 3-kinase and Akt activities. A and C, Serum-deprived 3T3-L1 cells were incubated in medium containing 2× amino acids mixture as found in MEM for 1 h. Vehicle (0.01% DMSO) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated or not with 10 nM insulin for the last 30 min of incubation, rinsed twice in ice-cold PBS, and fractionated as described in *Materials and Methods*. A, IRS-1 abundance in cytosol and LDM and association of the p85 subunit of PI 3-kinase with IRS-1. C, Phosphorylation of Akt on Ser473 at the PM, LDM and cytosol were determined by Western blotting as described in *Materials and Methods*. B, Cells were labeled with ^3H -myo-inositol as described in *Materials and Methods* and incubated in medium containing 2× amino acids mixture as found in MEM for 1 h. Vehicle (0.01% DMSO) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated or not with 10 nM insulin for the last 30 min of incubation. Measurement of $\text{PI}(3,4)\text{P}_2$ and $\text{PI}(3,4,5)\text{P}_3$ were determined as described in *Materials and Methods*. For A and C, results are one representative experiment repeated at least three times. The means \pm SE from three individual experiments are shown (for B). *, $P < 0.05$ vs. corresponding cells treated without rapamycin.



3-kinase must be in, or closely associated with, a membrane compartment to get easy access to its lipid substrate. Accordingly, whereas 30 min of incubation with a relatively low dose of insulin (10 nM) barely increased $\text{PI}(3,4,5)\text{P}_3$ levels, cotreatment with rapamycin markedly potentiated insulin action on $\text{PI}(3,4,5)\text{P}_3$ levels.

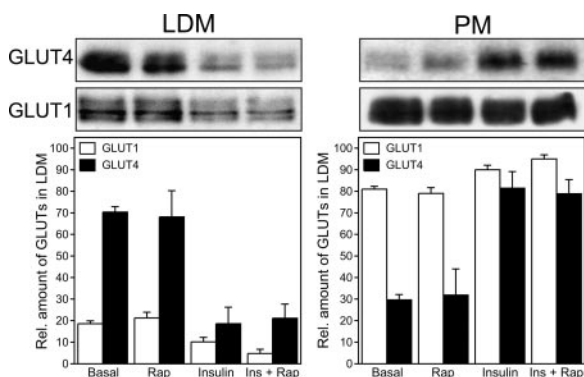


FIG. 6. Effect of mTOR/S6K1 activation on GLUT4 and GLUT1 subcellular distribution. Serum-deprived 3T3-L1 cells were incubated in medium containing 2× amino acids mixture as found in MEM for 1 h. Vehicle (0.01% DMSO) or rapamycin (25 nM) was added during the 1-h incubation. Cells were stimulated or not with 10 nM insulin for the last 30 min of incubation, rinsed twice in ice-cold PBS, and fractionated as described in *Materials and Methods*. GLUT4 and GLUT1 abundance in LDM and PM were determined by Western blotting as described in *Materials and Methods*. The means \pm SE from three individual experiments are shown. *, $P < 0.05$ vs. corresponding cells treated without rapamycin. Rel., Relative.

The observation that insulin-induced Akt phosphorylation in rapamycin-treated cells was not only enhanced in the LDM, where IRS-1/PI 3-kinase was segregated, but also in the cytosol and at the PM, raised the possibility that compartmentalization of PI 3-kinase is not the sole mechanism that may help to understand the dissociation between PI 3-kinase and Akt activities. For instance, it is possible that mTOR inhibition reduced susceptibility of $\text{PI}(3,4,5)\text{P}_3$ for degradation by the lipid phosphatases SHIP-2 and/or phosphatase and tensin homolog deleted on chromosome 10, either by modulation of their activity or by altering their cellular localization. More studies will be needed to test these potential mechanisms.

Despite the fact that rapamycin increased $\text{PI}(3,4,5)\text{P}_3$ levels and Akt activation in insulin-stimulated adipocytes, it failed to further enhance the ability of the hormone to induce GLUT4 or GLUT1 translocation to the PM, as assessed by subcellular fractionation. However, it is important to note that a large part of GLUT4 and GLUT1 was already translocated by treatment of insulin alone, leaving only 10–20% of transporters in the LDM intracellular compartment. Moreover, a significant effect of mTOR inhibition on insulin-stimulated glucose transport and Akt activation was not observed before 5 or 10 min, respectively, a time at which the recruitment of GLUT4 and GLUT1 to the cell surface by insulin is almost, if not entirely, completed in rat and 3T3-L1 adipocytes (46, 47). The reported lag between translocation of GLUT4 and GLUT1 and stimulation of glucose transport by insulin has been proposed to represent intermediate steps

in the recruitment of active glucose transporters at the cell surface, including docking and fusion of transporter-containing vesicles as well as activation of the transporter proteins (46–49). Our data thus suggest that mTOR might regulate one or more of these intermediate steps in the exocytosis of active glucose transporters at the cell surface. The precise molecular mechanism by which insulin-mediated glucose transport is controlled by mTOR will require further investigation of detailed kinetics of GLUT4 trafficking as well as determination of GLUT1 and GLUT4 intrinsic activity in purified PM vesicles.

In contrast to our previous study in muscle cells (24), IRS-1 serine phosphorylation failed to decrease PI 3-kinase activity but impaired Akt activation in 3T3-L1 adipocytes. Our data suggest that serine phosphorylation of IRS-1 in adipocytes also leads to a dysregulated PI 3-kinase activation, although not detectable when the kinase activity is measured in total cell lysates. In adipocytes, it appears that the mTOR-dependent impairment in PI 3-kinase activation is more subtle and rather involves a subcellular redistribution of the IRS-1/PI 3-kinase complex in the cytosol. Another point of divergence between muscle and fat cells is that Akt activation was not found to be inhibited after stimulation of the mTOR pathway in the former cell type. The reason(s) for this cell type-specific impairment in Akt activation remains to be uncovered. However, the literature is now filled with reports of normal Akt activity in the face of impaired PI 3-kinase activity in skeletal muscle (50–52). It was argued that Akt only requires partial PI 3-kinase activity to be fully activated in muscle cells, and this may not be the case for adipocytes, where we observed defective Akt with subtle alterations in PI 3-kinase activity. Thus it may be possible that PI 3-kinase and Akt are differently coupled to glucose transport in muscle and adipose cells, explaining the distinct impact of activation of the mTOR pathway on these signaling molecules. Another notable difference between adipocytes and myocytes is their different sensitivity *vis-à-vis* amino acid availability. Indeed, in L6 muscle cells, amino acids were absolutely required to fully activate the mTOR downstream target S6K1 and mediate desensitization of insulin action (24). In marked contrast, we found that insulin was almost as potent as the combination of amino acids + insulin at activating S6K1 in 3T3-L1 adipocytes. Because the mechanism by which mTOR senses the presence of amino acids in cells is still ill-defined, one can only propose that this system is not as sensitive (less expression or less activity) in adipocytes when compared with muscle cells.

In summary, we have demonstrated the involvement of the mTOR pathway in the acute regulation of insulin-stimulated glucose transport in 3T3-L1 and human adipocytes. The inhibitory effect of mTOR on insulin-induced glucose uptake results from a rapid down-regulation of PI(3,4,5)P₃ levels and reduced Akt phosphorylation and activation, which represents a novel mechanism of acute regulation of insulin-mediated glucose metabolism by the mTOR pathway. These results emphasize the important role of mTOR/S6K1 on glucose metabolism, not only as a chronic inducer of insulin resistance but also as an integral component of a negative physiological feed-back loop in

insulin action operating in both murine and human adipocytes.

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