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Amino acids and leucine allow insulin activation of the PKB/mTOR pathway in normal adipocytes treated with wortmannin and in adipocytes from *db/db* mice

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ABSTRACT

Amino acids are nutrients responsible for mammalian target of rapamycin (mTOR) regulation in mammalian cells. The mTOR protein is mainly known for its role in regulating cell growth, notably via protein synthesis. In addition to amino acids, mTOR is regulated by insulin via a phosphatidylinositol 3-kinase (PI 3-kinase)-dependent pathway. mTOR mediates crosstalk between amino acids and insulin signaling. We show that in freshly isolated rat adipocytes, insulin stimulates the phosphorylation of mTOR on serine 2448, a protein kinase B (PKB) consensus phosphorylation site. This site is also phosphorylated by amino acids, which in contrast to insulin do not activate PKB. Moreover, insulin and amino acids have an additive effect on mTOR phosphorylation, indicating that they act via two independent pathways. Importantly, amino acids, notably leucine, permit insulin to stimulate PKB when PI 3-kinase is inhibited. They also rescue glucose transport and the mTOR pathway. Further, leucine alone can improve insulin activation of PKB in *db/db* mice. Our results define the importance of amino acids in insulin signaling and reveal leucine as a key amino acid in disease situations associated with insulin-resistance in adipocytes.

Key words: glucose metabolism • phosphorylation • signaling • insulin-resistance

The regulation of cell functions by amino acids in mammalian cells has become a major focus of interest. For example, it is now recognized that certain amino acids are involved in hepatic autophagy (1) and in muscle protein synthesis (2), or that they facilitate multicellular clustering of adipocytes (3). All of these amino acid-regulated processes are inhibited by rapamycin, an immunosuppressive and antiproliferative agent.

Until recently, the only signaling molecule known to be regulated by amino acids was the mammalian target of rapamycin (mTOR), first identified by its specific inhibition by rapamycin (4–6). The mTOR protein, also named FRAP or RAFT1, is structurally related to the phosphatidylinositol kinases but functions as a serine/threonine kinase. The principal known

function of mTOR is the regulation of cell growth, especially via the regulation of protein synthesis, where it phosphorylates the ribosomal protein S6 kinase (p70S6 kinase; 7) and the translational repressor PHAS-I (also known as 4EBP-1; 8). Phosphorylation at the appropriate sites activates p70S6 kinase, which then phosphorylates ribosomal protein S6 to promote regulation of ribosome biogenesis (9). PHAS-I phosphorylation results in the release of eukaryotic initiation factor 4E (eIF4E), thereby increasing cap-dependent mRNA translation (10). The mTOR protein is activated by free amino acids and more precisely by neutral ones with aliphatic chains (3, 11). Among these amino acids, leucine is the most potent activator of the mTOR pathway notably in adipocytes (12).

mTOR is not only regulated by amino acids availability, but also by insulin, growth factors, and cytokines. For insulin, it seems that phosphatidylinositol 3-kinase (PI 3-kinase) and protein kinase B (PKB) are involved in mTOR activation (13, 14). PKB has been shown in vitro to phosphorylate mTOR on serine 2448, a site phosphorylated in response to PKB activation (14–16) and to amino acids (15, 17). The precise role of serine 2448 phosphorylation is not known, but it may be involved in mTOR functioning. Concerning PKB, it is still not settled whether this kinase can activate mTOR directly or whether one or more intermediates are involved. Recently, several reports have implicated the heterodimer formed by the tumor suppressors TSC1 and TSC2 (hamartin and tuberin, respectively; 18). TSC2 encodes a putative GTPase-activating protein (GAP), while TSC1 contains coil–coil domains but has no known enzymatic activity. The results point to TSC2 as a direct PKB substrate and as a primary effector of PKB-mediated signaling to mTOR/p70S6k/PHAS-I pathway (18).

mTOR constitutes a checkpoint for cellular growth regulated by growth factors and amino acids. For example, insulin action on p70S6 kinase and PHAS-I is strongly attenuated if the medium does not contain amino acids. In contrast, these molecules are activated if amino acids are present (11, 19, 20). These studies show that amino acids are the nutrients responsible for mTOR regulation in mammalian cells and that they have a permissive effect on insulin-stimulated protein synthesis.

Although the positive role of mTOR and amino acids in insulin-stimulated protein synthesis is well established, little is known concerning their contribution to other effects of the hormone, especially in adipocytes. Adipose tissue is one of the main sites of insulin action and plays a central role in the control of the energetic balance of the organism. Therefore, we were interested in the effect of amino acids and insulin on the mTOR pathway in freshly isolated adipocytes. Moreover, we investigated the contribution of amino acids to the metabolic effects of insulin in adipocytes.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA, Fraction V) was purchased from Intergen (Purchase, CO), and collagenase was purchased from Sigma-Aldrich (St. Louis, MO). Cell culture solutions and supplements, reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein A sepharose were from Life Technologies (Carlsbad, CA). Recombinant human insulin was from Novo-Nordisk (Copenhagen, Denmark). Amino acids mixture (50×) in

MEM was from Gibco (Cergy Pontoise, France), and amino acids concentration in a 1× mixture was as follows: Arg 0.6 mM, Cys 0.2 mM, Gln 2 mM, His 0.2 mM, Ile 0.4 mM, Leu 0.4 mM, Lys 0.4 mM, Met 0.1 mM, Phe 0.2 mM, Thr 0.47 mM, Trp 0.05 mM, Tyr 0.2 mM, and Val 0.4 mM. L-glutamine (2 mM) is added to amino acids mixture before use. Rapamycin was obtained from Calbiochem (La Jolla, CA) and Wortmannin from Sigma (St. Louis, MO). [His_6]PHAS-I was expressed in bacteria and purified as described previously (21). L-α-Phosphatidylinositol was from Sigma (St. Louis, MO), TLC silica plates from Merck (Darmstadt, Germany), Crosstide from Upstate Biotechnology (Lake Placid, NY), polyvinylidene difluoride (PVDF) membranes for immunoblotting from Millipore (Bedford, MA), ECL reagents, [γ-^{32}P]-ATP (>5000 Ci/mmol), D-[2-^{3}H]-glucose (11.3 Ci/mmol), 2-Deoxy-D-[1-^{3}H]glucose (9.70 Ci/mmol) from Amersham Pharmacia Biotech (Uppsala, Sweden). All other chemicals were of highest analytical grade.

Antibodies

The mTab2 antibody was directed against a central portion (1272–1290) of mTOR (22), and the phosphospecific antibody was directed against phosphoserine 2448 (17). The antibody to PHAS-I was directed against a C-terminal peptide of PHAS-I, and the phosphospecific antibody was directed against phosphothreonine 36/45 (23). Antibodies to mTOR and to PHAS-I and [His_6]PHAS-I were a gift from J.C. Lawrence Jr. (Charlottesville, VA). The antibody to PKB used for immunoblotting was directed against a C-terminal peptide (466–473) of PKB (Cell Signaling Technology, Beverly, MA). To immunoprecipitate PKB, we used a PKBβ antibody directed against a C-terminal peptide (455–469) of PKBβ protein (Upstate Biotechnology, Lake Placid, NY). Phosphospecific antibodies to PKB were directed against phosphoserine 473 or phosphothreonine 308 (Cell Signaling Technology). The antibody to p70S6k was directed against a C-terminal peptide of S6K1 (Santa Cruz, CA), and the phosphospecific antibody was directed against phosphothreonine 389 (Cell Signaling Technology). The antibody to IRS-1 was produced in our laboratory by immunizing rabbits with a synthetic peptide corresponding to the C-terminal portion (1223–1235) of IRS-1. Secondary anti-rabbit antibody conjugated to horseradish peroxidase was from Jackson Laboratories (Copenhagen, Denmark).

Adipose tissue fractionation

Adipocytes were isolated from epididymal fat pads of 160–200 g male Wistar rats (“Elevage Janvier,” France) by collagenase digestion, as described (24). All animals were fed ad libitum and were sacrificed by cervical dislocation. Briefly, the fat pads were digested with collagenase (1 mg/ml) in Krebs-Ringer bicarbonate 30 mM HEPES, buffer pH 7.4 (KRBH; 120 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, 0.75 mM $CaCl_2$, 10 mM $NaHCO_3$, 0.3 mM glucose), with 1% (wt/vol) BSA and without amino acids for 45 min at 37°C with constant shaking. The disrupted tissues were filtered through a 250 μm nylon mesh, and cells were washed three times by flotation with KRBH buffer without glucose and BSA. The floating top layer of adipocytes was diluted at appropriate adipocrit (vol/vol) with KRBH with 1% (wt/vol) BSA. After 15 min at 37°C without shaking, cells were treated with different reagents as described in the figure legends.

Adipose tissue culture

Fat pads from male *db/+* and *db/db* mice at 8–10 weeks of age ("Elevage Janvier," France) were dissected as described above, washed in KRBH buffer, minced, and incubated (1 ml of media per 70–90 mg of tissue) in 12-well culture plates containing KRBH with glucose and BSA, pH 7.4. The plates were incubated at 37°C under 5% (v/v) CO2.

Immunoprecipitation of mTOR and measurement of its kinase activity

At the end of the incubation, the adipocytes [70% vol/vol in KRBH 1% (wt/vol) BSA] were solubilized and homogenized with a Potter Thoma C (10 strokes) at 4°C in buffer A (50 mM β glycerophosphate, 10 mM KH_2PO_4, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, pH 7.4) containing 0.2% (vol/vol) Tween-20, 1 mM DTT and anti-proteases (1 mM vanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM AEBSF [4-(2-aminoethyl)-benzene-sulfonylfluoride HCL)]. Lysates were centrifuged for 10 min at 12 000 x *g*, and the supernatants (500 µg) were immunoprecipitated for 3 h at 4°C with mTab2 or anti-pSer2448 (5 µg) coupled to protein A-Sepharose beads. For kinase activity, immune pellets were washed twice with buffer A containing 0.2% (vol/vol) Tween-20, 1 mM DTT, twice with buffer A containing 0.2% (vol/vol) Tween-20, 1 mM DTT, 0.5 mM NaCl, then twice with 50 mM Tris, 1 mM EDTA, 1 mM EGTA, and finally washed twice with phosphorylation buffer containing 10 mM HEPES, 1 mM DTT, 50 mM NaCl, 0.1 mM EGTA, and 50 mM β glycerophosphate, pH 7.4. The protein kinase assay was measured as described previously (14). For immunoprecipitation only, pellets were washed three times with buffer A containing 0.2% (vol/vol) Tween-20, 1 mM DTT, three times with buffer A containing 0.2% (vol/vol) Tween-20, 1 mM DTT, 0.5 mM NaCl, and suspended in 3% (wt/vol) SDS sample buffer.

Measurement of PKB kinase activity

PKB activation was determined either for immunoblotting described below with phosphospecific antibodies [and a shift in its electrophoretic mobility due to its phosphorylation (25)] or by measuring Crosstide phosphorylation (26). At the end of the incubations, the cell suspensions were solubilized for 45 min at 4°C in buffer B (25 mM HEPES, 75 mM NaCl, 5 mM EDTA, 5 mM $Na_4P_2O_7$, 50 mM NaF, 10 mM β glycerophosphate) containing 1% (vol/vol) Nonidet-P40 and anti-proteases. Lysates were centrifuged for 10 min at 12 000 x *g*, and the supernatants (400 µg) were immunoprecipitated for 2 h at 4°C with antibodies to PKBβ (2 µg) coupled to protein A-Sepharose beads. Immune pellets were washed twice with buffer B containing 1% (vol/vol) Nonidet-P40 and 1 mM vanadate; twice with buffer B containing 1% (vol/vol) Nonidet-P40, 1 mM vanadate, and 0.5 mM NaCl; and twice with 20 mM Tris, pH 7.4, 10 mM $MgCl_2$, and 1 mM DTT. The kinase assay was performed on the immune pellets by addition of 20 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 4.5 µM [γ-^{32}P]ATP (2.5 µCi/reaction), and 30 µM Crosstide. After 30 min at room temperature with constant shaking, samples were adsorbed on phosphocellulose p81 paper, extensively washed in 1% (vol/vol) orthophosphoric acid solution, and paper-associated radioactivity was counted.

Measurement of PI 3-kinase activity

At the end of the incubations, the adipocytes [70% vol/vol in KRBH 1% (wt/vol) BSA] were solubilized and homogenized with a Potter Thoma C (10 strokes) at 4°C in buffer A containing 1% (vol/vol) Nonidet-P40 and anti-proteases. Lysates were centrifuged for 10 min at 12000 x *g*, and the supernatants (500 μg) were incubated for 3 h at 4°C with antibodies to IRS-1 (5 μg) coupled to protein A-Sepharose beads. Immune pellets were washed twice in phosphate-buffered saline (PBS) containing 1% (vol/vol) Nonidet-P40, 0.2 mM vanadate; then twice with 0.5 M LiCl, 0.1 M Tris, and 0.2 mM vanadate; and twice with 10 mM Tris, 100 mM NaCl, 1 mM EDTA, and 0.2 mM vanadate. Lipid kinase assays were performed basically as described previously (27). Phosphorylated lipids were detected by autoradiography on Kodak X-Omat films or by phosphorimaging (STORM 840, Molecular Dynamics, Amersham Pharmacia Biotech, Orsay).

Glucose transport and metabolism

Glucose transport in the adipocytes [70% (vol/vol) in KRBH 1% (wt/vol) BSA] was assessed by the uptake of 2-deoxy-[^{3}H]glucose (0.1 mM, 0.5 μCi/reaction) during 90 s at 37°C with constant shaking. The reaction was stopped with 40 μM cytochalasin B. Incorporation of [^{3}H]-2-DOG was measured with a β counter. Glucose metabolism was determined from lipogenesis measured by the incorporation of glucose into lipids. At the end of the incubations, the adipocyte suspensions [10% (vol/vol) in KRBH 1% (wt/vol) BSA] were incubated for 1 h at 37°C with [2-^{3}H]glucose (0.3 mM, 0.2 μM/reaction) with constant shaking. [^{3}H]glucose incorporation into lipids was measured with a β counter after the addition of toluene containing 11 mM Butyl PBD (2-(4-t-butylphenyl)-5-(4-biphenylyl)1,3,4-oxadiazole).

Protein analysis

For freshly isolated adipocytes following the different incubations, cells (50% (vol/vol) in KRBH 1% (wt/vol) BSA) were centrifuged through dinonylphthalate and the cell cakes were solubilized in 3% (wt/vol) SDS sample buffer. For adipose tissue explants after treatment with different reagents, samples were washed with KRBH buffer and lysed in buffer B containing 1% (vol/vol) Nonidet-P40 and anti-proteases. After homogenization and 15 min shaking at 4°C, lysates were centrifuged for 10 min at 12 000 x *g*. The supernatants were collected and eluted in 3% (wt/vol) SDS sample buffer. Protein concentration was assayed by bicinchoninic acid technique (BCA protein assay kit, Pierce). Samples (50 μg) were separated by SDS-PAGE using the method of Laemmli (28), before proteins were transferred to PVDF membranes and immunoblotted with antibodies. Immunoreactive proteins were revealed by enhanced chemiluminescence using ECL. For the mTOR kinase assay, ^{32}P-labeled [His_6]PHAS-I was detected by autoradiography after drying the gels.

Statistical analysis

Results are presented as mean ± SE of number of experiments indicated in the figure legends. In each experiment, all determinations were performed at least in triplicate. Statistical significance was assessed using Student's *t*-test.

RESULTS

Amino acids and insulin have an additive effect on mTOR pathway

Little is known about the role of mTOR in freshly isolated adipocytes. Therefore, we investigated the phosphorylation and the kinase activity of the enzyme in response to amino acids and insulin. We examined mTOR phosphorylation on serine 2448, a PKB consensus phosphorylation site that has been implicated in mTOR activation (14). Adipocytes were freshly isolated and incubated with or without vehicle (0.001% Me_2SO) or wortmannin (100 nM). After 30 min, adipocytes were incubated with or without insulin (100 nM) or/and amino acids (1×) for 80 min. To study mTOR phosphorylation, cells were lysed and equal quantities of protein were immunoprecipitated with the mTab2 antibody. Immune pellets were analyzed by immunoblotting with the antibody to phosphoserine 2448. At a basal state mTOR is already phosphorylated, but insulin or amino acids increase serine 2448 phosphorylation (Fig. 1*A*, lanes 1–3). Furthermore, amino acids have an additive effect on insulin stimulation (Fig. 1*A*, lane 4). Wortmannin, a PI 3-kinase inhibitor, totally prevents insulin's effects and those of amino acids but blocks only partially the combination of both (Fig. 1*A*, lanes 5–8). mTOR reactivity with the mTab2 antibody was not changed by different reagents. In parallel, we analyzed p70S6k phosphorylation on threonine 389, a rapamycin-sensitive site, as a reflection of its activity (29). Phosphorylation of threonine 389 is more pronounced when adipocytes are stimulated by insulin plus amino acids than by either one separately. This is also reflected by the reduced electrophoretic mobility of p70S6k (Fig. 1*B*, lanes 1–4). Wortmannin completely inhibits p70S6k phosphorylation induced by insulin or amino acids, but part of the phosphorylation induced by the combination of both reagents persists (Fig. 1*B*, lanes 5–8). We also analyzed PHAS-I phosphorylation on phosphothreonine 36/45, two rapamycin-sensitive sites (23). Both insulin and amino acids are needed to robustly phosphorylate threonine 36/45, and wortmannin only partially inhibits PHAS-I phosphorylation on these sites. PHAS-I phosphorylation is also reflected by an increase in the amount of the gamma species and a decrease in the α species (Fig. 1*B*). Finally, we performed in vitro kinase assays after immunoprecipitation of phosphorylated mTOR with the phosphoserine 2448 antibody and with PHAS-I as substrate, as described in Materials and Methods. Insulin and amino acids alone stimulate PHAS-I phosphorylation, and amino acids have an additive effect on insulin stimulation (Fig. 1*C*, Lanes 1–4). With wortmannin, the effects of amino acids or insulin are totally blocked, whereas the inhibition reaches only 30% when insulin is combined with amino acids (Fig. 1*C*, Lanes 5–8). This profile of mTOR kinase activity is tightly correlated with the one observed for the phosphorylation of mTOR on serine 2448 and of its substrates (Fig. 1*A*, *B*). Because the kinase assay was performed with a phosphospecific antibody, increased activity reflects a larger quantity of immunoprecipitated mTOR. These results show that in freshly isolated adipocytes, insulin and amino acids increase mTOR phosphorylation on serine 2448 in an additive manner and that phosphorylated mTOR is active. Importantly, insulin and amino acids together prevent the inhibitory effect of wortmannin on mTOR pathway.

Amino acids allow insulin to activate PKB in the presence of wortmannin

Because of evidence that mTOR is controlled by PKB (14–17, 30) and to better understand the action of amino acids in insulin signaling, we investigated their effect on PKB activity in freshly isolated adipocytes. PKB activation was determined either by immunoblotting with

phosphospecific antibodies to assess changes in serine 473 or threonine 308 phosphorylation or by measuring crosstide phosphorylation as described in Materials and Methods. As expected, insulin induces serine 473 and threonine 308 phosphorylation of PKB (Fig. 2*A*, lanes 1 and 2). These two sites become phosphorylated when PKB binds phospholipid products of the PI 3-kinase reaction, and phosphorylation of both sites is required for full kinase activation (31). Amino acids alone do not lead to PKB phosphorylation (Fig. 2*A*, lane 4), and they do not or only weakly modulate insulin-stimulated PKB phosphorylation on both sites in comparison with the total amount of immunodetected PKB (Fig. 2*A*, lane 3). Wortmannin inhibits insulin-induced PKB phosphorylation on both sites (Fig. 2*A*, lane 6). Surprisingly, this inhibitory effect of wortmannin on insulin-stimulated PKB phosphorylation no longer occurs in the presence of amino acids (Fig. 2*A*, lane 7). We observed the same profile of PKB phosphorylation with LY294002, another PI 3-kinase inhibitor (data not shown). In parallel, we performed in vitro kinase assays after specific immunoprecipitation of the β isoform of PKB (Fig. 2*B*). Among the mammalian isoforms of PKB, α, β, and γ, PKBβ is the main isoform in rat adipocytes and its insulin-stimulated activity is twice that of PKBα (32). Insulin stimulates PKBβ activity ~10-fold (Fig. 2*B*). Amino acids do not modulate the insulin-stimulated activity of PKBβ (Fig. 2*B*). Interestingly, wortmannin inhibits insulin's effect by 80%, whereas with amino acids its inhibition reaches only 60%. In summary, amino acids and insulin can rescue PKB phosphorylation and partially restore its activity despite the presence of wortmannin. In the same conditions, the mTOR pathway is still active (Fig. 1*A–C*, lane 8).

Amino acids inhibit insulin-stimulated PI 3-kinase activity

As PI 3-kinase is localized upstream of PKB in insulin signaling and is the target of wortmannin, we next examined the effect of amino acids on this enzyme. It has been shown in L6 skeletal muscle cells that amino acids inhibit PI 3-kinase activity associated with IRS-1 due to IRS-1 hyperphosphorylation on serine and threonine induced by mTOR (33). Therefore, we measured PI 3-kinase activity associated with IRS-1 in our experimental conditions, as described in Materials and Methods. Insulin increases PI 3-kinase activity ~10-fold (Fig. 3*A*, lane 2). Amino acids alone have no effect, whereas they decrease insulin-stimulated PI 3-kinase activity by 45% (Fig. 3*A*, lanes 3 and 4). Wortmannin inhibits 60% of insulin's effect (Fig. 3*A*, lane 6), and 45% of the one seen with amino acids plus insulin (Fig. 3*A*, lane 8). Similar data were obtained with LY294002 (data not shown). In parallel, we analyzed IRS-1 by immunoblotting after its immunoprecipitation (Fig. 3*B*). In the presence of insulin or amino acids, we observe a small IRS-1 upward mobility shift, which is much more pronounced with the combination of both agents (Fig. 3*B*, lanes 1–4). Wortmannin inhibits the IRS-1 shift induced by insulin or/and amino acids (Fig. 3*B*, lanes 5–8). Thus, amino acids inhibit the insulin-stimulated PI 3-kinase activity associated with IRS-1 in freshly isolated adipocytes. Moreover, their effect is correlated with an upward shift of IRS-1, which reflects its increased serine/threonine hyperphosphorylation.

Effects of amino acids on insulin-stimulated glucose transport and lipogenesis

Studies have shown that amino acids inhibit insulin-stimulated glucose transport in L6 skeletal muscle cells (33) and in 3T3-L1 adipocytes (34). We tested whether this effect could be observed in freshly isolated adipocytes. Glucose transport was assessed from the uptake of 2-deoxy-[^{3}H]glucose (0.1 mM, 0.5 μCi/reaction) and in parallel phosphorylation of PKB on serine 473 and of p70S6k was determined. Insulin stimulates glucose transport by fivefold (Fig. 4*A*) as well

as phosphorylation of PKB and p70S6k (Fig. 4*B*, lane 2). Amino acids, either alone or in the presence of insulin, have no significant effect on glucose transport in correlation with results observed for PKB (Figs. 2 and 4*B*, lanes 3 and 4). However, amino acids induce a p70S6k upward shift (Fig. 4*B*, lane 4), reflecting mTOR pathway activation. Moreover, they have an additive effect on insulin action (Fig. 4*B*, lane 3). Rapamycin does not affect glucose transport or PKB phosphorylation even in the presence of insulin or/and amino acids (Fig. 4*B*, lanes 5–8). In contrast, rapamycin completely inhibits p70S6k phosphorylation induced by insulin and amino acids showing that mTOR signaling is blocked (Fig. 4*B*, lane 7). As expected, wortmannin totally inhibits insulin-stimulated glucose transport (Fig. 4*A*), PKB, and p70S6k phosphorylation (Fig. 4*B*, lanes 9 and 10). However, with amino acids, wortmannin inhibits insulin-stimulated glucose transport only by half; this is correlated with persisting PKB phosphorylation (Fig. 4*B*, lane 11, and Fig. 2*A*, lane 7). We next investigated the effect of amino acids on lipogenesis directly related to glucose metabolism (Fig. 4*C*). We measured the incorporation of glucose into lipids as described in Materials and Methods. Insulin stimulates lipogenesis by 10-fold contrary to amino acids that have no effect. However, amino acids inhibit insulin-stimulated lipogenesis by 20%. This inhibitory effect is not prevented by treatment with rapamycin, which suggests that it is not mediated through the mTOR pathway. Rapamycin has no effect on insulin stimulation. In contrast, wortmannin strongly inhibits insulin-stimulated lipogenesis except in the presence of amino acids where the inhibition only reaches 15%. To conclude, amino acids inhibit insulin-stimulated lipogenesis in a rapamycin-insensitive manner. However, the combination of amino acids with insulin prevents the inhibitory action of wortmannin on glucose transport and metabolism, as well as on PKB activation.

Leucine allows insulin to activate PKB and glucose transport when PI 3-kinase is blocked

Since we showed that amino acids mixture allows insulin to activate PKB despite the presence of wortmannin, we wished to determine which amino acids could be involved in this unexpected effect. We tested different amino acids such as leucine, lysine, and cysteine. In the presence of 4 mM leucine, insulin can phosphorylate PKB on serine 473 and threonine 308 despite the presence of wortmannin (Fig. 5*A*, lane 8). This effect is also observed at a 10 times lower leucine concentration (0.4 mM), which corresponds to the leucine concentration in the amino acids mixture (Fig. 5*A*, lane 7). In contrast, even at high concentration, lysine, and cysteine cannot rescue insulin-induced PKB phosphorylation in the presence of wortmannin (Fig. 5*A*, lanes 9–12). Because leucine is a strong mTOR activator (12), we looked at the rapamycin effect on PKB phosphorylation rescued by amino acids mixture or leucine when insulin signaling is inhibited by wortmannin (Fig. 5*B*). Rapamycin alone does not modulate PKB phosphorylation induced by insulin regardless the presence of amino acids mixture or leucine but inhibits the p70S6k shift induced by the combination of insulin with amino acids or leucine (Fig. 5*B*, lanes 12–14). Wortmannin blocks insulin's effect even in the presence of rapamycin (Fig. 5*B*, lane 9). Moreover, rapamycin does not prevent PKB phosphorylation stimulated by insulin and amino acids mixture or leucine with wortmannin (Fig. 5*B*, lanes 10 and 11). We also analyzed leucine's effect on insulin-stimulated glucose transport (Fig. 5*C*). Neither at 4 mM nor at 0.4 mM does leucine modulate significantly insulin-induced glucose transport. Interestingly, leucine at both concentrations diminishes the inhibitory effect of wortmannin on insulin-induced glucose transport. In summary, these results show that despite the presence of wortmannin, leucine allows insulin to stimulate PKB phosphorylation and partially restores glucose transport. However, rapamycin does not inhibit the positive effect of amino acids or leucine on PKB when

insulin signaling is inhibited by wortmannin, suggesting that this effect is independent of the mTOR pathway.

Leucine improves insulin-induced PKB phosphorylation in adipose tissue explants of *db/db* mice

After having shown that amino acids, notably leucine, permit insulin to activate PKB despite chemically induced PI 3-kinase inhibition, we wondered whether such effect occurs in the setting of physiopathologic impairment of insulin signaling. To investigate putative involvement of amino acids in insulin resistance associated with obesity and type 2 diabetes, we analyzed their effect on insulin-stimulated PKB phosphorylation in adipose tissue of *db/db* mice (35; Fig. 6). Adipose tissue explants from *db/+* (control) or *db/db* mice at 8–10 weeks of age were incubated with or without insulin or/and amino acids mixture or/and leucine (4 mM) for 80 min. Insulin stimulates PKB phosphorylation in *db/+* and *db/db* adipose tissue explants, but insulin sensitivity is slightly reduced in *db/db* mice (Fig. 6 lanes 3 and 4). Amino acids, either alone or in the presence of insulin, have no significant effect on PKB phosphorylation in *db/+* and *db/db* adipose tissue explants (Fig. 6 lanes 5–8). Leucine alone does not or only weakly phosphorylates PKB in both strains (Fig. 6 lanes 9 and 10). Interestingly, leucine seems to potentiate insulin-stimulated PKB phosphorylation in *db/db* and *db/+* adipose tissue explants (Fig. 6 compare lanes 3 and 11, and lanes 4 and 12). However, quantification of several experiments shows that only the increase observed for *db/db* adipose tissue explants is significant (1.7-fold, Fig. 6 histogram). Thus, leucine seems to improve insulin sensitivity in the genetic *db*/*db* insulin-resistant mouse model.

DISCUSSION

The mTOR kinase has been studied only marginally in adipocytes, and the signaling pathway upstream of mTOR is not clearly defined. Many steps remain to be elucidated, especially the regulation of the kinase activity of mTOR. To this end, we were interested in the phosphorylation of mTOR on serine 2448, a PKB consensus phosphorylation site, and in its kinase activity in response to insulin or/and amino acids. Our results show that in freshly isolated adipocytes, insulin increases the phosphorylation of mTOR on serine 2448. In this case, PKB is phosphorylated on serine 473 and threonine 308, which is necessary for its maximal activity. Amino acids alone also increase mTOR phosphorylation on this residue in a wortmannin-sensitive manner. However, they activate neither PI 3-kinase nor PKB. Moreover, amino acids have an additive effect on insulin-stimulated mTOR phosphorylation, reinforcing the idea that both agents transduce the signal through two independent pathways, a PI 3-kinase/PKB pathway stimulated by insulin and an as-yet-unidentified pathway activated by amino acids.

We investigated mTOR kinase activity after immunoprecipitation of phosphorylated mTOR from freshly isolated adipocytes. We show that when insulin and amino acids stimulate serine 2448 phosphorylation, mTOR is active. Phosphorylation on this residue does not seem to modulate mTOR kinase activity, or at least not negatively. The precise role of this phosphorylation is still unclear, and the observations are controversial. Indeed, Scott et al. have shown in 3T3-L1 cells that insulin activates mTOR by promoting serine 2448 phosphorylation via a signaling pathway that includes PKB (14). In contrast, Sekulic et al. did not observe differences between the effects of mTOR protein mutated on serine 2448 (serine 2448 replaced by alanine) and the wild-type

mTOR protein on the phosphorylation of p70S6k in HEK293 cells (16). However, this study used forms of mTOR rendered rapamycin-resistant by mutation of serine 2035 into isoleucine. Recently McMahon et al. show that this mutation inhibits mTOR ability to phosphorylate PHAS-I in vitro (36). Moreover, the regulation of the kinase activity seems to be more complicated than anticipated by the recent identification of raptor (regulatory associated protein of mTOR). Indeed, the interaction of raptor with mTOR seems to regulate its kinase activity, depending on amino acids availability (37). In our experimental conditions we do not know the status of the association between raptor and mTOR, but we can assume that the association occurs since we do see phosphorylation of PHAS-I by mTOR in our kinase assay. Additional studies will be necessary to establish the role of phosphorylation on serine 2448 in the function of mTOR. In adipocytes, the phosphorylation on this residue could have a physiological role. Indeed, Reynolds et al. have shown in skeletal muscle that decreases and increases of serine 2448 phosphorylation are associated with atrophy and hypertrophy, respectively. This finding suggests that modulation of this site may have an important impact on protein synthesis (17). In this context, mTOR phosphorylation could have a similar role in adipocyte protein content.

We were interested in the impact of amino acids on two key metabolic effects of insulin, glucose transport, and lipogenesis. We show that amino acids have no effect on insulin-stimulated glucose transport in freshly isolated rat adipocytes. These results, apparently in contradiction with results obtained in L6 muscle cells (33) or in 3T3-L1 adipocytes (34, 38), could be explained by differences in experimental conditions and/or in cellular models. Indeed, it has been proposed that the mechanism of amino acids action is different in freshly isolated adipocytes compared with cell lines (39). However, we observed that amino acids inhibit insulin-stimulated PI 3-kinase activity associated with IRS-1. This inhibition is likely to be due to mTOR activation by amino acids, which exerts a negative feedback by hyperphosphorylation of IRS-1 on serine residues (33, 34). Nevertheless, the inhibitory effect of amino acids is not observed for insulin-induced PKB activity. This result suggests that the residual insulin-stimulated PI 3-kinase activity in the presence of amino acids is sufficient to activate PKB and to stimulate glucose transport (40).

In parallel, we show that amino acids inhibit insulin-stimulated lipogenesis but that their effect is not reversed by rapamycin. This indicates that amino acids act either through an mTOR-independent pathway, or via an mTOR pathway insensitive to rapamycin. Indeed, the complex formed between rapamycin and its intracellular receptor, the FKBP12 protein (FK506 Binding Protein 12), binds directly to mTOR, but its mechanism of action is not clearly defined. It has been shown that rapamycin does not inhibit directly mTOR kinase activity but seems to form an inactive complex that partially blocks its function (36). The inhibitory effect of amino acids on lipogenesis is unlikely to be due to a decrease in glucose transport, because we show that they do not modulate insulin-stimulated glucose transport in freshly isolated adipocytes. Therefore, they could act by modulating either the activity of the lipogenic enzymes and/or their expression.

We found here, as previously shown (41)**,** that wortmannin, a pharmacologic PI 3-kinase inhibitor, blocks insulin-stimulated glucose transport and lipogenesis in freshly isolated adipocytes. Interestingly, we demonstrate, for the first time to the best of our knowledge, that this wortmannin-induced inhibition is partially reversed by amino acids. This positive effect of amino acids is correlated with the rescue of the phosphorylation of PKB, mTOR p70S6k and PHAS-I. However, amino acids alone cannot activate PKB or to stimulate glucose transport and

lipogenesis. Taken together, our data highlight a PI 3-kinase-independent pathway that would require insulin and amino acids (Fig. 7). Although the precise molecular mechanism of this newly identified effect remains to be unraveled, we observe that leucine alone can rescue insulin-induced PKB phosphorylation and partially restore insulin-induced glucose transport despite the presence of wortmannin. Because leucine is known to be an activator of the mTOR pathway, we analyzed the effect of rapamycin on leucine-and amino acids-induced PKB phosphorylation. We found that, even in the presence of rapamycin, leucine and amino acids allow insulin to activate PKB despite the blockade of PI 3-kinase. Thus, this amino acid effect is not mediated via the mTOR pathway.

In most situations in humans and animal models associated with insulin-resistance impaired activation of several key signaling molecules has been reported, including the PI 3-kinase/PKB module (42). To see whether the amino acids/leucine action to enhance insulin's effect on PKB could exist in a naturally occurring insulin-resistance situation, we looked at the *db/db* mouse. This appears to be indeed the case, at least in part. Leucine, but not amino acids, improves insulin-stimulated PKB phosphorylation solely in adipose tissue explants of insulin resistant *db/db* mice but not in those of *db/+* mice. Considering our data as a whole, we reveal a pathway that is likely pre-existing in situations with normal insulin response but occurring at an imperceptible level. This pathway, which would be not solicited in normal situations, becomes necessary in insulin-resistance states, where it could substitute for the "classical" insulin-signaling pathway that is impaired. Further investigations will be needed to determine the real importance of leucine and amino acids in insulin action by using other models of insulin-resistance, such as the obese mouse after high-fat diet, which resembles the most common insulin-resistance form in humans.

To conclude, our present work demonstrates that amino acids and/or leucine, depending on the setup, can sensitize PKB to insulin's stimulatory effect under conditions of hormonal resistance at steps upstream of PKB. By doing so, amino acids also rescue glucose transport and metabolism as well as mTOR pathway activation in adipocytes (Fig. 7). Taken as a whole, our data shed new light on the mechanism of action of amino acids in adipocyte insulin signaling and disclose— especially for leucine—an intriguing role in disease situations associated with decreased insulin action. In this context, leucine, a "physiological" molecule, may turn out to be a propitious agent for treatment and/or prevention of insulin-resistance in humans.

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Fig. 1

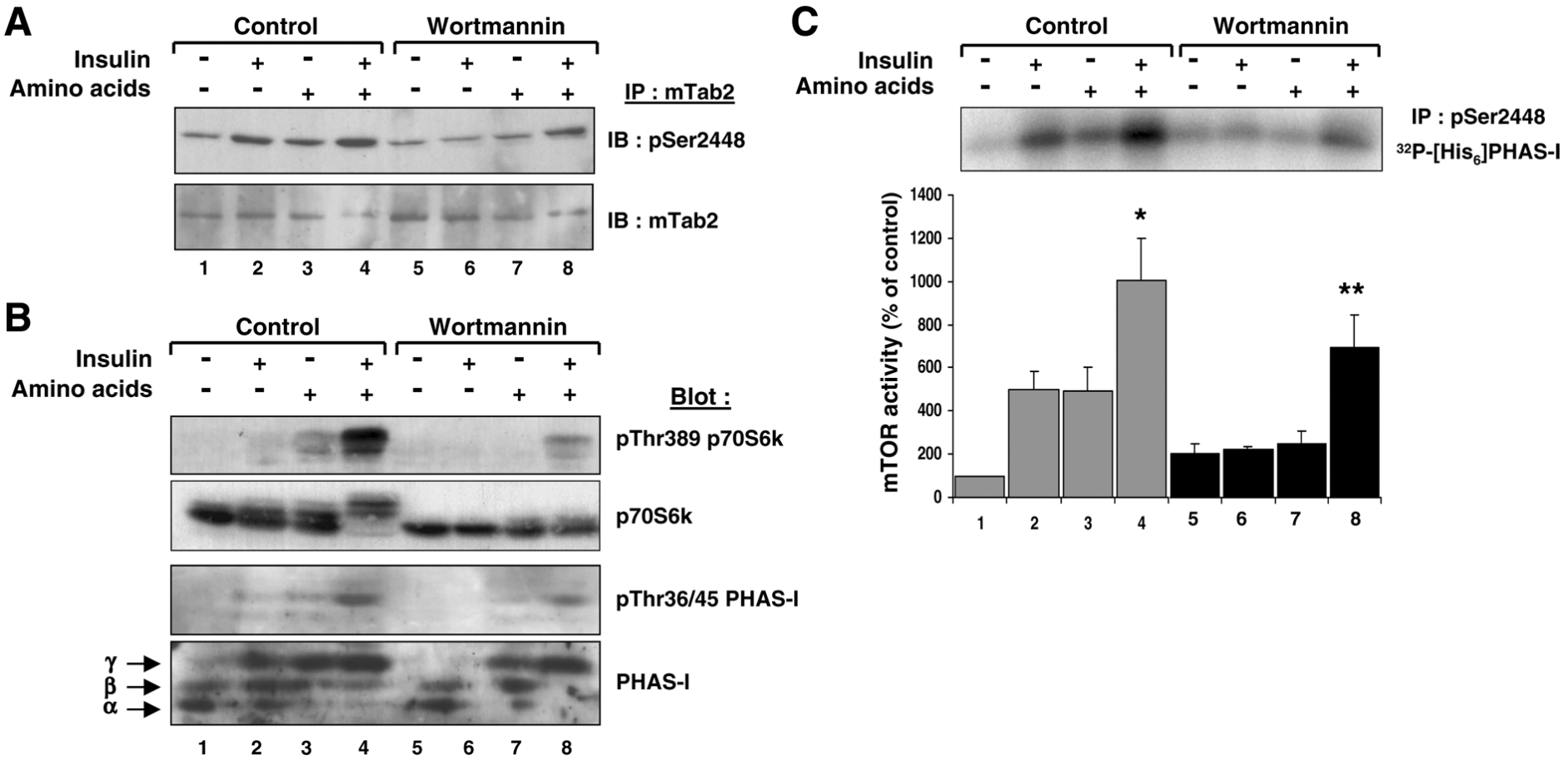

Figure 1. Effect of insulin and amino acids on mTOR pathway. Adipocytes were prepared as described in Materials and Methods and were treated with or without vehicle (0.001% Me_2SO) or wortmannin (100 nM). After 30 min, adipocyte suspensions were incubated with or without insulin (100 nM) and/or amino acids (1×) for 80 min. ***A*)** Adipocyte lysates were immunoprecipitated with mTab2 antibody. After washes, immune pellets were suspended in 3% (wt/vol) SDS sample buffer, separated by SDS-PAGE, and immunoblotted with antibody to phosphoserine 2448 or mTab2. ***B*)** In parallel, cell lysates were separated by SDS-PAGE and immunoblotted with phosphospecific p70S6k or PHAS-I antibodies and immunoblots were stripped and reprobed with p70S6k or PHAS-I antibodies. Representative immunoblots from experiments repeated at least three times are shown. ***C*)** For mTOR kinase assay, cell lysates were immunoprecipitated with antibody to phosphoserine 2448. After washes, beads were suspended in reaction mix prepared in phosphorylation buffer containing [His_6]PHAS-I (2 mg/reaction), [γ-^{32}P]-ATP (0.1 mM, 5 μCi/reaction), $MnCl_2$ (10 mM), and then incubated for 1 h as described in Materials and Methods. After the reaction was terminated, samples were subjected to SDS-PAGE and autoradiograms of the dried gels were prepared. ^{32}P incorporated in PHAS-I was quantified and expressed as a percentage of activity measured in control condition. Means ± SE from four individual experiments, each performed in duplicate, are shown. *P<0.05 vs. 2 or 3. **P<0.025 vs. 6 or 7.

Fig. 2

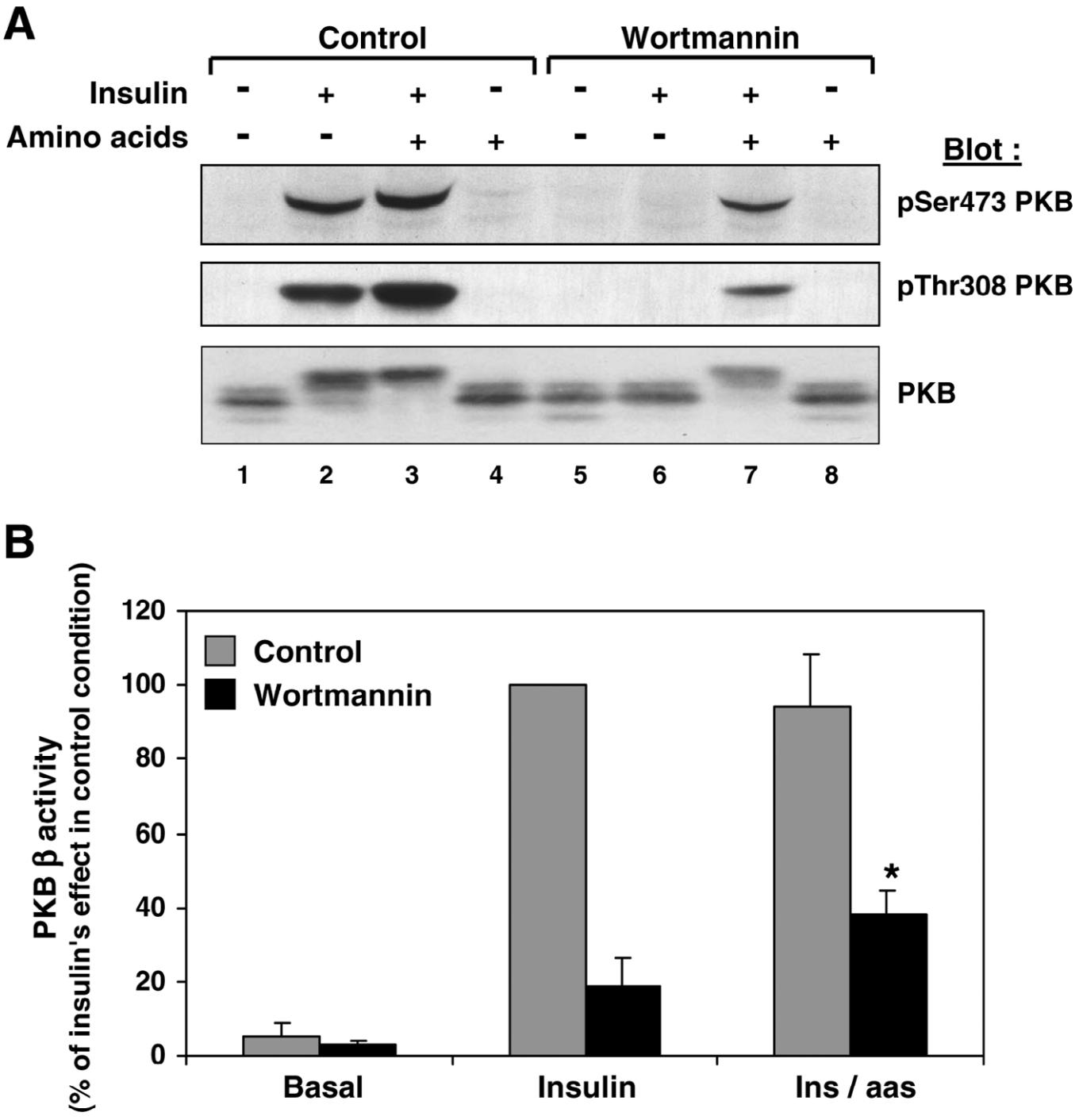

Figure 2. Amino acids allow insulin to activate PKB when PI 3-kinase activity is inhibited by wortmannin. Adipocytes were incubated as described in Figure 1. ***A*)** At the end of the incubations, adipocytes were centrifuged through dinonylphthalate, and the cell cakes were solubilized in 3% (wt/vol) SDS sample buffer. Samples were separated by SDS-PAGE and immunoblotted with phosphoPKB (Ser 473 or Thr 308) antibody, and immunoblots were stripped and reprobed with PKB antibody. Representative immunoblots are presented from experiments repeated at least four times. ***B*)** Adipocyte lysates were immunoprecipitated with antibody to PKBβ and were used to measure kinase activity toward Crosstide (30 μM), [γ-^{32}P]-ATP (4.5 μM, 2.5 μCi/reaction) over 30 min as described in Materials and Methods. ^{32}P incorporated in crosstide was quantified and expressed as a percentage of activity measured in presence of insulin. Means ± SE from three individual experiments, each performed in triplicate, are shown. **P*<0.05 vs. wortmannin and insulin.

Fig. 3

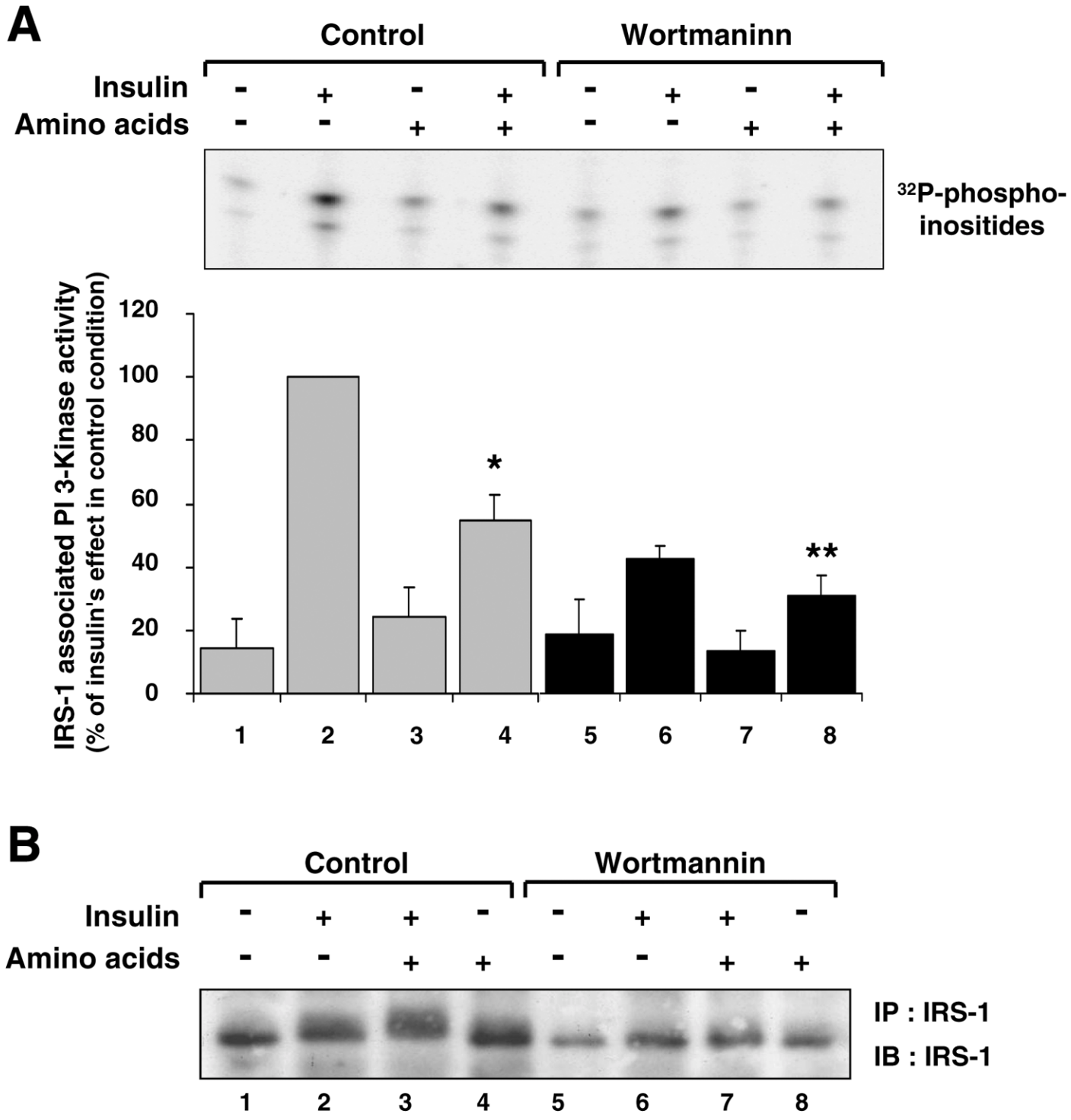

Figure 3. Amino acids inhibit insulin-stimulated PI 3-kinase activity. Adipocytes were incubated as described in Figure 1. ***A*)** For kinase assay, cell lysates were immunoprecipited with IRS-1 antibody and incubated after washes with phosphoinositides (10 μg/ml), [γ-^{32}P]-ATP (50 μM, 10 μCi/reaction) for 20 min as described in Materials and Methods. Samples were analyzed by chromatography. ^{32}P incorporated in phosphoinositides was quantified and expressed as a percentage of activity measured in presence of insulin. Means ± SE from three individual experiments, each performed in duplicate, are shown. **P*<0.005 vs. 2. ***P*< 0.05 vs. 4. ***B*)** Cell lysates were immunoprecipited with IRS-1 antibody for 2 h and washed. Immune pellets were then suspended in 3% (wt/vol) SDS sample buffer, separated by SDS-PAGE, and immunoblotted with IRS-1 antibody. One representative immunoblot of three individual experiments is presented.

Fig. 4

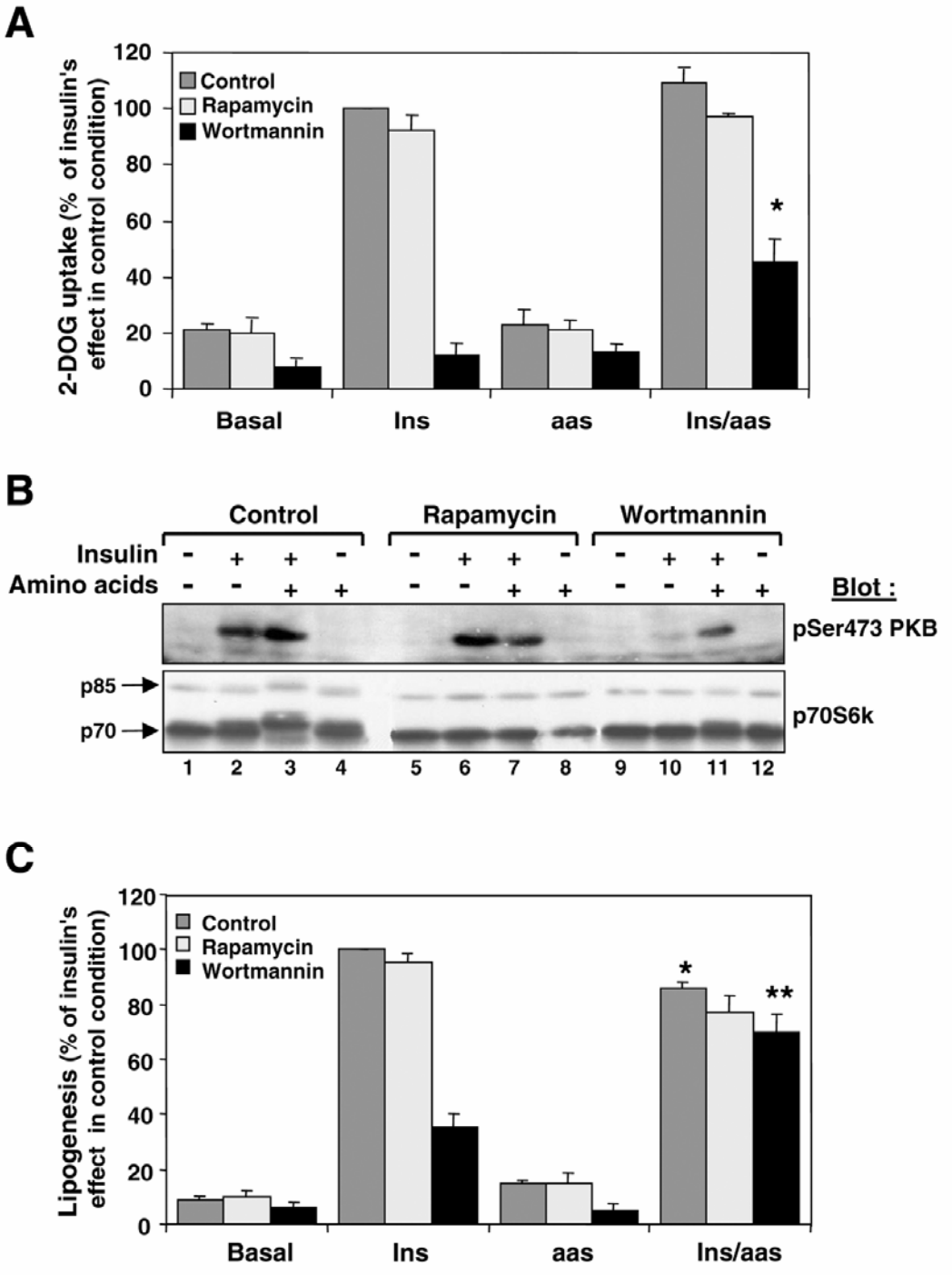

Figure 4. Effect of amino acids on insulin-stimulated glucose transport and lipogenesis. Adipocytes were treated with or without vehicle (0.005% Me_2SO), rapamycin (50 nM), or wortmannin (100 nM). After 30 min, adipocyte suspensions were incubated with or without insulin (100 nM) and/or amino acids (1×) for 80 min. ***A)*** Glucose transport was measured as described in Materials and Methods. ^3H incorporated in DOG was counted and expressed as a percentage of 2-DOG uptake in the presence of insulin. Means ± SE from three individual experiments, each performed in triplicate, are shown. **P*<0.025 vs. wortmannin and insulin. ***B)*** In parallel, cell lysates were separated by SDS-PAGE and immunoblotted with phosphoPKB (Ser 473) and p70S6k antibodies. Representative immunoblots of three individual experiments are presented. ***C)*** Lipogenesis was measured as described in Materials and Methods. ^3H incorporated in lipids was counted and expressed as a percentage of lipogenesis stimulated by insulin. Means ± SE from four individual experiments, each performed in triplicate, are shown. **P*<0.01 vs. insulin. ***P*<0.005 vs. wortmannin and insulin.

Fig. 5

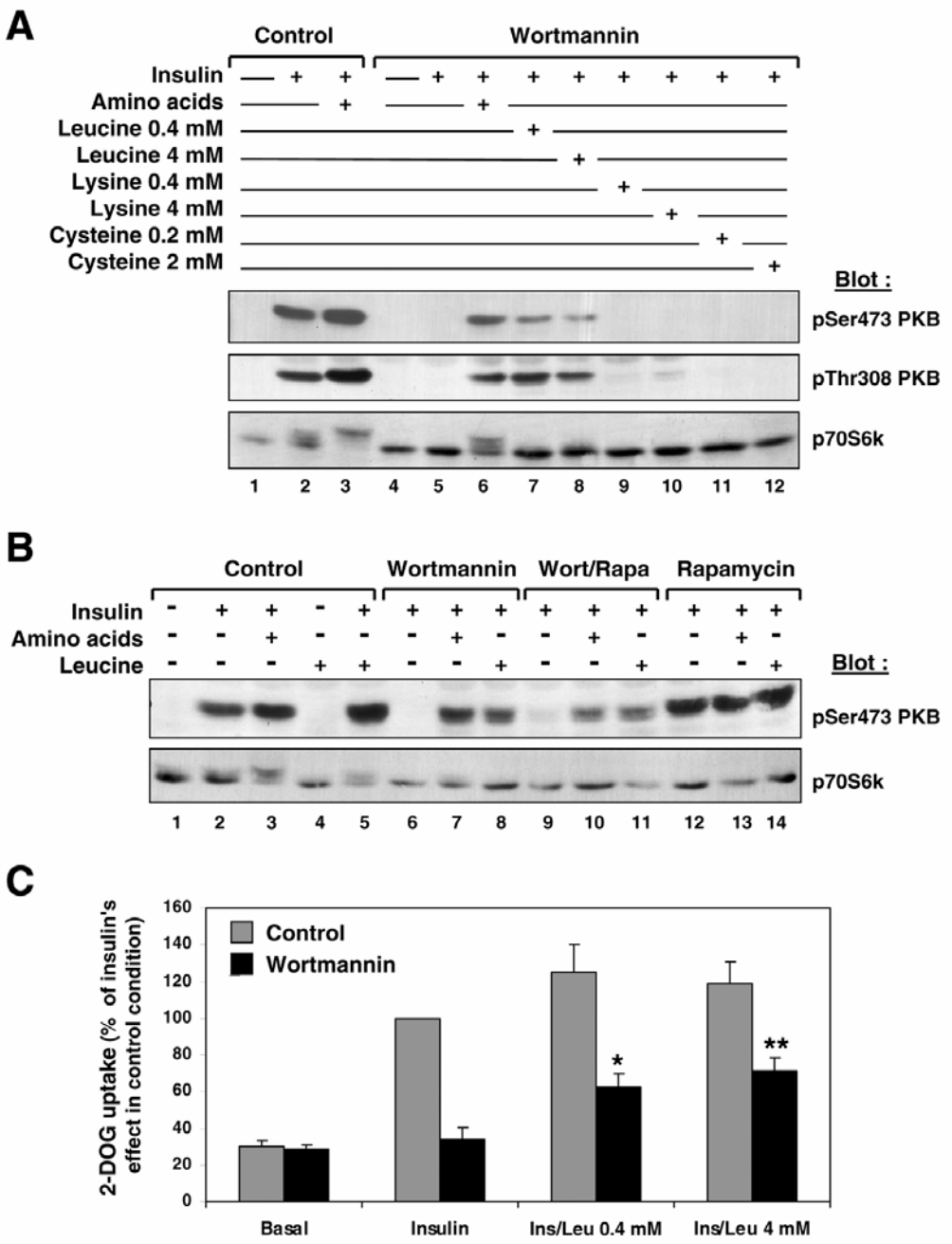

Figure 5. Leucine allows insulin to activate PKB and glucose transport when PI 3-kinase activity is inhibited. *A*) Adipocytes were incubated with or without vehicle or wortmannin for 30 min, then for 80 min in the following conditions: insulin or/and amino acids mixture or/and leucine (0.4 mM, 4 mM) or/and lysine (0.4 mM, 4 mM) or/and cysteine (0.2 mM, 2 mM). Samples were separated by SDS-PAGE and blotted with phosphoPKB (Ser 473 or Thr308) antibodies. Immunoblots were then stripped and reprobed with p70S6k antibodies. One representative immunoblot of three individual experiments is presented. ***B*)** Adipocytes were treated with or without vehicle, wortmannin, or/and rapamycin. After 30 min, cells were stimulated without or with insulin and/or amino acid mixture and/or leucine (0.4 mM) for 80 min. One representative immunoblot of three individual experiments with phosphoPKB (Ser 473) antibodies, stripped, and reprobed with p70S6k antibodies, is presented. ***C*)** [^{3}H]-2-DOG uptake was measured and expressed as described in Figure 4*A*) Adipocytes were incubated with or without vehicle or wortmannin for 30 min, then with or without insulin or/and leucine (0.4 or 4 mM) for 80 min. Means ± SE from 6 individual experiments, each performed in triplicate, are shown. **P*<0.01 or ***P*<0.005 vs. wortmannin and insulin.

Fig. 6

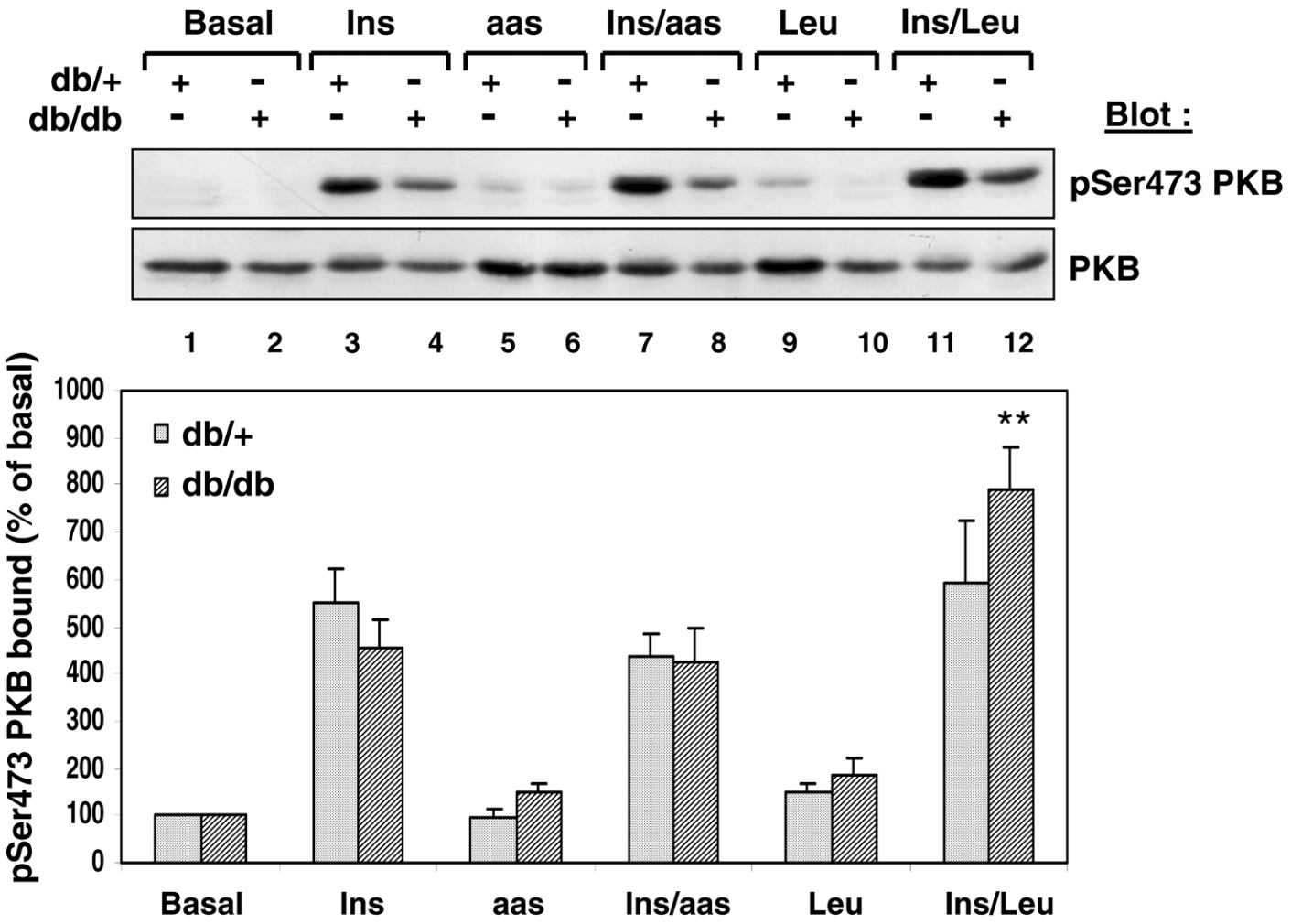

Figure 6. Leucine improves insulin-induced PKB phosphorylation in adipose tissue explants of *db/db* mice. Adipose tissue explants from male *db/+* or *db/db* mice at 8–10 weeks of age were incubated with or without insulin or/and amino acids mixture or/and leucine (4 mM) for 80 min, rinsed twice with ice-cold PBS, and lysed as described in Materials and Methods. Samples were separated by SDS-PAGE and immunoblotted with phosphoPKB (Ser 473) antibodies. Immunoblots were then stripped and reprobed with PKB antibodies. The results of phosphoPKB bound were quantified from five individual experiments in which one representative immunoblot is shown. The values were corrected for differences in the total amount of PKB recovered and expressed as a percentage of basal in *db/+* or *db/db* mice. $^{**}P<0.01$ vs. insulin of *db/db*.

Fig. 7

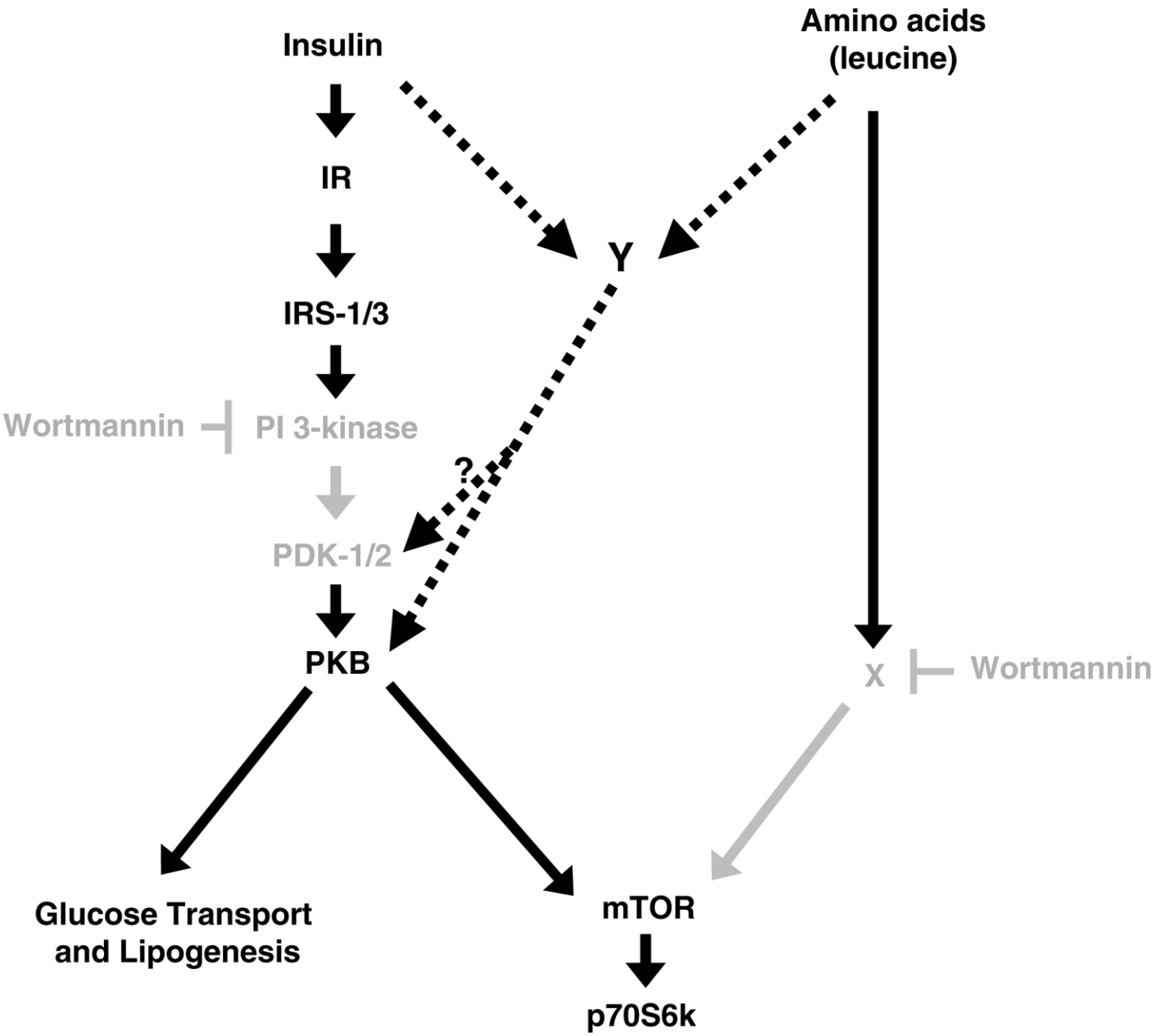

Figure 7. Schematic diagram of potential role of amino acids contribution in insulin signaling.