C. Hinault · I. Mothe-Satney
N. Gautier · E. Van Obberghen

Amino acids require glucose to enhance, through phosphoinositide-dependent protein kinase 1, the insulin-activated protein kinase B cascade in insulin-resistant rat adipocytes

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Abstract *Aims/hypothesis:* Amino acids are well known to activate the mammalian target of the rapamycin (mTOR) pathway in synergy with insulin to regulate cell functions. Despite recent important advances, the mTOR signalling pathway is poorly understood. Our previous results revealed a new pathway in which amino acids permit insulin-induced activation of the protein kinase B (PKB)/mTOR pathway in freshly isolated adipocytes when phosphatidylinositol 3-kinase (PI3K) is inhibited. The aim of this study was to further investigate this pathway at the molecular level. *Methods:* We studied the effect of amino acids on PKB phosphorylation in different cellular models or in freshly isolated adipocytes incubated in different buffers, after a time course of insulin and amino acids and in the presence of pharmacological inhibitors. To investigate the potential role of amino acids in insulin action, the effect on glucose transport in obese rat adipocytes following a high-fat diet was assessed. *Results:* Insulin-induced PKB phosphorylation is restored by amino acids in the presence of wortmannin in adipose tissue explants and freshly isolated adipocytes, but not in cultured adipocytes or hepatocytes. Moreover, amino acids require the presence of glucose to phosphorylate PKB and to partially rescue glucose transport in a PI3K-independent manner. The results also suggest that the amino acids act through the phosphoinositide-dependent protein kinase 1. In addition, amino acids were seen to improve insulin-stimulated glucose transport in adipocytes from high-fat-fed rats. *Conclusions/interpretation:* This study suggests that amino acids could enhance adipocyte insulin signalling in pathophysiological situations such as insulin resistance associated with obesity.

Keywords Adipocytes · Amino acids · Glucose transport · Insulin resistance · Obesity · Phosphorylation · PKB · Signalling

Abbreviations DOG: deoxyglucose · HFD: high-fat diet · mTOR: mammalian target of rapamycin · mTORC1: rapamycin-sensitive TOR complex 1 · mTORC2: rapamycin-insensitive TOR complex 2 · PDK1: phosphoinositide-dependent protein kinase 1 · PI3K: phosphatidylinositol 3-kinase · PKB: protein kinase B · PP2A: protein phosphatase 2A · Rheb: Ras homologue enriched in brain · TSC: tuberous sclerosis complex · UCN-01: 7-hydroxystaurosporine

Introduction

In addition to being substrates for different metabolic pathways, it is well recognised that amino acids activate the mammalian target of the rapamycin (mTOR) signalling pathway in synergy with insulin to regulate cell functions. Of the amino acids, leucine is the most potent activator of the mTOR pathway, most notably in adipocytes. Thus, mTOR mediates the crosstalk between amino acids and insulin signalling. The main function of mTOR is the control of cell growth, in particular via the regulation of protein synthesis. To achieve this regulation, mTOR phosphorylates the ribosomal protein S6 kinase (p70S6 kinase) and the translational repressor 4E-BP1 [1]. This signalling is mediated by the rapamycin-sensitive TOR complex 1 (mTORC1) formed by the association of mTOR with mLST8 and raptor proteins, necessary for nutrient-induced activation of the mTOR pathway [2–4]. More recently, a rapamycin-insensitive TOR complex 2 (mTORC2) formed by the association of mTOR with mLST8 and rictor protein has been identified in mammalian cells. mTORC2 does not seem to regulate protein synthesis, but, rather, is involved in the regulation of cytoskeleton organisation [5, 6].

mTOR is activated by insulin and amino acids via two independent pathways, a phosphatidylinositol-3 kinase

C. Hinault · I. Mothe-Satney (✉) ·
N. Gautier · E. Van Obberghen
INSERM Unit 145, IFR 50, Faculty of Medicine,
Avenue de Valombrose,
F-06107 Nice Cedex 2, France
e-mail: satney@unice.fr
Tel.: +33-4-93815447
Fax: +33-4-93815432

(PI3K)/protein kinase B (PKB) pathway stimulated by insulin [7] and an as-yet-unidentified PKB-independent pathway induced by amino acids [8, 9]. Several recent pieces of evidence have shown that intermediate proteins are involved between PKB and mTOR, such as tuberous sclerosis complex (TSC) formed by TSC1 and TSC2 (hamartin and tuberlin, respectively) [10, 11], and Ras homologue enriched in brain (Rheb) [12]. Rheb has been shown to bind and positively regulate mTOR [13, 14]. The action of Rheb is inhibited by TSC, which promotes the conversion of Rheb-GTP in Rheb-GDP. This inhibition of Rheb by TSC is blocked by PKB-induced TSC2 phosphorylation [15]. Despite these advances, the molecular mechanism of amino acid action in the regulation of the mTOR signalling is poorly understood and remains controversial. For instance, Gao et al. have published results indicating that TSC is involved in amino acid-TOR signalling [10]. Others have shown that the Rheb–mTOR interaction is regulated by amino acid sufficiency [14]. By contrast, Smith et al. have recently demonstrated that TSC2 is not required for the regulation of mTOR by amino acids. They have also shown that the binding of Rheb to mTOR is not disrupted by amino acid withdrawal [13]. Thus, further investigations are required to clearly identify how amino acids activate mTOR. An explanation could be provided by two recent studies presenting evidence that the class III PI3K hVps34 is a nutrient-regulated lipid kinase that integrates amino acid input to mTOR [16, 17].

Increasing interest is being taken in the role of amino acids in the modulation of insulin action in peripheral tissues. In adipose tissue, the mTOR-signalling pathway exerts tissue-specific actions on differentiation, morphogenesis, hypertrophic growth and leptin secretion [18]. We have previously shown in freshly isolated adipocytes that amino acids, notably leucine, permit insulin to stimulate PKB when PI3K is inhibited. They also rescue glucose transport and the mTOR pathway. Moreover, our previous results illustrated that leucine is able to improve insulin-stimulated PKB activation in insulin resistant *db/db* mice [8]. In the present study, we wanted to further investigate this positive effect of amino acids on insulin action. Firstly, we wanted to investigate whether amino acids can restore insulin-induced PKB activation despite the presence of wortmannin in different cellular models. In addition, it was desirable to better understand the molecular mechanism by which they act. Finally, to determine the role of amino acids in insulin resistance, their effect on glucose transport in adipocytes from high-fat-fed rats was investigated.

Materials and methods

Materials

BSA (Bovine Serum Albumin, Fraction V) was purchased from Interger (Purchase, NY, USA) and collagenase from Sigma-Aldrich (St Louis, MO, USA). Cell culture solutions, supplements and reagents for SDS-PAGE were from Life Technologies (Carlsbad, CA, USA). Recombinant

human insulin was from Novo-Nordisk (Copenhagen, Denmark). The amino acid mixture (50×) was from Gibco (Cergy Pontoise, France) and the concentration of amino acids in a 1× mixture was as previously described [8]. Wortmannin and okadaic acid were obtained from Sigma (St Louis, MO, USA). 2-Deoxy-D-[³H]-glucose (360 GBq/mmol) and [γ -³²P]-ATP (>185 TBq/mmol) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Leptin and insulin levels were quantified by an ELISA kit from Linco (St Charles, MO, USA). All other chemicals were of highest analytical grade.

Antibodies

PKB antibody was directed against a C-terminal peptide (466–473) of the PKB protein and detects total PKB α , PKB β and PKB γ protein levels (Cell Signalling Technology, Beverly, MA, USA). Phosphospecific antibodies to PKB were directed against the phosphoSer473 residue or phosphoThr308 residue of the three PKB isoforms and were purchased from Cell Signalling Technology, as well as the phosphospecific p70S6k antibody directed against the phosphoThr389 residue and the p70S6k antibody.

Animals

Animal care was performed in accordance with institutional guidelines. Male 3-week old Wistar rats ('Elevage Janvier', France) weighing 50–75 g were housed in pairs in plastic cages in animal quarters maintained at 22°C with a 12:12-h dark–light schedule. One group were fed a regular unrestricted diet for 3 weeks and killed by cervical dislocation to isolate adipocytes. Another group of male Wistar rats were fed an unrestricted regular diet (control) or high-fat diet (HFD) (ref. TD88137; HARLAN, Indianapolis, IN, USA) over a 10-week period, commencing 1 week after their arrival. This group of rats was killed following anaesthesia by ketamine:xylazine (87.5:12.5 mg/kg i.p.) to isolate adipocytes.

Cellular models

Adipose tissue fractionation Adipocytes were freshly isolated from epididymal fat pads of male Wistar rats (160–200 g) by collagenase digestion, as previously described [8]. Adipocytes were incubated in Krebs Ringer bicarbonate 30 mmol/l HEPES buffer, pH 7.4, (KRBH) (120 mmol/l NaCl, 4 mmol/l KH₂PO₄, 1 mmol/l MgSO₄, 0.75 mmol/l CaCl₂, 10 mmol/l NaHCO₃) with 0.3 mmol/l glucose and 1% (wt/vol) BSA. Primary rat preadipocyte cultures were prepared as previously described [19].

Adipose tissue culture Fat pads from Wistar rats were dissected, washed in KRBH buffer, finely minced and incubated (1 ml of media per 70–90 mg of tissue) in 12-well culture plates containing KRBH with BSA and

0.3 mmol/l glucose, pH 7.4. The plates were incubated at 37°C under 10% CO₂.

Adipocyte differentiation and primary culture of hepatocytes 3T3-L1 preadipocytes (ATCC CCL-92.1) and primary rat preadipocytes were cultured and differentiated by classical protocol [19]. Hepatocytes were isolated from male Wistar rats (160–200 g) by collagenase dissociation of the liver and cultured, as previously described [9].

Glucose transport

Glucose transport in freshly isolated adipocytes was assessed by the uptake of 2-deoxy-D-[³H]-glucose (0.3 mmol/l, 18.5 KBq/reaction) during 90 s at 37°C with constant shaking. The reaction was stopped by the addition of 40 µmol/l cytochalasin B. The incorporation of 2-deoxy-D-[³H]-glucose was measured with a beta-counter.

Measurement of PDK1 kinase activity

The activity of PDK1 in freshly isolated adipocytes was measured following immunoprecipitation from 500 µg of

lysate, employing the PDKtide substrate peptide as previously described [20]. The PDK1 antibody, the PDKtide peptide and the chemical inhibitor UCN-01 were a gift from D. Alessi (Dundee, Scotland).

Protein analysis

For freshly isolated adipocytes, following the different incubations, cells were centrifuged through dinonylphthalate, and the cell cakes were solubilised in 3% (wt/vol) SDS sample buffer. For the adipose tissue explants, after treatment with different reagents, samples were washed with KRBH buffer with or without insulin and/or amino acids, and lysed in buffer containing 0.2% (vol/vol) Tween-20, 1 mmol/l DTT, 1 mmol/l vanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mmol/l AEBSF. After homogenisation and 15 min shaking at 4°C, lysates were centrifuged for 10 min at 12,000×g. The supernatants were collected and eluted in sample buffer. For 3T3-L1 adipocytes, primary adipocytes, HepG2 cells and hepatocytes, cell monolayers were incubated for 2 h in KRB containing 1% (wt/vol) BSA and 0.3 mmol/l glucose after serum depletion overnight. Cells were then treated with different reagents at the concentration and for the duration given in the figure legends. After two washes with ice-cold

Fig. 1 Effect of amino acids on insulin-induced PKB phosphorylation in different cellular models. Adipocytes (a) or adipose tissue explants (b) were incubated with or without vehicle (0.001% Me₂SO) or wortmannin (100 nmol/l). After 30 min, cells were stimulated with or without insulin (100 nmol/l) or/and amino acid mixture (1×) or/and leucine (4 mmol/l) for 80 min. After overnight serum depletion, 3T3-L1 differentiated adipocytes (c) and primary differentiated adipocytes (d) were incubated for 2 h in KRBH containing 1% (wt/vol) BSA and 0.3 mmol/l glucose. Cells were

then incubated with or without vehicle (0.001% Me₂SO) or wortmannin (100 nmol/l). After 30 min, cells were stimulated with or without insulin (100 nmol/l) or/and amino acids (1×) for 80 min. After overnight serum depletion, HepG2 cells (e) and primary hepatocytes (f) were incubated as described above. Cell lysates were separated by SDS-PAGE and immunoblotted with phosphoPKB (Ser473) antibody. Immunoblots were then stripped and reprobed with PKB antibody. One representative immunoblot of three individual experiments is presented for each cell type

PBS, cells were scraped and lysed as described for adipose tissue explants. Each cellular extract was eluted with sample buffer. Samples (50 µg) were separated by SDS polyacrylamide gel electrophoresis using the Laemmli method, before proteins were electrophoretically transferred to PVDF membranes and immunoblotted with different antibodies. Immunoreactive proteins were revealed by enhanced chemiluminescence using ECL.

Statistical analysis

Results are presented as the means±SEM for number of experiments indicated in the figure legends. Statistical significance was assessed using the Student's *t*-test and accepted for $p < 0.05$.

Results

Effect of amino acids on insulin-induced PKB phosphorylation in different cellular models

We have previously shown that amino acids and leucine allow insulin activation of the PKB/mTOR pathway when PI3K is inhibited in adipocytes [8]. With these results in mind, we investigated whether this positive effect of amino acids on PKB when PI3K is inhibited by wortmannin, can be reproduced in other adipocyte models. The phosphorylation of PKB in rat adipose tissue explants was examined initially (Fig. 1b). Explants were incubated in the same experimental conditions as freshly isolated adipocytes. For each condition, we observe the same profile of PKB phosphorylation in adipose tissue explants (Fig. 1b)

Fig. 2 Amino acids require the presence of glucose to phosphorylate PKB and to partially rescue glucose transport when PI3K is inhibited in freshly isolated adipocytes. **a** Adipocytes were incubated in different buffers: KRBH containing 1% (wt/vol) BSA, with or without amino acids (1×) and with or without glucose (0.3 or 25 mmol/l), or DMEM containing 1% (wt/vol) BSA. Adipocytes were treated with or without vehicle (0.001% Me₂SO) or wortmannin (100 nmol/l). After 30 min, adipocyte suspensions were stimulated with or without insulin (Ins; 100 nmol/l) and with or without amino acids (1×) for 80 min. Cell lysates were separated by SDS-PAGE and immunoblotted with phosphoPKB (Ser473) antibody. Then, immunoblots were stripped and reprobed with PKB

antibody. Immunoblots shown are representative of at least three independent experiments. **b** Adipocytes were incubated in KRBH containing 1% (wt/vol) BSA with or without glucose (0.3 mmol/l), treated with or without vehicle (0.001% Me₂SO) or wortmannin (100 nmol/l) and then stimulated as described above. Glucose transport was measured by the uptake of 2-deoxy-D-[³H]-glucose. ³H incorporated in adipocytes was counted and expressed as a percentage of 2-deoxyglucose (DOG) uptake in basal in each condition. Means±SEM from three individual experiments, each performed in triplicate. White bars, no glucose; shaded bars, in the presence of 0.3 mmol/l glucose. * $p < 0.016$ vs wortmannin and insulin with glucose

compared to freshly isolated adipocytes (Fig. 1a). More precisely, an amino acid mixture or leucine also prevents the inhibition by wortmannin of insulin-induced PKB phosphorylation in adipose tissue explants. PKB phosphorylation in 3T3-L1 and stroma-vascular cells differentiated into adipocytes *in vitro* was then analysed. After overnight serum starvation, cells were incubated as described in the legend (Fig. 1c,d). In each cell type, insulin stimulates PKB phosphorylation as expected (Fig. 1c,d). Amino acids alone do not induce PKB phosphorylation and do not modulate the positive effect of insulin. Wortmannin inhibits insulin-stimulated PKB phosphorylation and this inhibition occurs even in the presence of amino acids both in 3T3-L1 and primary adipocytes. PKB phosphorylation in HepG2 cells and primary hepatocytes was also studied. The same pattern of PKB phosphorylation (Fig. 1e,f) as in 3T3-L1 or primary adipocytes was observed (Fig. 1c,d). Taken together, these results suggest that only in freshly isolated adipocytes and adipose tissue explants do amino acids and insulin manage to induce PKB phosphorylation despite the presence of wortmannin.

Amino acids require the presence of glucose to phosphorylate PKB and to partially rescue glucose transport when PI3K is inhibited

The following section of the study was carried out to better understand how amino acids permit PKB activation when PI3K is inhibited. Since the amino acid effect does not

occur in cultured cells, freshly isolated adipocytes were incubated in different buffers and PKB phosphorylation was analysed using the experimental conditions described (Fig. 2a). Freshly isolated adipocytes are usually incubated in KRBH containing 1% (wt/vol) BSA and 0.3 mmol/l glucose, and amino acids are added with insulin for 80 min after 30 min of wortmannin treatment. In the absence of glucose in KRBH, wortmannin inhibits insulin-induced PKB phosphorylation in the presence of amino acids. If amino acids are added in KRBH containing 1% (wt/vol) BSA and 0.3 mmol/l glucose from the beginning of the experiment, insulin-induced PKB phosphorylation persists despite the presence of wortmannin. Consequently, PKB phosphorylation is restored by amino acids even if they are added before wortmannin treatment. When glucose concentration in KRBH is increased up to 25 mmol/l (which is the concentration found in DMEM), wortmannin is still unable to inhibit insulin stimulation in the presence of amino acids. In contrast, when adipocytes are incubated in DMEM containing 1% BSA, wortmannin succeeds in inhibiting insulin-stimulated PKB phosphorylation. Since we have previously shown that amino acids can partially restore insulin-induced glucose transport in the presence of wortmannin, this effect was measured in freshly isolated adipocytes incubated in KRBH with or without glucose (0.3 mmol/l) (Fig. 2b). In the presence of glucose in the buffer, insulin induces glucose transport but the stimulation is 1.6× lower than in the absence of glucose. Interestingly, amino acids are able to significantly decrease wortmannin inhibition of insulin-induced glucose transport only when adipocytes are incubated in KRBH containing glucose,

Fig. 3 **a** Time course of insulin- and amino acid-stimulated PKB phosphorylation. Adipocytes were incubated with or without vehicle (0.001% Me₂SO) or wortmannin (0.1 µmol/l). After 30 min, adipocyte suspensions were stimulated with or without insulin (Ins; 100 nmol/l) or/and amino acids (1×) for 10, 30, 60 and 80 min. **b** Effect of different concentrations of wortmannin and amino acids on PKB phosphorylation. Adipocytes were incubated with or

without vehicle (0.001% Me₂SO) or wortmannin (Wort; 1 or 0.1 µmol/l). After 30 min, adipocyte suspensions were stimulated with or without insulin (100 nmol/l) or/and amino acids (1 or 2×) for 80 min. Cell lysates were separated by SDS-PAGE and immunoblotted with phosphoPKB (Ser473) antibody. Immunoblots were then stripped and reprobed with PKB antibody. Immunoblots shown are representative of at least three independent experiments

which is correlated with PKB phosphorylation (Fig. 2a). Thus, glucose is required for the effect of amino acids on PKB phosphorylation and on glucose transport.

PKB phosphorylation rescue by amino acids occurs rapidly but is sensitive to increased wortmannin concentration

Freshly isolated adipocytes were incubated with insulin and amino acids for increased periods of time, following treatment with or absence of wortmannin for 30 min, to analyse PKB phosphorylation (Fig. 3a). PKB is phosphorylated from 10 min after initiation of insulin stimulation with or without amino acids, up to 80 min. Interestingly, it was observed that 10 min of incubation with amino acids is sufficient to block the inhibition by wortmannin. We further investigated this effect of amino acids on PKB phosphorylation by increasing the concentration of wortmannin up to 1 $\mu\text{mol/l}$ (Fig. 3b). As expected, wortmannin inhibits insulin-induced PKB phosphorylation at both concentrations. Amino acids totally block the effect of wortmannin when used at 0.1 $\mu\text{mol/l}$. However, their effect is much less pronounced when the wortmannin concentration reaches 1 $\mu\text{mol/l}$. Adding twice the quantity of amino acids does not improve PKB phosphorylation. Therefore, the rescue of PKB phosphorylation by amino acids is sensitive to high wortmannin concentration.

PDK1, but not PP2A, is required for PKB phosphorylation by amino acids when PI3K is inhibited

In another attempt to understand how PKB is phosphorylated by insulin and amino acids in the presence of wortmannin, it was hypothesised that, in these conditions, the phosphatase PP2A is inhibited. PKB phosphorylation under our experimental conditions was analysed with or without vehicle (0.01% Me_2SO) or okadaic acid (1 $\mu\text{mol/l}$), an inhibitor of PP2A. Okadaic acid alone stimulates PKB phosphorylation (Fig. 4). Moreover, okadaic acid increases insulin stimulation on PKB phosphorylation since both act via two different pathways. Amino acids do not affect okadaic acid stimulation with or without insulin. The inhibition of insulin-induced PKB phosphorylation by

wortmannin is eliminated in the presence of okadaic acid. Moreover, okadaic acid increases the effect of amino acids and insulin in the presence of wortmannin on PKB phosphorylation. These results suggest that the phosphorylation of PKB by amino acids is independent of the PP2A pathway. Finally, we wondered whether PDK1, located upstream of PKB, is involved in the rescue of PKB phosphorylation by amino acids. To this end, a chemical inhibitor of PDK1, UCN-01 (7-hydroxystaurosporine) was used [21]. We performed an *in vitro* kinase assay in our experimental conditions with or without UCN-01 after immunoprecipitation of PDK1 and with the PDKtide substrate peptide as previously described [20] (Fig. 5a). PDK1 is already active at the basal state and the addition of insulin with or without amino acids has no significant effect. Wortmannin is unable to inhibit the *in vitro* kinase activity of PDK1. By contrast, UCN-01 strongly inhibits the PDK1 activity in the absence as well as in the presence of insulin and amino acids. Both PKB phosphorylation sites, Ser473 and Thr308, were also analysed (Fig. 5b, upper panel). UCN-01 inhibits insulin-induced PKB phosphorylation on Thr308, since this site is known to be phosphorylated by PDK1. Conversely, UCN-01 has no effect on Ser473 in the presence or the absence of amino acids in comparison with control conditions. Interestingly, UCN-01 inhibits insulin-induced PKB phosphorylation restored by amino acids in the presence of wortmannin. In these conditions, UCN-01 totally inhibits PKB phosphorylation on Thr308 and only partially on Ser473. In parallel, we analysed p70S6k phosphorylation on Thr389, which is a rapamycin-sensitive site and reflects its activity [22]. Phosphorylation of Thr389 is more pronounced when adipocytes are stimulated by insulin plus amino acids than by insulin alone. This is also reflected by an increased electrophoretic mobility of p70S6k (Fig. 5b, lower panel). Wortmannin completely inhibits p70S6k phosphorylation induced by insulin, but Thr389 phosphorylation induced by the combination of both reagents persists as shown in our previous study [8]. UCN-01 alone does not or only weakly affects p70S6k phosphorylation on Thr389 induced by insulin plus amino acids. This is not surprising because PDK1 has been described to directly phosphorylate p70S6k on Thr229 [23]. However, in the presence of wortmannin, UCN-01 diminished Thr389 phosphorylation induced by insulin plus amino acids. This observation correlates with the inhibition of PKB phosphorylation in

Fig. 4 Effect of okadaic acid on PKB phosphorylation. Adipocytes were incubated with or without vehicle (0.001% Me_2SO) or wortmannin (100 nmol/l). After 30 min, adipocyte suspensions were stimulated with or without insulin (100 nmol/l) or/and amino acids (1 $\times$) for 80 min, and 40 min before the end of the incubation,

vehicle (0.01% Me_2SO) or okadaic acid (1 $\mu\text{mol/l}$) was added. Cell lysates were separated by SDS-PAGE and immunoblotted with phosphoPKB (Ser473) antibody. Then, immunoblots were stripped and reprobed with PKB antibody. Immunoblots shown are representative of at least three independent experiments

Fig. 5 Effect of UCN-01 on PKB phosphorylation. Adipocytes were incubated with or without vehicle (0.01% Me₂SO) or UCN-01 (1 μmol/l) during 1 h, and 30 min before the end of the incubation vehicle (0.001% Me₂SO) or wortmannin (Wort; 100 nmol/l) was added. Adipocyte suspensions were then stimulated with or without insulin (Ins; 100 nmol/l) or/and amino acids (1×) for 80 min. **a** Adipocyte lysates (500 μg) were immunoprecipitated with antibody to PDK1 and used to measure kinase activity towards the PDKtide substrate peptide (10 μmol/l), [γ-³²P]-ATP (0.1 mmol/l, 18.5 KBq/

reaction) over 30 min at 30°C. ³²P incorporated in the substrate was counted. Means±SEM from three individual experiments, each performed in duplicate, are shown. *White bars*, control; *black bars*, wortmannin; *dark grey bars*, wortmannin plus UCN-01; *light grey bars*, UCN-01. **b** Cell lysates were separated by SDS-PAGE and immunoblotted with phosphoPKB (Ser473 and Thr308) and phosphospecific p70S6k antibodies (Thr389). Immunoblots were stripped and reprobed with PKB or p70S6k antibodies. Immunoblots shown are representative of at least three independent experiments

the same conditions. To summarise, amino acids require active PDK1 to stimulate PKB when PI3K is inhibited in adipocytes.

Amino acids improve insulin-stimulated glucose transport in adipocytes of HFD rats

At this point in the investigation the question arose of whether the positive effect of amino acids could occur in

the setting of physiologic impairment of insulin signalling. Our previous results have shown that leucine improves insulin-induced PKB phosphorylation in adipose tissue of *db/db* mice, a monogenic insulin resistant mouse model [8]. Thus, we wanted to further investigate the importance of amino acids in pathophysiological states by using other models of insulin resistance such as the obese rat fed with an HFD. This polygenic model resembles the most common form of insulin resistance in humans. Firstly, different parameters were measured in order to verify diet

Table 1 Characteristics of mice fed a normal or high-fat diet

	Weight (g)	Perididymal fat (g/kg)	Leptinaemia (ng/ml)	Glycaemia (pmol/l)	Insulinaemia (pmol/l)
Control rats	466±11 (n=11)	14.4±1.0 (n=11)	6.6±1.0 (n=3)	4.88±0.17 (n=11)	209±29 (n=6)
HFD rats	529±18 (n=15)	29±1.7 (n=15)	13.2±0.6 (n=5)	4.92±0.14 (n=15)	390±64 (n=9)

Male Wistar rats were fed with a regular diet (control rats) or a high fat diet (HFD rats) over 10 weeks. Each rat was weighed before being killed. Peri-testicular fat pads were weighed and normalised by body weight. Leptinaemia and insulinaemia were measured in serum from rats fasted overnight and for 6 h, respectively

Fig. 6 Amino acids improve insulin-stimulated glucose transport in freshly isolated adipocytes from HFD rats. Adipocytes were isolated from male rats fed a regular diet or a high-fat diet (for characteristics see Table 1) and incubated with or without insulin (Ins; 100 nmol/l) or/and amino acids (1×) for 80 min. Glucose transport was measured as described in the legend of Fig. 2b and expressed as a percentage of 2-DG uptake in basal condition in each group. Means±SEM of experiments, each performed in quadruples, from two independent groups of rats (five control rats [white bars], and nine HFD rats [black bars]) are shown. * $p < 0.022$ vs insulin in HFD rats. ** $p < 0.016$ vs insulin in control rats

efficiency (Table 1). As expected, rats fed with an HFD for 10 weeks became obese and had twice as much perididymal fat. Circulating leptin levels double in HFD rats and are proportionally correlated with established perididymal fat. HFD rats are non-diabetic with a fasted glycaemia similar to control rats, but they have a significantly higher insulinaemia ($p < 0.024$), a typical characteristic of insulin resistance, which is also confirmed by a glucose tolerance test (data not shown). The effect of amino acids on glucose transport in freshly isolated adipocytes from rats fed with regular diet ($n=5$) or HFD ($n=9$) (Fig. 6) was then measured. Insulin stimulates glucose transport in control rats, but insulin response is significantly reduced in HFD rats confirming their insulin resistance state. Amino acids alone stimulate glucose transport to the same extent as insulin in HFD rats. Interestingly, amino acids permit insulin to induce glucose transport in adipocytes from HFD rats—similar to the effect seen in control rats. To conclude, our results show that amino acids enhance insulin action on glucose transport in adipocytes from obese rats.

Discussion

We have previously determined a novel role for amino acids, notably leucine, in insulin action within freshly isolated adipocytes. Indeed, amino acids permit insulin-induced PKB activation when PI3K is inhibited, whereas alone they are unable to activate PKB. This effect is observed with two different PI3K inhibitors, wortmannin and LY294002 (data not shown). By generating this effect, amino acids also partially allow glucose transport and metabolism as well as the mTOR pathway to resume [8]. Therefore, the present study further investigates this newly

disclosed effect of amino acids. Our results firstly show that amino acids are able to rescue insulin-induced PKB phosphorylation in the presence of wortmannin only in freshly isolated adipocytes and in adipose tissue explants. This effect does not occur in 3T3-L1 and primary adipocytes, nor in hepatocytes. Moreover, PKB phosphorylation is not observed when PI3K is inhibited in intact muscles or in HEK293 cells, or when glucose or amino acid concentrations were increased (data not shown). Thus, the data suggest that amino acids allow insulin to activate the PKB pathway in a manner independent of PI3K only in freshly isolated adipocytes or in adipose tissue. It also appears that the adipocyte environment is important since the effect of amino acids does not occur in *in vitro* differentiated adipocytes and is dependent on the composition of the incubation buffer. Indeed, amino acids can rescue, at least in part, PKB activation and glucose transport when insulin-induced PI3K is inhibited, only in the presence of glucose. However, glucose concentration does not appear to be critical. This suggests that glucose is crucial for the integration of multiple signalling inputs rather than for the activation of signalling molecules. Taken as a whole, the results show that amino acids can sensitise PKB and glucose transport to insulin stimulation in the presence of wortmannin only in adipocytes under physiological conditions.

To explain this PI3K-independent effect, it was deduced that amino acids inhibit the action of phosphatases on PKB phosphorylation, notably the action of PP2A. Indeed, PP2A has recently been shown to be the main phosphatase involved in the regulation of PKB in freshly isolated adipocytes [24]. This hypothesis was tested by using okadaic acid, a PP2A inhibitor within our experimental conditions. It was shown that okadaic acid does indeed increase amino acid-stimulated PKB phosphorylation when insulin signalling is inhibited by wortmannin. This result suggests that the activation of PKB by amino acids is independent of the PP2A pathway. However, amino acids could act via other phosphatases such as PHLPP recently identified as a phosphatase that directly dephosphorylates PKB [25].

PKB needs to be phosphorylated on Ser473 and Thr308 for full kinase activation [26]. PDK1 was originally identified as the kinase that catalyses PKB phosphorylation at Thr308. Consequently, adipocytes were incubated in the presence or absence of UCN-01, a chemical inhibitor of PDK1. The activity of PDK1 was measured and the phosphorylation of PKB then analysed on both sites. The results show that PDK1 is constitutively active and remains active in the presence of wortmannin. Interestingly, UCN-01 inhibits the activity of the kinase and blocks the rescue of insulin-induced PKB phosphorylation on Thr308 by amino acids when PI3K is inhibited. Therefore, this pathway induced by insulin and amino acids requires PDK1.

We have previously measured PI3K activity associated with IRS-1 in our experimental conditions and showed that amino acids inhibit 45% of insulin-induced PI3K activity, which is most likely due to the negative feedback generated

from activated mTOR [8]. Moreover, we confirmed that wortmannin inhibits by 60% insulin-stimulated PI3K activity. This inhibition is further increased by amino acids. To explain PKB phosphorylation in the presence of insulin, amino acids and wortmannin, one possibility is that a residual PI3K activity could produce enough phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to allow the recruitment of PKB and PDK1. If this is the case, insulin could also phosphorylate PKB in the absence of amino acids despite the inhibition by wortmannin. This hypothesis can be excluded since no PKB phosphorylation is observed when wortmannin inhibits insulin-induced PI3K activity. Thus, the mechanism of amino acid-induced recruitment of PKB and PDK1 remains to be resolved. Furthermore, we have studied only class Ia PI3K, well known to be activated by insulin, but other classes of PI3Ks exist. Indeed, the availability of amino acids has recently been shown to regulate the mTOR pathway through hVps34, the class III PI3K [16, 17]. It is possible that hVps34 could take over from class Ia PI3K when the latter is inhibited by wortmannin or is defective.

Concerning Ser473 phosphorylation of PKB, the rapamycin-insensitive mTORC2 has recently been reported to be the “PDK2” notably in 3T3-L1 adipocytes [27, 28]. Considering our previous results where it was shown that rapamycin fails to inhibit PKB phosphorylation, this recent finding suggests that amino acids could rescue the phosphorylation of PKB on Ser473 through mTORC2 activation. It is not yet known whether this complex is regulated by amino acids. However, our study found that the effect of amino acids is sensitive to a high concentration of wortmannin. This reinforces the possible involvement of mTOR since Brunn et al. have published results showing that 1 μ mol/l of wortmannin irreversibly inhibits mTOR autokinase activity [29]. In conclusion, when PI3K is inhibited, amino acids and insulin could activate PKB by phosphorylating Thr308 through PDK1, and Ser473 through mTORC2. It is also conceivable that this effect occurs via hVps34. Nevertheless, further investigations will be required to elucidate this view.

In most situations in humans and animal models associated with insulin resistance, impaired activation of several key signalling molecules has been reported including the PI3K/PKB module [30]. To investigate whether the amino acid action which enhances insulin stimulation of PKB occurs in pathophysiological states, our study was extended to a model of insulin resistance which resembles the most common insulin resistance form in humans—the obese rat after HFD. To do so, groups of rats were fed without restriction with a regular diet or with an HFD for 10 weeks duration. These insulin resistant rats were used to study the effects of amino acids on insulin-induced glucose transport which is known to be impaired in such a model. Amino acids were shown to stimulate glucose transport in adipocytes from HFD rats, but not in adipocytes from control rats. Furthermore, amino acids are able to restore glucose transport stimulated by insulin in adipocytes from obese rats. These results are in agreement

with those obtained in the *db/db* mouse model [8]. Indeed, our previous work has shown that leucine improves insulin-stimulated PKB phosphorylation solely in adipose tissue explants of insulin resistant *db/db* mice [8].

To conclude, our present work brings new insight into how amino acids sensitise PKB to insulin’s stimulatory effect under conditions of hormonal resistance. Indeed, our results show that this positive effect of amino acids requires the presence of glucose and occurs in adipocytes under physiological conditions. Moreover, amino acids and insulin appear to act through PDK1 to restore PKB activation in the presence of wortmannin. Finally, our data reinforce the idea that amino acids enhance adipocyte insulin signalling in models of insulin resistance. It would be of interest to determine whether amino acid supplementation could be a novel approach to prevent or treat metabolic diseases associated with dysfunction of adipose tissue.

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