Potential role of 3-phosphoinositide-dependent protein kinase 1 (PDK1) in insulin-stimulated glucose transporter 4 translocation in adipocytes

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Abstract Insulin stimulation of Glut 4 translocation requires the activation of phosphatidylinositol 3-kinase (PI 3-kinase) but the downstream pathway remains ill-defined. We demonstrated that the overexpression of PDK1 (3-phosphoinositide-dependent protein kinase 1), a downstream effector of PI 3-kinase, stimulated Glut 4 translocation in adipocytes. This effect does not require the PH domain of PDK1, but expression of the pleckstrin homology domain-deleted PDK1 inhibits the effect of insulin, but not okadaic acid, on Glut 4 translocation. These results support a role of the PDK1 pathway in the transmission of insulin signal to Glut translocation.

Key words: Insulin effect; Okadaic acid; Glut 4 translocation; Adipocyte; 3-Phosphoinositide-dependent protein kinase 1

1. Introduction

In muscle and adipose cells insulin involves the translocation of specific vesicles containing the glucose transporter Glut 4 from an intracellular storage compartment to the plasma membrane [1]. It is now well established that phosphatidylinositol 3-kinase (PI 3-kinase) activation is required for this insulin effect. Indeed, expression of a constitutively active form of PI 3-kinase mimics insulin effects on Glut 4 translocation [2–4] while inhibition of PI 3-kinase activity prevents this translocation [5–7]. However, it is unclear how activation of PI 3-kinase relays the insulin signal to Glut 4 translocation. The protein serine/threonine kinase B (PKB) and the atypical protein kinases C λ or ζ (aPKC) have been shown to be downstream effectors of PI 3-kinase. Activation of these kinases requires their phosphorylation on both threonine 308 and serine 473 for PKB and threonine 410 for PKC ζ by the serine/threonine kinase PDK1 (3-phosphoinositide-dependent protein kinase 1) [8,9]. PDK1, like PKB, possesses a pleckstrin homology (PH) domain which binds phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) or phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3) and which is involved in the regulation of PDK1 localization and/or activity [10–12]. However, it is not clear whether PDK1 and its downstream effectors, PKD or aPKC, are involved in Glut 4 translocation. Indeed, both constitutively active forms of PKB [4,13] or PKC λ/ζ [14–16] increase Glut 4 translocation in adipocytes and 3T3-L1 adipocytes. However, some studies show that dominant negative mutants of PKB inhibit insulin-induced Glut 4 translocation [17,18] while our studies found no effect of these mutated forms of PKB [19] or found that dominant negative mutants of PKC λ/ζ inhibit glucose transporter translocation [14–16].

In this paper we assess the role of the PDK1 pathway in Glut 4 translocation in freshly isolated adipocytes. Our results indicate that overexpression of PDK1 partially mimics the insulin effect and that a mutated form of PDK1 deleted of its PH domain prevents insulin stimulation of Glut 4 translocation. We propose that PDK1 plays a role in the regulation of insulin-stimulated Glut 4 translocation.

2. Materials and methods

2.1. Antibodies

Monoclonal antibody to the HA epitope (clone 12CA5) was purchased from Roche Diagnostics (Meylan, France). Antibody against the myc epitope was raised in rabbits with the peptide AEEQKLISEEEDLLK coupled to keyhole limpet hemocyanin. Purified Ig was obtained by affinity chromatography.

2.2. Mammalian expression vectors

2.2.1. pCIS Glut 4 myc. pCIS is an expression vector containing a cytomegalovirus promoter and enhancer with a generic intron located upstream of the multiple cloning site (a gift of Genentech). The cDNA encoding rat Glut 4 with the myc epitope inserted in the first exofacial loop (Glut 4 myc) was subcloned into this vector as previously described [20].

2.2.2. pSG HA-PKB. The cDNA encoding bovine PKBα (a gift of Dr. Coffer, Utrecht, The Netherlands) was subcloned into the mammalian expression vector pSG5. The protein was tagged at the NH_2-terminus with an influenza virus hemagglutinin epitope.

2.2.3. pCMV5 PDK1myc and pCMV5 ΔPH PDK1myc. PDK1 (a gift of Dr. Alessi, Dundee, UK) cloned from the human MCF7 cDNA library was subcloned into the vector pCMV5. ΔPH PDK1 was obtained by deleting the PH domain of PDK1 and was subcloned into the pCMV5 vector. Both proteins were tagged at their NH_2-terminus with a myc epitope.

Plasmid DNAs were obtained using a maxi kit (Qiagen SA, Courteboeuf, France), and their concentrations were determined by measuring the OD at 260 nm.

2.3. Measurement of PKB activity

Adipose cells were isolated from epididymal and perirenal fad pats of fed male Wistar rats (200–220 g) by collagenase digestion. Adipocytes were transfected by electroporation using a double electric shock with 1 μg of pSG HA-PKB and 10 μg of pCMV5 PDK1myc [4]. After 16–20 h, electroporated adipocytes were washed twice, resuspended as a 20% (v/v) suspension in Krebs-Ringer bicarbonate buffer containing 30 mM HEPES, pH 7.4 (KRBH) and 1% (w/v) bovine serum albumin (BSA) and incubated for 10 min at 37°C with or without 100 nM insulin. After washing, cells were solubilized for 45 min at 4°C in 20 mM Tris pH 7.4, 150 mM NaCl, 10 mM EDTA, 2 mM sodium orthovanadate, 100 mM NaF, 10 mM $Na_4P_2O_7$, containing a protease inhibitor cocktail (Complete Roche Diagnostics, Meylan, France). Lysates were centrifuged for 15 min at 13 000×*g* and pro-

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Abbreviations: PDK1, 3-phosphoinositide-dependent protein kinase 1; PH, pleckstrin homology; PtdIns, phosphatidylinositol

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teins (150–200 μg) were incubated with monoclonal anti-HA antibodies coupled to protein G Sepharose beads. After 3 h at 4°C, the beads were washed three times with 25 mM HEPES pH 7.4, 0.1% BSA, 10% glycerol, 1% Triton, 1 M NaCl and twice with 20 mM Tris pH 7.4, 10 mM $MgCl_2$, 0.5 mM DTT. Assay for PKB activity was performed on the immune pellet using Crosstide as a substrate [21].

2.4. Assay for cell surface epitope-tagged Glut 4 measurement

Adipocytes were co-transfected by electroporation with pCIS Glut 4 myc (1 μg) and with pCMV5 PDK1myc (10 μg) or pCMV5 ΔPH PDK1myc (10 μg). 16–20 h following the transfection, they were resuspended as a 10% (v/v) suspension in KRBH buffer containing 1% (w/v) BSA. Cells were then incubated for 30 min at 37°C in the absence or presence of 100 nM insulin. Following insulin stimulation, adipose cells were treated with 2 mM potassium cyanide to prevent Glut 4 redistribution. The level of cell surface Glut 4 myc was determined using rabbit antibodies to the myc epitope in conjunction with ^{125}I-radiolabeled protein A [4]. Radioactivity was normalized by measuring protein concentration in each sample using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Cells transfected with empty vector alone were used to determine non-specific binding, which represented 30% of the total binding observed in cells transfected with pCIS Glut 4 myc in the absence of insulin stimulation. This value was subtracted from all values.

3. Results and discussion

To test whether overexpression of PDK1myc activates PKB, freshly isolated adipocytes were cotransfected with pSG HA-PKB and pCMV5 PDK1myc and were treated or not with insulin before the measurement of HA-PKB activity using Crosstide as a substrate. Insulin induced a 8–9-fold increase in PKB activity in cells transfected with pSG HA-PKB alone (Fig. 1A, control). PDK1myc overexpression partially activates PKB and insulin treatment did not further increase this activity compared to the insulin-treated control cells (Fig. 1A). This result is in agreement with previous data showing that overexpression of PDK1 in 293 cells induces only the phosphorylation of PKB on its threonine 308 and its partial activation [10]. Indeed, phosphorylation of both threonine 308 and serine 473 was necessary for the full activation of PKB [22–24]. To study whether PDK1 could play a role in Glut 4 translocation, adipocytes were transfected with pCIS Glut 4 myc and pCMV5 PDK1myc, and the amount of Glut 4 myc at the plasma membrane was measured by the binding of an anti-myc antibody to the intact cells. This approach makes it possible to determine specifically the amount of transporters at the cell surface of transfected adipocytes. In control cells, expressing Glut 4 myc alone, insulin induced a 4-fold increase in the amount of Glut 4 myc at the cell surface (Fig. 1B). In the absence of insulin stimulation, PDK1 overexpression increased the number of Glut 4 myc at the plasma membrane by 2-fold compared to control cells (Fig. 1B). Following insulin stimulation, the amount of Glut 4 myc at the cell surface was similar in control adipocytes or in PDK1-transfected adipocytes. These results show that an increase in PDK1 activity partially mimics the effect of insulin. It has been reported that the PH domain of PDK1 could play a role in the activation and/or the localization of the protein [10–12] leading us to study the effect of the deletion of this domain on the ability of PDK1 to stimulate Glut 4 translocation. Adipocytes were transfected by electroporation with pCIS Glut 4 myc alone (Fig. 2, control) or in combination with pCMV5 ΔPH PDK1myc (Fig. 2, ΔPH-PDK1). As shown in Fig. 2, ΔPH PDK1 expression induced an increase in Glut 4 translocation in the absence of insulin stimulation indicating that the PH

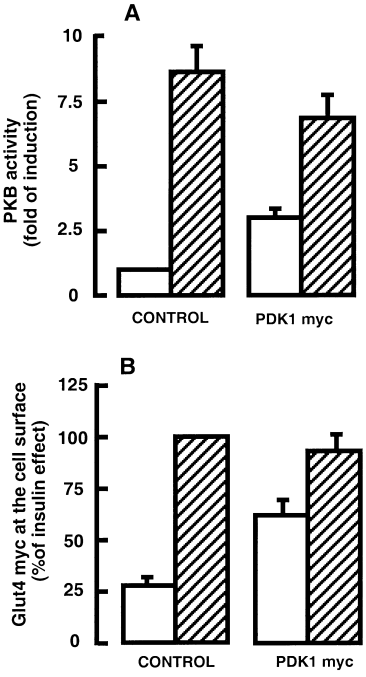

Fig. 1. PDK1 overexpression promotes PKB activation and Glut 4 myc translocation in adipocytes. A: Adipocytes were transfected as described in Section 2 with pSG HA-PKB alone (CONTROL) or in combination with pCMV5 PKD1myc. After 24 h, cells were incubated for 10 min in the absence (open bars) or presence (hatched bars) of insulin (100 nM). HA-PKB was immunoprecipitated with anti-HA antibodies, and kinase assays were performed on immune pellets as described in Section 2. Results are expressed as fold induction and represent the mean ± S.E.M. of five independent experiments. B: Adipocytes were transfected with pCIS Glut 4 myc alone (CONTROL) or in combination with pCMV5 PDK1myc. After 24 h, cells were incubated without (open bars) or with (hatched bars) 100 nM insulin for 30 min. The amount of Glut 4 myc at the cell surface was measured by the binding of antibodies to the myc epitope as described in Section 2. Results are expressed as percent of insulin stimulation. Values are presented as the mean ± S.E.M. of six independent experiments.

domain of PDK1 is not necessary for this effect. It has been shown that PDK1 without its PH domain phosphorylates PKB on threonine 308 leading to a partial activation of this protein [10]. This could explain why the expression of ΔPH PDK1 partly stimulates Glut 4 translocation. Interestingly, while expression of ΔPH PDK1 per se stimulated Glut 4 translocation, it markedly reduced the insulin-stimulated Glut 4 translocation (Fig. 2). This effect is not due to a change in Glut myc expression (data not shown) and seems specific for insulin since ΔPH PDK1 expression did not modify the effect of okadaic acid (Fig. 2), an inhibitor of protein phosphatase 2A, which stimulates glucose transport and PKB activity independently of the PI 3-kinase/PDK1 pathway [21,25]. These data suggest that the PH domain of PKD1 is required to allow the insulin stimulation of Glut 4 translocation and that ΔPH PDK1 behaves as a dominant negative mutant which interferes with the insulin signaling pathway. A recent report demonstrated that PDK1 can also phosphorylate PKB on its serine 473 but, unlike its threonine 308 phosphorylating activity, this serine phosphorylation was increased by PtdIns(3,4,5)P_3 production suggesting that the PH domain was required [26]. It is thus conceivable that ΔPH PDK1 could not phosphorylate PKB on serine 473 and prevent the

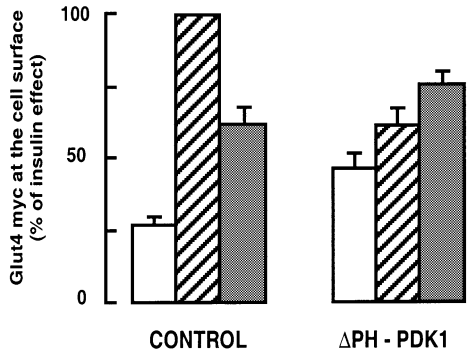

Fig. 2. ΔPH PDK1 expression inhibits insulin-stimulated Glut 4 myc translocation in adipocytes. Isolated adipocytes were transfected with pCIS Glut 4 myc alone (CONTROL) or in combination with pCMV5 PDK1myc. Twenty-four hours after transfection, cells were treated without (open bars) or with (hatched bars) 100 nM insulin or with 1 μM okadaic acid (gray bars) for 30 min. The amount of Glut 4 myc present at the cell surface was measured by binding of antibodies to the myc epitope. Results are expressed in percent of insulin effect and represent the mean ± S.E.M. of six independent experiments.

serine 473 phosphorylation of endogenous PKB by insulin. The mechanism of this effect remains to be determined. However, since it has been shown that PDK1 can form a complex with its downstream effectors such as PKC ζ/λ [27], it would be possible that overexpressed ΔPH PDK1, by titrating these proteins, could blunt their activation.

In summary, our results show that the PDK1 pathway is involved in the insulin-induced translocation of Glut 4. Further the PH domain of PDK1 seems necessary for the insulin stimulation of this translocation.

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