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Rapid communication

Impaired phosphorylation and insulin-stimulated translocation to the plasma membrane of protein kinase B/Akt in adipocytes from Type II diabetic subjects

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Abstract

Aims/hypothesis. To examine protein kinase B/Akt distribution and phosphorylation in response to insulin in different subcellular fractions of human fat cells from healthy subjects and subjects with Type II (non-insulin-dependent) diabetes mellitus.
Methods. We prepared subcellular fractions of plasma membranes (PM), low density microsomes and cytosol and examined gene and protein expression as well as serine and threonine phosphorylation in response to insulin.
Results. Protein kinase B/Akt mRNA as well as total protein kinase B/Akt protein in whole-cell lysate and cytosol were similar in both groups. Insulin increased protein kinase B/Akt translocation to the the plasma membrane about twofold [($p < 0.03$) in non-diabetic cells but this effect was impaired in diabetic cells (~ 30 %; $p > 0.1$)]. In both groups, protein kinase B/Akt threonine phosphorylation considerably increased in low density microsomes and cytosol whereas serine phosphorylation was predominant in the plasma membrane. Phosphatidylinositol-dependent kinase 1, which partially activates and phosphorylates protein kinase B/Akt on the specific threonine site, was predominant in cytosol but it was also recovered in low density microsomes. Serine phosphorylation in response to insulin was considerably reduced (50–70 %; $p < 0.05$) in diabetic cells but threonine phosphorylation was less reduced (~ 20 %). Wortmannin inhibited these effects of insulin supporting a role for PI3-kinase activation.
Conclusion/interpretation. Insulin stimulates a differential subcellular pattern of phosphorylation of protein kinase B/Akt. Furthermore, insulin-stimulated translocation of protein kinase B/Akt to the plasma membrane, where serine phosphorylation and full activation occurs, is impaired in Type II diabetes. Threonine phosphorylation was much less reduced. This discrepancy may be related to differential activation of phosphatidylinositol 3-kinase in the different subcellular compartments and phosphatidylinositol-dependent kinase 1 having high affinity for phosphatidylinositol phosphate 3. [Diabetologia (2000) 43: 1107–1115]

Keywords PKB/Akt, PI3-kinase, insulin action, Type II diabetes, GLUT-4.

Insulin resistance, i. e. an impaired ability of insulin to elicit its various metabolic effects including glucose uptake in the target tissues of fat, liver and muscle, is an important cause of Type II (non-insulin-dependent) diabetes mellitus and cardiovascular disease [1–3]. Insulin stimulates glucose uptake through the translocation of the GLUT-4 glucose transporters from intracellular site(s) to the plasma membrane [4, 5]. Insulin resistance is probably caused by defects in the insulin signal transduction pathways [6, 7] but reduced expression of GLUT-4 or impaired translocation or both also play a part in Type II diabetes [8, 9].

The insulin signalling pathways responsible for GLUT-4 translocation are still not clearly defined al-

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Abbreviations: PK: Protein kinase, PM: plasma membranes, LDM: low density microsomes, PDK1: phosphatidylinositol-dependent kinase 1, PI3: phosphatidylinositol-3, IRS, insulin receptor substrate, PIP, phosphatidylinositol phosphate

though much progress has been made recently [10, 11]. Activation of the insulin receptor, tyrosine kinase, leads to the phosphorylation of the key docking proteins, insulin receptor substrate 1 and 2 (IRS-1 and 2), for proteins containing Src homology 2 (SH2) like the p85 regulatory subunit of phosphatidylinositol-3 (PI3)-kinase. Activation of PI3-kinase is a key step for initiating several of insulin's metabolic effects, including glucose uptake and GLUT-4 translocation, as evidenced by the inhibitory effects of introducing a dominant negative p85 regulatory subunit as well as by the relatively specific PI3-kinase inhibitor, wortmannin [12, 13].

Although activation of PI3-kinase is necessary to elicit the glucose transport stimulated by insulin, it is not sufficient on its own [14]. Several down-stream serine/threonine kinases have been identified as targets of PI3-kinase. These include protein kinase (PK)B/Akt [15], PKCζ and PKCλ [16]. There is much evidence to support a role for PKB/Akt but contradictory results have also been found. Expressing constitutively active PKBα/Akt 1 in 3T3-L1 adipocytes was found to lead to GLUT-4 translocation to the plasma membrane [17]. Similarly, introduction of dominant-negative and kinase-dead PKB/Akt constructs in L6 cells inhibited insulin-stimulated glucose transport [18]. Furthermore, microinjection of a PKB/Akt substrate peptide or an antibody to PKB/Akt also inhibited the effect of insulin [19]. In contrast, another PKB/Akt dominant-negative mutant, where the two phosphorylation sites had been mutated, did not prevent insulin-stimulated GLUT-4 translocation although other inhibitory effects were found [20]. The reason for these inconsistent results is not clear but could relate to the cells used, the complex regulation of PKB/Akt activity and cell-specific expression of the PKB/Akt isoforms [11, 21]. A role for PKB/Akt is also supported by our recent findings with an inhibitor of PKB/Akt kinase activity which, albeit not selective, did not inhibit PI3-kinase activation. Insulin-stimulated glucose transport and GLUT-4 translocation were, however, completely inhibited [22].

Insulin-stimulated PKB/Akt activity and serine phosphorylation in whole-cell lysates were recently found to be reduced in fat cells from Type II diabetic subjects [23], probably due to upstream signalling defects leading to impaired PI3-kinase activation. In contrast, PKB/Akt activation in muscle in response to an insulin infusion was found in one study [24] to be normal in Type II diabetic subjects but in another, where a high insulin concentration was added in vitro, to be impaired [25]. The reason for this discrepancy is not clear. Human fat cells, similar to rat and differentiated 3T3-L1 adipocytes, mainly express the PKBβ/Akt 2 isoform and hardly any PKBα/Akt 1 [23] whereas muscle predominantly expresses PKBα/Akt 1 and PKBγ/Akt 3 [21]. Similar to PI3-kinase activation, possibly, however, of most importance is not total PKB/Akt activity in whole cells but the need for activation in the relevant subcellular compartments. It has been found that PKBβ/Akt 2 is associated with the vesicles containing GLUT-4 [26] similar to PI3-kinase [27].

The present concept of PKB/Akt activation suggests that phosphatidylinositol phosphates (PIPs), the products of PI3-kinase, recruit PKB/Akt as well as phosphatidylinositol-dependent kinase 1 (PDK1), which phosphorylates PKB/Akt on threonine 308 (PKBα/Akt 1) or 309 (PKBβ/Akt 2), to the plasma membrane where PKB/Akt becomes phosphorylated. Full activation also, however, requires the phosphorylation of serine 473 (PKBα/Akt 1) or 474 (PKBβ/Akt 2) in the plasma membrane by the still unidentified PDK2 [11, 21].

We examined the PKB/Akt gene and protein expression as well as phosphorylation of the protein in response to insulin in different subcellular fractions from non-diabetic and Type II diabetic fat cells.

Materials and methods

Materials. Human insulin was from Novo Nordisk (Copenhagen, Denmark). Bovine serum albumin (BSA) (fraction V), collagenase, wortmannin and other fine chemicals were from Sigma (St. Louis, Mo., USA). Nonidet P-40 was purchased from USB (Cleveland, Ohio, USA). Antibodies specifically recognizing the serine 473/474 and threonine 308/309 phosphorylated regions of PKBα/Akt 1 and PKBβ/Akt 2 as well as a control antibody recognizing the same, but non-phosphorylated, region were purchased from New England Biolabs (Beverly, Mass., USA). Anti-GLUT-4 antibodies were a kind gift from Dr. S. W. Cushman (NIH, Bethesda, Md., USA). Protein A/G-Sepharose was from Santa Cruz (Santa Cruz, Calif., USA).

Subjects and source of adipose tissue. Specimens of human subcutaneous adipose tissue were obtained by needle biopsies or surgery for non-malignant disorders from the abdominal region of non-diabetic subjects ($n = 16$) and Type II diabetic subjects ($n = 12$). The cell proteins from the latter subjects were pooled into three groups. The Type II diabetic subjects had been diabetic for 10 ± 2 years and their mean HbA_{1c} value was 7.7 ± 0.3 % (reference 3.5–5.3 %). They were treated with oral agents alone ($n = 6$) or combined with insulin ($n = 6$). The biopsy specimens were obtained in the fasting state in the morning and at least 10 h after the subjects had taken their last medication. The biopsy specimens were placed in medium 199 at 37 °C containing 25 mmol/l HEPES, 4 % BSA with 5.5 mmol/l glucose. The tissue was immediately transported to the laboratory for further processing. Informed consent was obtained from the subjects and the study was approved by the ethics committee of the Gothenburg University.

Preparation of isolated human adipose cells. Adipose cells were essentially prepared as described previously [28]. Briefly, the tissue was cut into small fragments visibly free of connective tissue. About 0.6 g of tissue was incubated at 37 °C in a shaking water bath with medium 199 containing 25 mmol/l HEPES, 4 % BSA, 5.5 mmol/l glucose and 0.8 mg/ml collage-

nase. After approximately 50 min, liberated cells were filtered through a nylon mesh with a pore size of 400 μm and washed four times in a new medium of the same composition but excluding collagenase and finally resuspended. Cell size and number were measured as described previously [28]. Cells were then incubated with the additions indicated in the Results in the presence of saturating adenosine concentrations (200 nmol/l adenosine).

Cell lysate and immunoblotting. All cell incubations were carried out at 37 °C in medium 199 buffered with 25 mmol/l HEPES and with 200 nmol/l adenosine and 4 % BSA. Isolated human adipocytes were distributed between plastic vials (12–15 % cell suspension) in a final incubation volume of 500 μl. Cells were incubated as described with or without 6.9 nmol/l insulin and 1 μmol/l wortmannin for 10 min. This latter inhibitor was always present for 5 min before insulin when combined with it. Cells were immediately separated by centrifugation through silicone oil and lysed in 0.4 ml lysis buffer containing 25 mmol/l TRIS-HCl, pH 7.4, 0.5 mmol/l EGTA, 25 mmol/l NaCl, 1 % Nonidet P-40, 1 mmol/l Na_3VO_4, 0.1 mmol/l okadaic acid, 10 mmol/l NaF, 0.2 mmol/l leupeptine, 1 mmol/l benzamidine and 0.1 mmol/l 4-(2-aminoethyl)-benzenesulphonylfluoride hydrochlorine (AEBSF) and rocked for 40 min at 4 °C. Detergent-insoluble material was sedimented by centrifugation at 12,000 × *g* for 10 min at 4 °C and the supernatants collected. Whole-cell lysates were boiled for 5 min in Laemmli buffer containing 0.5 mmol/l TRIS-HCl, pH 6.8, 10 % SDS, 0.1 % bromophenol blue and 55 mmol/l dithiothreitol. The samples were analysed by electrophoresis on 7.5 or 10 % SDS-polyacrylamide gels, the proteins transferred from the gel to nitrocellulose sheets and blocked in 5 % fat-free milk. The blots were probed with the different primary antibodies according to the manufacturer's recommendations and the proteins detected by enhanced chemiluminescence using horseradish peroxidase-labelled secondary antibodies (Amersham, Buckinghamshire, UK). The intensity of the bands was quantified by a laser densitometer Phospho Imager (Molecular Dynamics, Sunnyvale, Calif., USA) and expressed in arbitrary units. Proteins were identified by the bicinchoninic acid method [29], using a commercial kit (Pierce Chemical Co., Rockford, Ill., USA).

PKB/Akt gene expression. The RNA was extracted from the cells with guanidinium thiocyanate as described [30]. Northern blots were done on total cellular RNA (30 μg) with a labelled cDNA probe against *β*-actin as housekeeping gene and with a PCR fragment against PKB*β*/Akt 2 (bp 282–1130, Accession Nr 1495936) in a common sequence for PKBα/Akt 1, PKB*β*/Akt 2 and PKB*γ*/Akt 3. The 5' sequence CGAGAGGCCGCGACCCAACAC and 3' sequence AGGCGGCCGCACATCATCTCGTA were used as primers.

Subcellular fractionation. Subcellular fractions, enriched in cytosol, plasma membranes and intracellular microsomes, were obtained after homogenisation and differential centrifugation essentially as described previously [31, 32]. The membranes were resuspended in TES [20 mmol/l TRIS-HCl, 1 mmol/l EDTA, 0.25 mol/l Sucrose). Aliquots (30 μg of protein) were mixed with sample buffer, loaded on SDS-PAGE gels, electrophoretically transferrred onto nitrocellulose sheets and immunoblotting was done using the different anti-PKB/Akt and anti-GLUT-4 antibodies.

Statistical analysis. Statistical significance was calculated using student's *t* test, a *p* value of less than 0.05 was considered as statistically significant.

Results

Gene and protein expression of PKB/Akt. Expression of the *PKB/Akt* gene was measured on RNA extracted from healthy and Type II diabetic cells. The PCR fragment used in the northern blots recognized a major 3.3 kb transcript, also previously reported for *PKB/Akt* [33], and a minor 2.8 kb transcript. As the PCR fragment was based on common sites for all known isoforms, the smaller transcript could represent the low expression of the *PKB*α/Akt 1 gene. The 3.3 and the 2.8 kb transcripts were, however, both equally expressed in healthy and diabetic cells (Fig. 1).

In agreement with our previous report, PKB/Akt protein expression was similar in whole-cell lysates (not shown and [23]) and cytosol from the two patient groups (Fig. 2). Thus, the impaired insulin-stimulated activation and phosphorylation in diabetic cells (see below) cannot be accounted for by differences in protein expression.

PKB/Akt protein in subcellular fractions [cytosol, plasma membranes (PM) and low density microsomes (LDM)] and mobility shift in response to insulin. Total cellular PKB/Akt was most abundantly expressed in the cytosol as this fraction represents five to ten times the protein content of the subcellular fractions. Two bands were seen in the cytosol in both patient groups in non-stimulated cells and in cells incubated with insulin and wortmannin (Fig. 2). In the presence

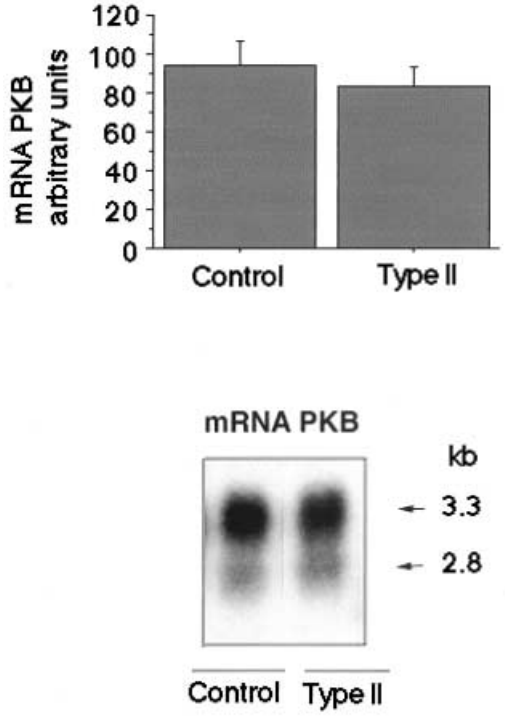


Fig. 1. PKB/Akt mRNA expression in fat cells from non-diabetic ($n = 16$) and Type II diabetic ($n = 10$) subjects

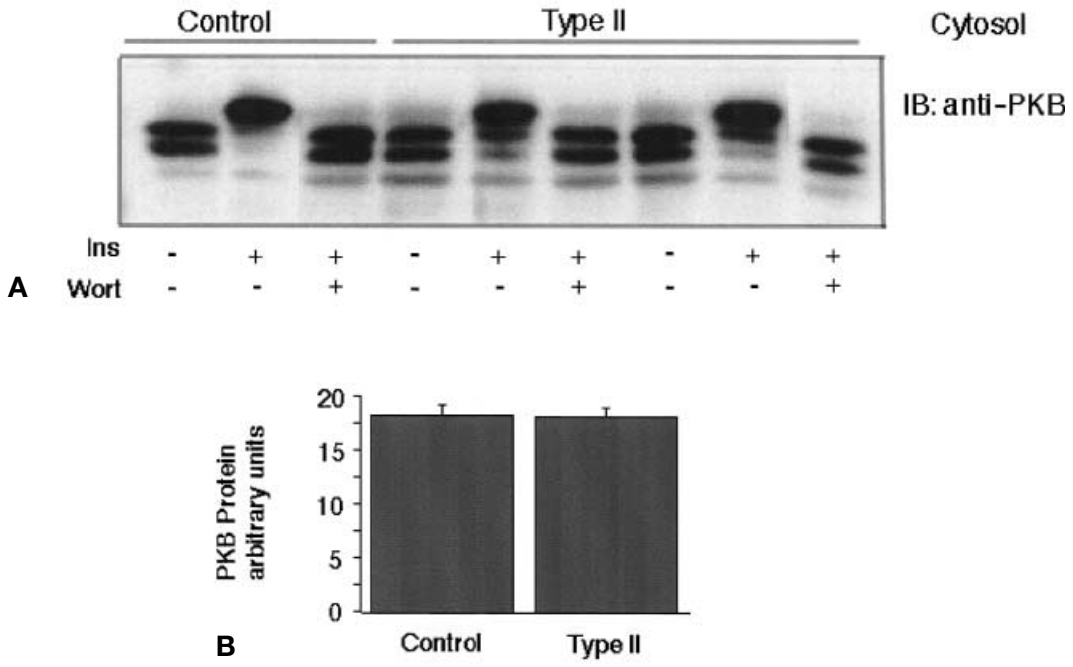


Fig. 2 A, B. PKB/Akt protein expression and mobility shift in response to insulin and wortmannin. The cells were incubated with or without wortmannin (1 μmol/l) for 5 min before 6.9 nmol/l insulin was added for 20 min. After homogenization, the subcellular fractions were prepared by differential ultracentrifugation. Protein (30 μg) from the cytostol fraction was run on 10% SDS-PAGE, transferred and immunoblotted with anti-PKB antibody (**A**). The protein concentrations from basal cells (i.e. without insulin and wortmannin) were scanned with a Phospho Imager and expressed in arbitrary units (**B**). $n = 3$ for both groups. Ins = insulin, Wort = wortmannin

of insulin, a pronounced mobility shift was, however, seen converting the two lower bands to one retarded band. This shift was less pronounced in the cytosol from the diabetic cells suggesting a lower degree of phosphorylation (Fig. 2). Wortmannin inhibited the shift in mobility in response to insulin in both groups (Fig. 2) showing the importance of PI3-kinase activation.

Figure 3A shows PKB/Akt protein in PM and LDM in response to insulin in both patient groups and Figure 3B shows the scanned data. Similar to the cytosolic fractions, at least two major bands were seen in LDM in both groups in non-stimulated cells. Insulin further retarded the upper band suggesting enhanced phosphorylation. The PKB/Akt protein concentration in LDM of non-stimulated cells was slightly (~ 20%) lower in the diabetic cells (Fig. 3B) but this difference was not statistically significant. Surprisingly, insulin produced a decrease in LDM PKB/Akt concentrations in both groups (Fig. 3B) although this difference did not reach statistical significance.

In contrast to LDM, only one major PKB/Akt band was seen in PM in non-stimulated cells (Fig. 3A). Insulin increased, however, the PKB/Akt concentration in PM from non-diabetic cells by about 100% (Fig. 3B) and also produced an additional retarded band suggesting that it was phosphorylated. The PKB/Akt translocation to PM was, as expected, inhibited by wortmannin (data not shown).

The diabetic cells also had only one major PKB/Akt band in PM in the non-stimulated state (Fig. 3A). This band was, however, slightly retarded compared with the control cells suggesting an increased phosphorylation in the basal state but this did not involve the sites phosphorylated by insulin because they were not recognized by the specific phosphopeptide antibodies used (Figs 4A, 5A). Furthermore, the protein increase in PM in response to insulin was smaller (~ 30%) and not significant (Fig. 3B) and the mobility shift was also less prominent (Fig. 3A) in the diabetic cells.

Taken together, these data show a twofold increase in PKB/Akt protein in the PM in response to insulin in control cells, suggestive of translocation from LDM or cytosol or both, but this effect was considerably reduced in diabetic cells. Furthermore, two major bands were seen in cytosol and LDM in non-stimulated cells but only one major band in PM. Insulin further increased the PKB/Akt concentrations in PM, but not LDM, and also produced an additional, and probably phosphorylated, band in both PM and LDM.

The concentrations of GLUT-4 were also measured in the same subcellular fractions (Fig. 3A – bottom). A clear translocation of GLUT-4 to PM and reduction in LDM in response to insulin was seen in the control cells. The GLUT-4 concentrations in the same membranes were considerably lower (~ 80%) in the diabetic cells and smaller reciprocal changes

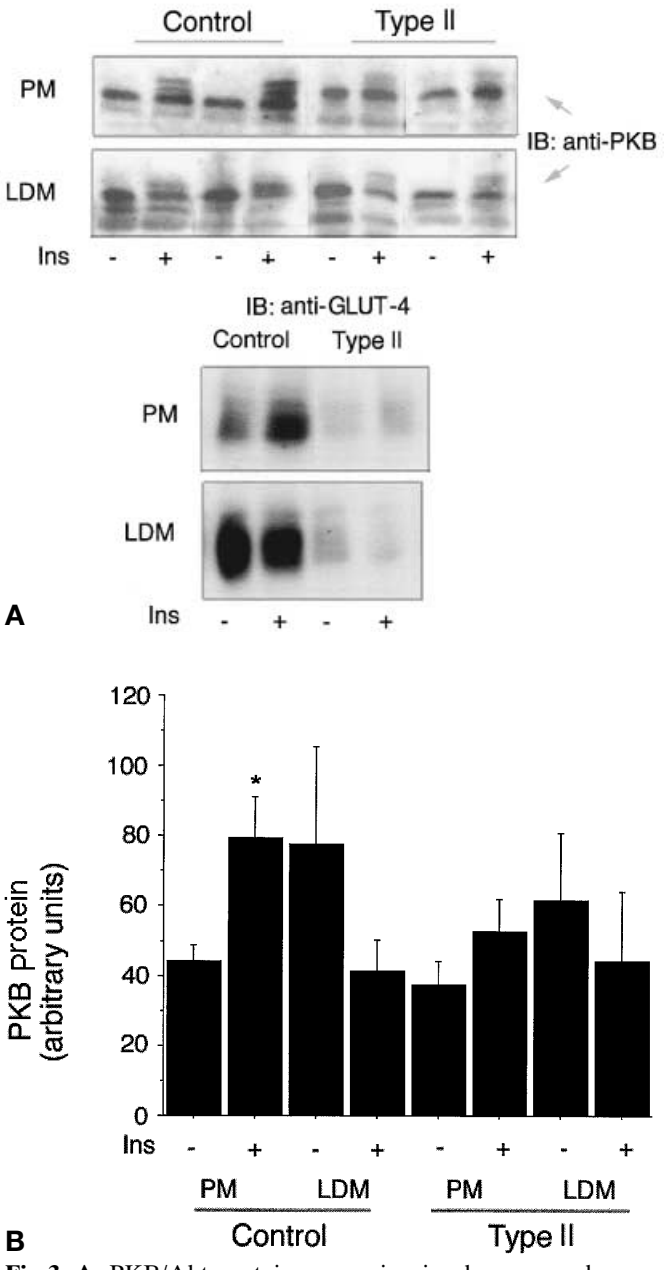


Fig. 3. A PKB/Akt protein expression in plasma membranes (PM), low density microsomes (LDM) and mobility shift in response to insulin (top). GLUT-4 protein expression and translocation from LDM to PM in response to insulin. The cells were incubated and fractionated as in Figure 2 (bottom). **B** Results of scanning the protein concentrations in **A** with a Phospho Imager (arbitrary units). * $p < 0.03$ for the effect of insulin vs basal. Ins = insulin

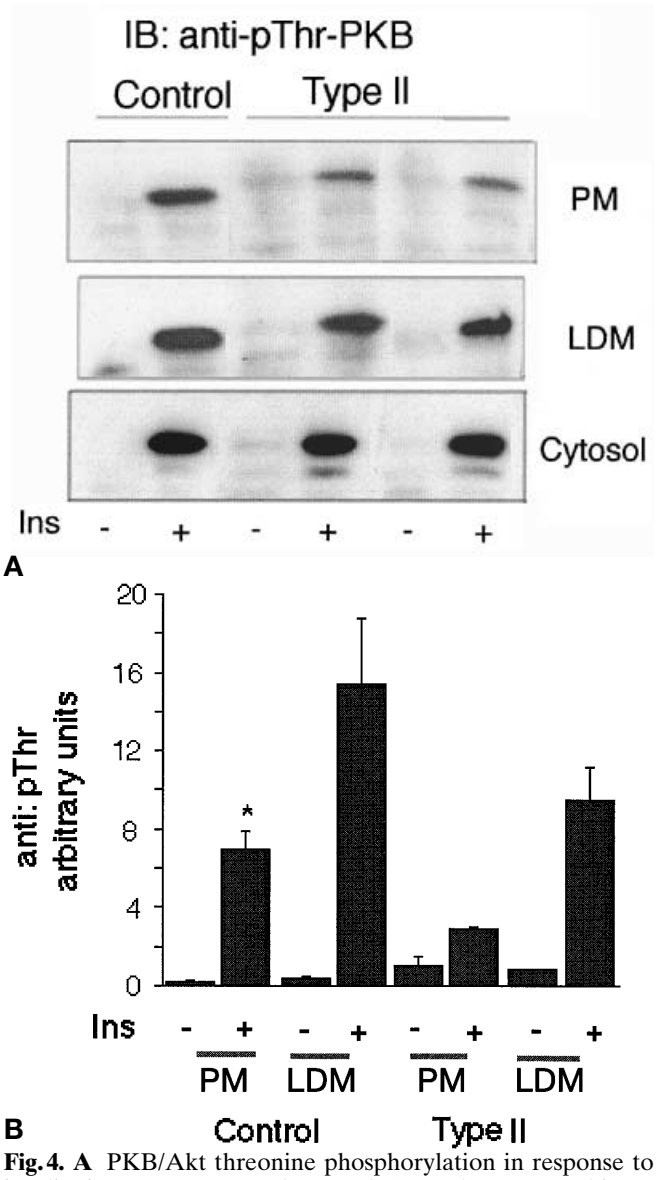
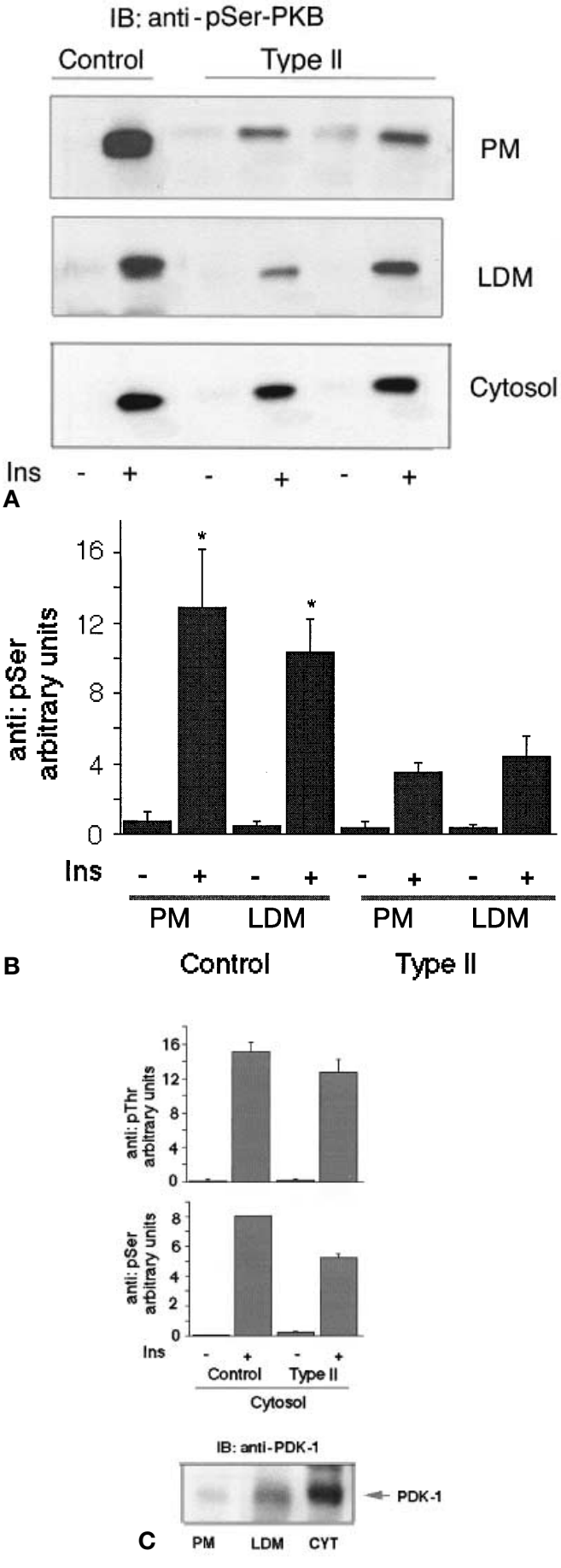


Fig. 4. A PKB/Akt threonine phosphorylation in response to insulin in PM, LDM and cytosol from the same subjects. **B** The relative intensity of the bands was quantified with a Phospho Imager. *$p < 0.01$ for threonine phosphorylation in PM from control vs diabetic cells. Ins = insulin

were also seen in the subcellular compartments in these cells in response to insulin.

Serine and threonine phosphorylation in the subcellular fractions in response to insulin. Insulin increased phosphorylation of threonine and serine in all three subcellular fractions in a wortmannin-sensitive manner (Figs. 4, 5 and data not shown). A single serine-phosphorylated band was seen in all fractions in both groups (Fig. 5A). This was also true for threonine-phosphorylated PKB/Akt but an additional smaller phosphorylated band was seen in the diabetic cells in LDM and cytosol (Fig. 4A), probably reflecting the incomplete mobility shift in response to insulin in these cells (Figs. 2, 3).

There were, however, considerable differences in the relative phosphorylation of serine and threonine between the subcellular fractions (Figs. 4, 5) and also between the groups. The comparisons between the different subcellular fractions and the patient groups were done on immunoblots run together, loaded with the same amount of protein and using the same antibodies. In both groups, relative threo-



nine phosphorylation was most pronounced in the cytosol and LDM (~twofold greater than in PM) (Fig. 4). This finding was not related to differences in PKB/Akt protein content because, in the same blots, more protein was present in PM than LDM in the presence of insulin (Fig. 3B). Phosphorylation of PKB/Akt serine in the subcellular fractions from the same cells showed an opposite pattern; the most pronounced phosphorylation was found in PM followed by LDM and cytosol (Fig. 5). Considering the differences in insulin-stimulated protein content in LDM and PM (Fig. 3B), the relative threonine phosphorylation was four to fivefold greater in LDM than in PM.

Taken together, these data show that relative threonine phosphorylation in response to insulin is predominant in LDM and cytosol whereas serine phosphorylation is predominant in PM. The difference in threonine phosphorylation could be accounted for by the presence of PDK1, the key kinase for threonine phosphorylation of PKB/Akt in response to insulin, in LDM. Most PDK1 was recovered in the cytosol, (Fig. 5C, bottom) in agreement with previous findings [34]. We also found a clear expression in LDM but much less in PM. We found, however, that PDK1 was also present in LDM from non-stimulated cells but no evidence of an insulin-induced translocation of PDK1 to LDM (data not shown).

Although serine and threonine phosphorylations were reduced in diabetic compared with non-diabetic cells, the most pronounced difference was seen in insulin-stimulated serine phosphorylation (~ 70 % reduction in PM). This can only partly be accounted for by the reduced PKB/Akt translocation to PM. Even considering the differences in protein translocation (Fig. 3B), PKB/Akt serine phosphorylation was still reduced about 50 % in both PM and LDM and about 40 % in cytosol compared with that in non-diabetic cells. The differences in threonine phosphorylation were much smaller; 10–20 % in cytosol and 20–30 % in PM and LDM.

Fig. 5. A PKB/Akt serine phosphorylation in response to insulin in PM, LDM and cytosol from the same subjects (also the same as in Fig. 4). **B** The relative intensity of the bands was quantified with a Phospho Imager. $*p < 0.05$ for serine phosphorylation in PM and LDM, respectively, comparing control and diabetic cells. **C** PKB/Akt serine and threonine phosphorylation of the cytosol from control and diabetic cells (top). PDK1 expression in PM, LDM and cytosol from the same subjects. The cells were incubated and fractionated as in Fig. 2 (bottom). CYT = cytosol, Ins = insulin

Discussion

The salient findings of this study are, firstly, differential insulin-stimulated phosphorylation patterns of PKB/Akt in LDM and cytosol compared with PM where threonine phosphorylation was predominant in the former and serine phosphorylation in the latter. Secondly, PDK1, the key kinase for threonine phosphorylation of PKB/Akt [21], was recovered in LDM and cytosol in basal and insulin-stimulated cells. Thirdly, evidence was obtained of an insulin-stimulated translocation (and enhanced serine phosphorylation) of PKB/Akt to the PM. Fourthly, there was a reduced insulin-stimulated translocation of PKB/Akt to PM in diabetic cells associated with an about 70% reduction in serine phosphorylation. The reduced insulin-stimulated PKB/Akt translocation to PM cannot, however, alone account for the pronounced reduction in serine phosphorylation. Even when the differences in protein content are considered the diabetic cells still have an about 50% reduction in serine phosphorylation.

We have previously found that PKB/Akt activity and protein serine phosphorylation in whole-cell lysates in response to insulin are reduced about 50% in diabetic cells [23]. Furthermore, similar to recent reports in rat fat cells and differentiated 3T3-L1 cells [19, 21], PKBβ/Akt 2 is highly expressed but PKBα/Akt 1 is almost absent in human fat cells [23]. We have preliminary evidence that PKBγ/Akt 3 is also expressed in human fat cells but its abundance and role are under current investigation.

Although PKB/Akt was expressed as a single band in PM, similar to recent results in differentiated 3T3-L1 adipocytes [19], at least two bands were found in cytosol and LDM; the subcellular fractions where insulin-stimulated threonine phosphorylation was predominant. The reason for this discrepancy is not clear but could relate to the constitutive phosphorylation of PKBβ/Akt 2. A recent study suggests that constitutive phosphorylation of Thr 450 and, possibly, Ser 124 [35] primes PKBα/Akt 1 for activation by growth factors when expressed in NIH 3T3 cells [35]. Thus, constitutive phosphorylation of one or both of these sites (Thr 451 and Ser 125 in PKBβ/Akt 2) could account for the different mobility patterns in these subcellular fractions. If constitutive phosphorylation is important for subsequent activation by insulin and growth factors, our data suggest that this is particularly important for PDK1 and the phosphorylation of Thr 309 of PKBβ/Akt 2. The single serine-phosphorylated and threonine-phosphorylated PKB/Akt band seen in PM also migrated as the most retarded (shifted) band suggesting that only the basally phosphorylated band became phosphorylated on the activation sites (Ser 474 and Thr 309). Similar findings were recently reported in differentiated 3T3-L1 cells [19]. Note, a PKBα/Akt 1 mutant of Thr 308 fails to become active even following forced membrane translocation [36].

The single PKB/Akt band in PM was further retarded in non-stimulated diabetic cells suggesting an increased constitutive phosphorylation. The specific phosphopeptide antibodies to the Thr 309 and Ser 474 sites did not provide any evidence that these sites were phosphorylated in the diabetic cells in the absence of insulin. Furthermore, basal PKB/Akt activity is not increased in diabetic cells [23] providing additional evidence that the activating sites were not phosphorylated in the absence of insulin.

The current concept of PKB/Akt activation is that of a three-step model [11, 21, 36, 37]: (1) a PH-domain-dependent step where constitutive phosphorylation of thr 450/451 and possibly ser 124/125 (PKBα/Akt 1 and PKBβ/Akt 2, respectively) makes it responsive to subsequent activation, (2) a hormone-stimulated and PI3-kinase dependent membrane-translocation step and (3) a hormone-stimulated and PI3-kinase-dependent phosphorylation of thr 308/309 and ser 473/474.

Out data from insulin-responsive human fat cells with a large intracellular pool of vesicles containing GLUT-4 show, however, that threonine phosphorylation in LDM was increased about fourfold relative to PM. This suggests that phosphorylation by PDK1 takes place in the LDM that enclose the GLUT-4 vesicles. We also identified PDK1 in LDM, albeit at a much lower concentration than in the cytosol. Whether activation of PKB/Akt also takes place in the cytosol, which seems less likely, or if the activated protein (re)cycles from LDM to PM and cytosol is not unclear.

There was no evidence of a further increase of threonine phosphorylation of PKB/Akt after the translocation to PM suggesting that this mainly occurs in LDM and, possibly, the cytosol. In contrast, serine phosphorylation was increased in PM relative to LDM and cytosol after its translocation. The PKB/Akt associated with LDM showed a similar low degree of serine phosphorylation relative to threonine as the cytosol supporting that PKB/Akt is also recycled from the cytosol to LDM.

The threonine phosphorylation was also increased about threefold in LDM and cytosol, relative to PM, in the diabetic cells. The most striking differences between control and diabetic cells were the impaired translocation and the about 70% reduction in total serine phosphorylation. These findings are consistent with the concept that PKB/Akt becomes serine phosphorylated following its translocation to the PM [21]. Because insulin-stimulated threonine phosphorylation was much less reduced in the diabetic cells (~ 20%), these data provide further support for a differential phosphorylation in LDM compared with PM.

One possible reason for the impaired translocation and serine phosphorylation of PKB/Akt in diabetic cells is the pronounced reduction (~ 70 %) in IRS-1 protein expression and PI3-kinase activation by insulin [38]. Because the maximum insulin-stimulated PI3-kinase activity is reduced by about 50–70 % in Type II diabetic cells [38], a major reduction in the activation of PDK2 and serine phosphorylation is expected in these cells. This concept seems, however, to be incongruent with the finding that the threonine phosphorylation and consequently activation of PDK1 (also dependent on PI3-kinase), is much less reduced. One possible explanation is that PDK1 is present in LDM and that sufficient PIP_3 is locally generated for its activation by insulin. We also recovered PDK1 in LDM but found no evidence of an insulin-stimulated translocation to LDM; PDK1 was present in LDM in both stimulated and non-stimulated cells. Previous studies have also shown that PDK1 has a high affinity for PIP_3 and could even be constitutively active when bound to membrane [34]. Thus, it is likely that sufficient phosphatidylinositol [3, 4, 5] and [3, 4] phosphates are generated by PI3-kinase in LDM in both diabetic and control cells to allow activation of PDK1 and thus threonine phosphorylation of PKB/Akt. It is now well established that insulin stimulates the translocation of both IRS-1 and IRS-2 from the cytosol to LDM [39, 40] where the associated PI3-kinase is activated. Recent experiments with insulin in rat adipocytes have shown that about 70 % of PI3-kinase activity associated with IRS 1 and 2 occurs in LDM and only about 10 % in PM [40]. Because diabetic cells have a normal IRS-2 expression and associated PI3-kinase activity [38] and PDK1 has a high affinity for PIP_3 it is not unreasonable to expect a normal or close to normal threonine phosphorylation of PKB/Akt. The attenuated PI3-kinase activity might, however, not generate sufficient PIP_3 to bind to the PH-domain of PKB/Akt and allow a normal translocation to PM. Our data provide direct support for the importance of differential PI3-kinase activation in different subcellular compartments.

Although our findings are consistent with such a differential activation of PI3-kinase in different subcellular compartments, we cannot exclude other possibilities for the decreased serine phosphorylation such as an increased expression of a truncated form of PKB/Akt, which does not become serine-phosphorylated, in diabetic cells similar to that reported for PKBγ/Akt 3 in rodent cells [21]. An alternative possibility is that PDK2 expression or insulin-stimulated activity or both is considerably reduced in diabetic cells.

Studies in insulin-stimulated PKB/Akt activity in skeletal muscle from Type II diabetic patients have produced differing results. One [25] found reduced PKB/Akt activity in human skeletal muscle after incubation with a high insulin concentration, consistent with our findings. Another [24], measuring PKB/Akt activity after a euglycaemic insulin infusion, found similar levels of activation in spite of a concomitant reduction in PI3-kinase activity of about 50 % in Type II diabetic muscle. It is not clear whether this dicotomy represents a difference between fat cells and muscle in insulin concentration and PIP_3 generation required for PKB/Akt translocation and full activation or to other differences in experimental design. Our data suggest, however, similar to PI3-kinase activation, PKB/Akt phosphorylation and activation varies in specific subcellular compartments. The differential phosphorylation pattern could also have functional consequences such as regulating the binding or targetting or both of specific substrates to PKB/Akt.

Our data show that the relative serine and threonine phosphorylation of PKBβ/Akt 2 varies in the different subcellular fractions; threonine is predominant in LDM and cytosol whereas serine is predominant in PM. Furthermore, in Type II diabetic cells, PKB/Akt translocation to PM and subsequent serine phosphorylation is considerably reduced (~ 70 %) and the threonine phosphorylation is much less impaired (~ 20 %). As serine and threonine phosphorylation are required for full activation of PKB/Akt, these findings probably explain the reduced PKB/Akt activation by insulin in fat cells from Type II diabetic subjects [23].

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