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Rapid communication

Impaired glucose transport and protein kinase B activation by insulin, but not okadaic acid, in adipocytes from subjects with Type II diabetes mellitus

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Abstract

Aims/hypothesis. To study the effects of insulin and okadaic acid, a serine/threonine phosphatase inhibitor which does not increase PI3-kinase activity, on the rate of glucose transport and protein kinase B activation in adipocytes from healthy subjects and subjects with Type II (non-insulin-dependent) diabetes mellitus.
Methods. Adipocytes were incubated with or without insulin or okadaic acid or both and glucose transport, protein kinase B activity, phosphorylation and protein expression measured.
Results. Insulin and okadaic acid alone increased glucose uptake to a similar degree in adipocytes from healthy subjects and, when combined, exerted a partial additive effect. The effect of insulin was reduced by about 60 % in adipocytes from Type II diabetic patients, whereas the effect of okadaic acid was essentially unchanged and no further increase was seen when okadaic acid and insulin were combined. Okadaic acid increased protein kinase B activity to a greater extent (two to threefold) than insulin but only slightly increased the serine phosphorylation of protein kinase B. Adipocytes from Type II diabetic subjects exhibited both an impaired sensitivity as well as a reduced total serine phosphorylation and activation of protein kinase B in response to insulin but protein kinase B activity in response to okadaic acid was intact.
Conclusion/interpretation. These results show that the ability of insulin to increase glucose transport and activate protein kinase B is reduced in fat cells from Type II diabetic subjects. Protein kinase B can, however, be activated by agents like okadaic acid which bypass the upstream defects in the insulin signalling pathway in Type II diabetic cells and, thus, increase glucose uptake. [Diabetologia (1999) 42: 819–825]

Keywords PKB, insulin, okadaic acid, Type II diabetes, glucose transport.

Type II (non-insulin dependent) diabetes mellitus is characterized by insulin resistance in the major target tissues coupled with insufficient insulin secretion [1] producing an impaired uptake and metabolism of glucose. The mechanisms for insulin resistance in Type II diabetes are not well understood, although the potential causes could be defects in the early and intermediate steps of the insulin signalling pathway. In animal models of Type II diabetes, there is considerable evidence for alterations in the early steps of insulin action. *Ob/ob* mice and other insulin-resistant models of Type II diabetes have decreased insulin binding, decreased insulin receptor tyrosine kinase activity, decreased IRS-1 and IRS-2 protein content and tyrosine phosphorylation as well as decreased PI3-kinase activity leading to an impaired glucose transporter 4 (GLUT-4) translocation and glucose transport [2–5]. In addition,

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Abbreviations: PKB, Protein kinase B; GLUT-4, glucose transporter 4; PDK, phosphatidylinositol-dependent kinase.

GLUT-4 protein content is reduced in some tissues including the adipose tissue. Recently, we have shown that adipocytes from Type II diabetic subjects have an impaired insulin-stimulated glucose transport as well as a large reduction in IRS-1 protein and tyrosine phosphorylation leading to a reduction in PI-3 kinase activity [6].

We have also shown that the serine/threonine phosphatase inhibitor, okadaic acid, that inhibits the activity of protein phosphatases 1 and 2A [7], exerts a full insulin-like effect in terms of increasing glucose transport activity through the translocation of GLUT-4 to the plasma membrane in human adipocytes [8]. This effect is independent of PI3-kinase activation, suggesting that alternate pathways exist to stimulate glucose uptake. Results from studies in skeletal muscle support this concept [9].

Recently, protein kinase B (PKB) (also known as Rac protein kinase or c-Akt) was recognized as a downstream target of PI3-kinase [10–12]. Protein kinase B was found to mediate some cellular responses induced by insulin and growth factors such as the inhibition of glycogen synthase kinase 3 [13], inhibition of apoptosis [14] and stimulation of glucose transport [15–17]. Furthermore, it has recently been shown that the PKBβ isoform, which is the predominant isoform in adipocytes, is closely associated with the GLUT4-containing intracellular vesicles in rat fat cells [18]. Although the activation of PKB by growth factors and insulin is mediated through the activation of PI3-kinase [12], activation of PKB by protein phosphatase inhibitors, such as okadaic acid, is insensitive to wortmannin and independent of PI3-kinase [19].

In this study, we investigated the effects of insulin and okadaic acid on glucose transport, PKB phosphorylation and activity in adipocytes from healthy and Type II diabetic subjects. In addition, we examined whether insulin-stimulated (PI3-kinase dependent) PKB activity is changed in Type II diabetic adipocytes and, if so, whether it could be overcome by okadaic acid and the PI3-kinase – independent pathway.

Materials and methods

Materials. Human insulin was from Novo Nordisk (Copenhagen, Denmark) and okadaic acid from LC (Woburn, Mass., USA). Bovine serum albumin (BSA) (fractionV), collagenase, wortmannin and other fine chemicals were from Sigma (St. Louis, Mo., USA). Radiochemicals were from Amersham (Buckinghamshire, UK). Anti PKB polyclonal antibodies recognizing both PKBα and PKBβ or specific for PKBβ were purchased from Upstate Biotechnology (Lake Placid, N.Y., USA). Antibodies recognizing the serine phosphorylated region of both PKBα and PKBβ as well as a control antibody recognizing the same but non-phosphorylated region were purchased from New England Biolabs (Beverly, Mass., USA). Protein A and A/G-Sepharose were from Santa Cruz (Santa Cruz, Calif., USA).

Subjects and source of adipose tissue. Specimens of human subcutaneous adipose tissue were obtained from the abdominal region of non-diabetic subjects ($n = 9$) and Type II diabetic subjects ($n = 14$). Both groups were of similar age and degree of obesity (healthy subjects; age = 50 ± 5 years, BMI = 29 ± 3 kg/m^2 and Type II diabetic subjects; age = 54 ± 4 years, BMI = 32 ± 2 kg/m^2). The subjects with Type II diabetes 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 or combined with insulin ($n = 6$). The biopsy specimens were obtained in the fasting state in the morning and before the subjects took their regular medication. The last medication was taken at least 10 h before the biopsy. 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 prepared according to methods previously described [6, 8]. The tissue was cut into small fragments visibly free of connective tissue and blood. About 0.6 g of tissue was incubated at 37 °C in Medium 199 containing 25 mmol/l HEPES, 4 % BSA, 5.5 mmol/l glucose and 0.8 mg/ml collagenase in a shaking water bath. After approximately 50 min, liberated cells were filtered through a nylon mesh with a pore size of 400 μm and washed four times in fresh BSA-containing medium and finally resuspended at 2 % cytocrit. Cell size and number were measured as described previously [20]. Cells were then incubated with the indicated additions in the presence of 0.1 μmol/l N^6 (2-phenylisopropyl)-adenosine (PIA) and 1 U/ml of adenosine deaminase (Boehringer, Mannheim, Germany).

Glucose transport in human adipose cells. Cellular uptake of [^{14}C-U]-glucose was measured during 1-h incubation of cells in glucose-free medium containing PIA and adenosine deaminase as indicated above at lipocrit 3–5 % as described [8]. After pre-incubation in the absence or presence of okadaic acid for 5 min, the concentration of insulin (6.9 nmol/l) that stimulates to the maximum [6] was added to some tubes and after 15 min 0.86 μmol/l [^{14}C-U]-glucose was added. The cells were separated from the incubation medium after 1 h by centrifugation through silicone oil, the radioactivity associated with the cells was measured by scintillation counting and the rate of glucose transport calculated.

Protein kinase B phosphorylation. All cell incubations were carried out at 37 °C in Medium 199 buffered with 25 mmol/l HEPES, 0.1 μmol/l N^6 (2-phenylisopropyl) – adenosine (PIA) and 1 U/ml of adenosine deaminase, with the addition of 4 % BSA. Isolated human adipocytes were distributed into plastic vials (12–15 % cell suspension) in a final incubation volume of 500 μl. Cells were incubated with or without the indicated concentrations of okadaic acid for 5 min and then continued to be incubated in the presence or absence of 6.9 nmol/l insulin for 10 min. 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_3 VO_4, 10 mmol/l NaF, 0.2 mmol/l leupeptine, 1 mmol/l benzamidine and 0.1 mmol/l 4-(2-aminoethyl)-benzenesulfonylfluoride 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 in Laemmli buffer containing 55 mmol/l dithiothreitol for 5 min. The samples were analysed by electrophoresis on 10% SDS-polyacrylamide gels, 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 recommendations of the manufacturer and the proteins were detected by enhanced chemiluminescence using horseradish peroxidase-labelled secondary antibodies (Amersham, Buckinghamshire, UK), The intensity of the bands was quantified using a laser densitometer (Molecular Dynamics, Sunnyvale, Calif., USA).

Protein kinase B kinase activity. Detergent-insoluble material from the cell lysates was sedimented by centrifugation at 12000 × *g* for 10 min at 4 °C and the supernatants were incubated overnight with 5 μg/tube of affinity purified anti-sheep PKB kinase antibody recognizing both PKBα and PKB*β*. Subsequently, the incubation was continued for 1 h with 50 μl of protein A/G-Sepharose beads. The beads were washed twice with Lysis Medium and resuspended in 100 μl of PKB-kinase assay medium containing 25 mmol/l TRIS-HCl, pH 7.5, 0.5 mmol/l EGTA, 10 mmol/l $MgCl_2$, 1 mmol/l DTT and 0.1 mg/ml BSA. Protein kinase B kinase activity was assayed as described [12]. Briefly, 20 μl aliquots of the resuspended immunoprecipitates were incubated for 10 min in assay medium supplemented with 74 KBcq (2 μCi) [γ-^{32}P] ATP, 100 μmol/l ATP, 2 ng/ml of the peptide inhibitor of cAMP-dependent protein kinase and 150 μg/ml myelin basic protein (MBP) or 100 μmol/l glycogen synthase kinase 3 (GSK-3) peptide (Upstate Biotechnology, Lake Placid, N.Y., USA) at 30 °C in at total volume of 40 μl. Similar results were obtained with both substrates and, thus, the data were pooled. The reaction was terminated by spotting an aliquot of the samples on phosphocellulose paper filters (Whatman P81, Clifton, N.J., USA). After five washes in 1% (v/v) orthophosphoric acid incorporation of radiophosphate was determined by liquid scintillation counting.

Results

Effect of insulin and okadaic acid on glucose transport. Incubation of non-diabetic human adipocytes with a maximum concentration of insulin (6.9 nmol/l) [6] resulted in a 150% stimulation over basal transport rate (Fig. 1). A maximum concentration of okadaic acid (1 μmol/l) produced a similar or greater effect and when the cells were incubated with both okadaic acid and insulin a partial additive effect was seen. The effect of insulin in Type II diabetic adipocytes was reduced by about 60% ($p < 0.01$) compared with adipocytes from non-diabetic subjects whereas the effect of okadaic acid was not significantly ($p < 0.1$) reduced (Fig. 1). The effect of insulin and okadaic acid in combination was reduced by 50% ($p < 0.01$) in Type II diabetic adipocytes and was similar to that of okadaic acid alone. Thus, these findings support a preferential reduction in the effect of insulin on glucose transport in Type II diabetic cells but the effect of okadaic acid was essentially unchanged.

Protein kinase B phosphorylation in adipocytes from healthy and Type II diabetic subjects. We examined the effect of insulin or okadaic acid or both on the serine phosphorylation of PKB using anti-serine specific antibodies recognizing the serine phosphorylated sites of both PKBα and PKB*β* (serine 473 and 474, respectively). Insulin, in contrast to okadaic acid alone, considerably increased the serine phosphorylation of PKB in non-diabetic adipocytes (Fig. 2A). The combination of these two agents produced an even greater effect. Densitometric scanning of all immunoblots showed that the serine phosphorylation of PKB in response to insulin alone was considerably impaired in adipocytes from Type II diabetic subjects by about 50% (relative intensity 6.25 ± 0.54 vs 11.59 ± 1.86 in Type II diabetic vs control, means ± SEM, $p < 0.01$) (Fig. 2A). In addition, the dose-response curve for this effect of insulin was noticeably shifted to the right in Type II diabetic (EC_{50} ~ 1.3 nmol/l) compared with control (EC_{50} ~ 0.2 nmol/l) cells documenting an impairment in the upstream signalling and activation (Fig. 2B). Densitometric scanning of these data (Fig. 2B-bottom) also showed the considerable reduction in serine phosphorylation in response to supramaximum insulin concentrations. To determine whether the reduction in serine phosphorylation observed in Type II diabetic adipocytes was due to changes in PKB protein expression, the same membranes were reprobed using a control anti-PKB antibody made against the same sequence. Scanning the immunoblots showed that total PKB protein expression (both PKBα and PKB*β*) was similar in the adipocytes from healthy and Type II diabetic subjects (data not shown).

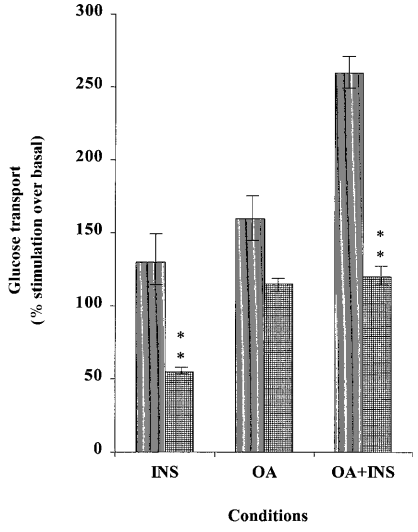

Fig. 1. Glucose transport in response to 6.9 nmol/l insulin or 1 μmol/l okadaic acid (OA) or both in fat cells from healthy or Type II diabetic subjects. Results are means ± SEM of six separate studies done in duplicate. Significances of differences between control and Type II diabetic cells are also shown; $^{**}p < 0.01$. Non-stimulated (basal) glucose transport was similar in both groups. ▨ Healthy, ▦ Type II diabetic

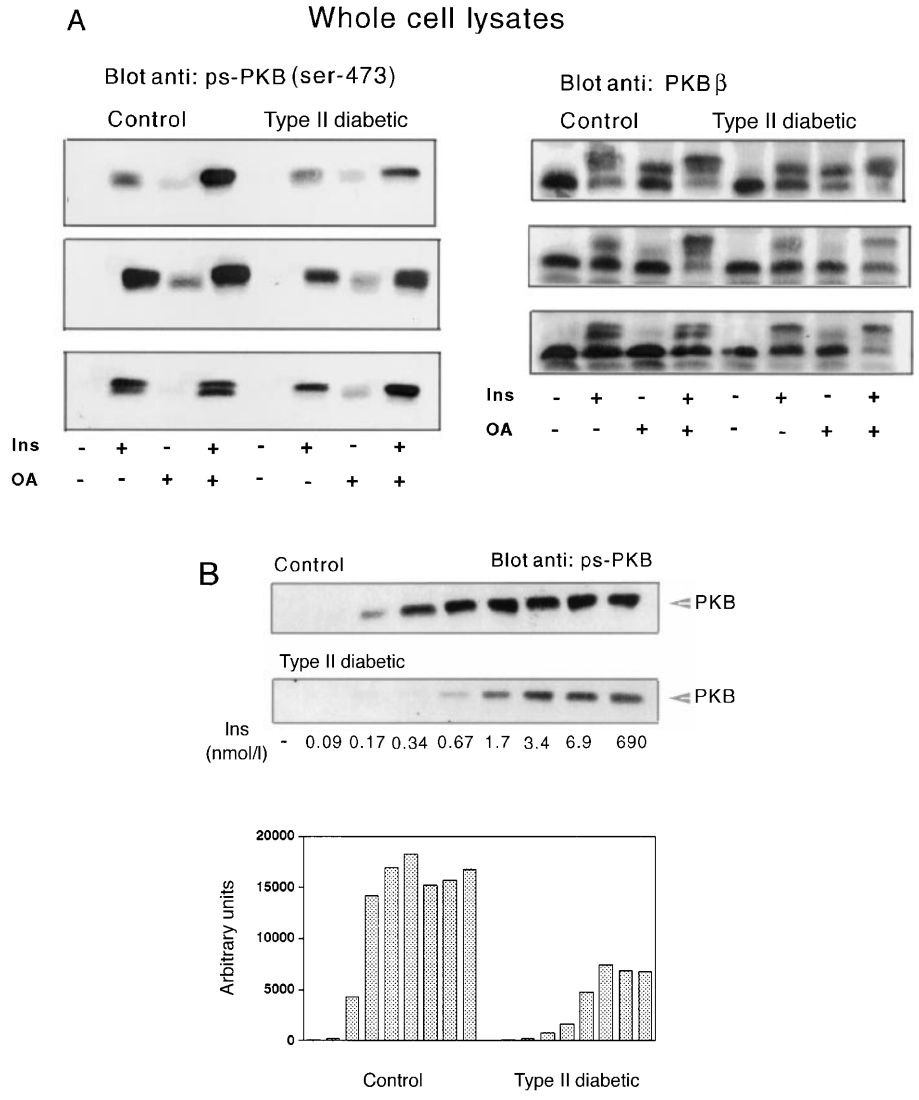

Fig. 2A Serine phosphorylation (both PKBα and PKBβ) (left) and electrophoretic mobility (right) of the PKBβ isoform in lysates of fat cells from healthy and Type II diabetic subjects. The cells were preincubated for 10 min with 6.9 nmol/l insulin (INS) or 1 μmol/l okadaic acid (OA) or both. The proteins (80 μg in each lane) were separated on 10% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with antibodies against the indicated proteins. **B** The dose-response curve for the ability of insulin to increase serine phosphorylation of PKB in healthy and Type II diabetic cells is also shown (top). A supramaximum insulin concentration (690 nmol/l) was used to verify a reduced insulin response. The results of scanning the intensity of the serine phosphorylation are also shown (bottom)

The protein expression was further examined by analysing the specific PKB isoforms in the fat cells. Protein kinase Bβ was the major isoform in both healthy and Type II diabetic cells accounting for around 70% of the PKB protein expression (data not shown). Cellular PKBβ protein expression, relative to total cell protein, was also similar in both groups as verified by densitometric scanning of the immunoblots (relative intensity 10.1 ± 2.0 and 12.4 ± 3.2 in Type II diabetic vs healthy cells, means $\pm$ SEM, $p > 0.1$) but the degree of phosphorylation, measured as intensity of mobility-shifted bands in response to insulin, was reduced around 30%. Okadaic acid alone and in combination with insulin produced a similar mobility shift in both groups suggesting a similar degree of phosphorylation. (Fig. 2A).

Protein kinase B kinase activity in Type II diabetic and non-diabetic cells. Surprisingly, in non-diabetic people, okadaic acid produced a greater increase in total PKB activity (both PKBα and PKBβ) than insulin alone whereas both agents together had a partial additive effect (Fig. 3), similar to that seen for glucose transport. The insulin and the insulin + okadaic acid effects were reduced by about 50% ($p < 0.01$) in adipocytes from Type II diabetic subjects compared with non-diabetic cells. The PKB kinase activity in response to okadaic acid alone was, however, not statis-

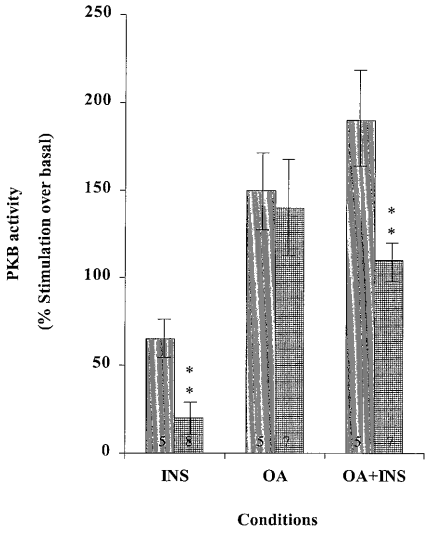

Fig. 3. Total PKB activity (both PKBα and PKBβ) in fat cells from non-diabetic and Type II diabetic cells preincubated for 10 min with 6.9 nmol/l insulin (INS) or 1 μmol/l okadaic acid (OA) or both. Protein kinase B activity was measured as described in the "Methods". Results are expressed as means ± SEM of the indicated number of subjects. $^{**}p < 0.01$. The subjects included in the PKB activity measurements were mostly the same as in Figure 1. ▨ Healthy, ▦ Type II diabetic

tically significantly different between non-diabetic and Type II diabetic adipocytes (Fig. 3).

Discussion

In this study, we provide evidence that insulin-stimulated PKB serine phosphorylation and total activity, but not protein expression, are considerably reduced in adipocytes from Type II diabetic compared with healthy subjects. This is not unexpected as we have shown previously that IRS-1 protein expression, insulin-stimulated tyrosine phosphorylation of IRS-1 and PI3-kinase activation are greatly reduced in Type II diabetic cells [6]. It is well established that PKB is a downstream target of PI3-kinase as dominant-negative mutants of the p85 regulatory subunit of PI3-kinase and inhibitors of PI3-kinase block insulin-stimulated PKB kinase activity [10–13]. Furthermore, overexpression of a constitutively active PI3-kinase activates PKB [21–23]. Another important finding of our study is that the PP1 and 2A phosphatase inhibitor, okadaic acid, bypasses and overcomes the upstream signalling defects in Type II diabetes producing a greater response than that of insulin with respect to both increasing PKB activity and glucose transport. Surprisingly, this occurs with only a minor increase in the serine phosphorylation of PKB. In addition, similar to recent findings with rat fat cells [18], PKBβ is the major isoform in human fat cells, accounting for around 70% of the PKB protein expression and it is normally expressed in adipocytes from Type II diabetic subjects.

Protein kinase B is activated through different mechanisms by insulin and okadaic acid. Protein kinase B stimulation by insulin is inhibited by wortmannin as PI3-kinase activation is required for the activation and translocation of PKB to the plasma membrane [10–12, 24, 25]. The PKB activation is due to the phosphorylation of threonine and serine sites [26, 27]. Okadaic acid-stimulated glucose transport and PKB kinase activity are independent of PI3-kinase activity [8, 19]. Interestingly, the extent of the serine phosphorylation was not directly correlated with PKB activity in response to okadaic acid. Okadaic acid induced a minor serine phosphorylation but a greater PKB activation than insulin alone. Consequently, activation of PKB in response to okadaic acid seems to be less dependent of serine phosphorylation of the kinase and thus probably occurs through threonine phosphorylation similar to what has been reported for vanadate [18] or through other pathways as suggested for isoproterenol [28].

A recent study showed that both insulin and IGF 1 induced PI3-kinase-dependent translocation of PKB to the plasma membrane [24]. The recently identified kinase phosphatidylinositol-dependent kinase (PDK)1 phosphorylates PKBα on Thr308 although another kinase, called PDK2, phosphorylates it on Ser473 producing the full activation of the kinase [29–31]. The kinase PDK2 has not yet been identified definitively but recent data suggest that it could be similar to the integrin-linked kinase [32]. In Type II diabetic adipocytes, both the serine phosphorylation and the activation of PKB in response to insulin are impaired. This can be attributable to an impairment in the activation of PDK2 kinase activity, also dependent on PI3-kinase, as well as to an impaired activation of PDK1. Interestingly, the dose-response curve for the ability of insulin to increase the serine phosphorylation of PKB was similar to the metabolic effect of insulin and was noticeably shifted to the right in Type II diabetic cells. These data are consistent with the upstream signalling defect in Type II diabetic cells that we reported previously [6]. Furthermore, these data support PKB being closely linked to insulin-stimulated glucose transport and lacks the redundancy found for other upstream kinases.

Okadaic acid activates both PKB and glucose transport in human adipocytes to a greater extent than insulin, in particular in Type II diabetic cells. These findings are consistent with the concept that PKB has a critical role in the activation of glucose transport and GLUT4 translocation. Our recent findings with a novel PKB inhibitor support this concept [33] although another study [34], using a dominant negative PKB mutant, did not find insulin-stimulated glucose transport to be abolished.

The present study also supports the concept that glucose transport can be activated through alternate pathways (PI3-kinase dependent/independent) presumably leading to a common end point such as PKB activation. Furthermore, it should be emphasised that a reduced GLUT4 protein content has been shown in Type II diabetic fat cells [6]. Although this probably reflects the reduced "maximum" rate of glucose transport seen in the presence of insulin + okadaic acid, the present data also clearly show that an impaired intracellular signalling mechanism(s) for insulin has an important role for the insulin resistance related to the activation of glucose transport in Type II diabetic fat cells. A recent study in human skeletal muscle also showed an impairment in PKB activation in response to a high insulin concentration [35] whereas in another study no difference was seen after infusion of a physiological insulin concentration [36]. Further studies are required to elucidate whether this inconsistency is due to differences in the patients included, such as degree of glucose control, or to other differences in the experimental conditions.

In conclusion, the present study shows that the effect of insulin, but not okadaic acid, on both glucose transport and PKB activation is severely impaired in adipocytes from Type II diabetic subjects. The data also provide further evidence for the existence of different pathways to stimulate glucose transport, probably through the activation of PKB.

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