

## EXPERIMENTAL STUDY

## High glucose and insulin in combination cause insulin receptor substrate-1 and -2 depletion and protein kinase B desensitisation in primary cultured rat adipocytes: possible implications for insulin resistance in type 2 diabetes

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### Abstract

**Objective:** The purpose of this study was to investigate the cellular effects of long-term exposure to high insulin and glucose levels on glucose transport and insulin signalling proteins.

**Design and methods:** Rat adipocytes were cultured for 24 h in different glucose concentrations with  $10^4$   $\mu$ U/ml of insulin or without insulin. After washing,  $^{125}$ I-insulin binding, basal and acutely insulin-stimulated D-[ $^{14}$ C]glucose uptake, and insulin signalling proteins and glucose transporter 4 (GLUT4) were assessed.

**Results:** High glucose (15 and 25 mmol/l) for 24 h induced a decrease in basal and insulin-stimulated glucose uptake compared with control cells incubated in low glucose (5 or 10 mmol/l). Twenty-four hours of insulin treatment decreased insulin binding capacity by  $\sim$ 40%, and shifted the dose–response curve for insulin's acute effect on glucose uptake 2- to 3-fold to the right. Twenty-four hours of insulin treatment reduced basal and insulin-stimulated glucose uptake only in the presence of high glucose (by  $\sim$ 30–50%). At high glucose, insulin receptor substrate-1 (IRS-1) expression was downregulated by  $\sim$ 20–50%, whereas IRS-2 was strongly upregulated by glucose levels of 10 mmol/l or more (by 100–400%). Insulin treatment amplified the suppression of IRS-1 when combined with high glucose and also IRS-2 expression was almost abolished. Twenty-four hours of treatment with high glucose or insulin, alone or in combination, shifted the dose–response curve for insulin's effect to acutely phosphorylate protein kinase B (PKB) to the right. Fifteen mmol/l glucose increased GLUT4 in cellular membranes (by  $\sim$ 140%) compared with 5 mmol/l but this was prevented by a high insulin concentration.

**Conclusions:** Long-term exposure to high glucose *per se* decreases IRS-1 but increases IRS-2 content in rat adipocytes and it impairs glucose transport capacity. Treatment with high insulin downregulates insulin binding capacity and, when combined with high glucose, it produces a marked depletion of IRS-1 and -2 content together with an impaired sensitivity to insulin stimulation of PKB activity. These mechanisms may potentially contribute to insulin resistance in type 2 diabetes.

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### Introduction

Insulin resistance is an important component of type 2 diabetes, and hyperglycaemia as well as hyperinsulinaemia can contribute to its deterioration (1–3). Insulin levels are increased to compensate for progressing insulin resistance, but on the other hand, hyperinsulinaemia leads to a further impairment in insulin sensitivity, partly via receptor downregulation (4). In addition, hyperglycaemia may cause and promote insulin resistance in type 1 and 2 diabetes, leading to decreased glucose uptake and glucose utilisation (1, 5, 6). The effects of hyperglycaemia may be partly

mediated by metabolites of the hexosamine pathway (7) or activation of different protein kinase C isoforms (8, 9).

Insulin binding to the insulin receptor initiates a cascade of intracellular signalling events, including receptor autophosphorylation and subsequent phosphorylation and/or activation of downstream molecules, including insulin receptor substrates (IRSs), phosphatidylinositol 3-kinase (PI3-K), protein kinase B (PKB) and probably other molecules of importance for activation of the glucose transport system. The final step in this signalling cascade is the translocation of glucose transporter 4 (GLUT4) from intracellular

compartments to the plasma membrane, thereby facilitating the entry of glucose into insulin-sensitive cells like muscle and fat cells. Glucose transport is considered to be the rate-limiting step in glucose metabolism in insulin-sensitive tissues like muscle and fat under most physiological conditions (10, 11). Various cellular defects resulting in insulin-resistant glucose transport have been proposed, including alterations in insulin receptor function, depletion of the GLUT4 transporter pool and alterations in the post-receptor signalling pathway. We have recently shown that insulin can exert an upregulating effect on GLUT4 mRNA level in 3T3-F442A adipocytes at low (5.6 mmol/l) but not high (25 mmol/l) glucose levels (12). These data stress the importance of evaluating possible interaction effects besides the isolated effects of high levels of glucose and insulin.

In the present work we have studied the effects of high glucose and insulin, alone or in combination, on glucose transport capacity and on the cellular content of insulin signalling proteins and GLUT4. We wished to mirror four different stages in the development and progression of type 2 diabetes, namely, in their usual order of appearance: (i) the normal state with low glucose and insulin levels, (ii) the hyperinsulinaemic state, (iii) the hyperinsulinaemic and hyperglycaemic state, and finally (iv) the hyperglycaemic state alone. For this purpose we have used primary cultured rat adipocytes as an established model of target cells of insulin. Most previous work on cultured adipocytes has employed high glucose concentrations, ~25 mmol/l, in the culture medium, but we studied several different glucose levels, with and without a high, saturating concentration of insulin. We assessed insulin-signalling proteins and, in particular, downstream events, i.e. activation of PKB and glucose transport.

## Materials and methods

### Animals

Male Sprague–Dawley rats were obtained from B&K (Sollentuna, Sweden). The Umeå Ethical Committee for Animal Research approved the study protocol.

### Chemicals

DMEM, fetal calf serum (FCS), penicillin/streptomycin and Medium 199 were obtained from Gibco BRL, Life Technologies (Paisley, Strathclyde, UK). Collagenase A and adenosine deaminase (ADA) were from Boehringer Mannheim (Mannheim, Germany). BSA (fraction V) and *N*<sup>6</sup>-(*R*-phenylisopropyl) adenosine (PIA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Human insulin (Actrapid) was from Novo Nordisk A/S (Copenhagen, Denmark). D-[U-<sup>14</sup>C]glucose (specific activity ~200–300 mCi/mmol), human monocomponent [<sup>125</sup>I-tyr<sup>14</sup>]insulin

(specific activity ~2000 Ci/mmol) and ECL-Plus-Blotting reagents were purchased from Amersham Pharmacia Biotech, Freiburg, Germany. Anti-IRS-1, IRS-2, p85 $\alpha$ / $\beta$  (PI3-K), Akt1/2 (PKB) and GLUT4 polyclonal antibodies as well as secondary antibodies were obtained from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA). The anti-phospho-Akt1 (Ser<sup>473</sup>) antibody was from New England Biolabs (Beverly, MA, USA).

### Isolation and primary culture of rat adipocytes

Male Sprague–Dawley rats (150–200 g), fed freely, were killed by decapitation and epididymal fat pads were immediately excised and minced. Isolated fat cells were obtained by shaking finely minced tissue in polypropylene containers at 37°C for 1 h in Medium 199 containing 5.6 mmol/l glucose with 40 mg/ml BSA and 0.6 mg/ml collagenase. Cells were then filtered through a nylon mesh and washed four times with fresh medium. Isolated adipocytes were placed in flasks containing DMEM with 5, 10, 15 or 25 mmol/l (initial concentrations) D-glucose, 10% FCS, penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml). The cells were incubated at 37°C for 24 h under a gas phase of 95% O<sub>2</sub> and 5% CO<sub>2</sub> in the absence or presence of insulin (10<sup>4</sup>  $\mu$ U/ml). Glucose concentrations decreased slightly after 24 h, e.g. to ~4 and ~12 mmol/l for cells cultured at initial glucose concentrations of 5 and 15 mmol/l respectively. Cell size was determined in isolated adipocytes as previously described (13) and it did not differ between the varying incubation conditions (data not shown). Cell viability was verified by trypan blue exclusion tests.

### Glucose uptake assay

After 24 h incubation of isolated adipocytes and washing four times, glucose uptake was assessed as previously described (14, 15). The washing procedure will remove all insulin in the medium and it dissociates >90% of any cell-bound insulin from the pretreatment period (16). In brief, adipocytes (lipocrit 3–5%) were incubated at 37°C in Medium 199 without glucose for 15 min with 4% BSA, ADA (1 U/ml), PIA (1  $\mu$ mol/l) and various insulin concentrations (0–1000  $\mu$ U/ml). After that, D-[U-<sup>14</sup>C]glucose (0.86  $\mu$ mol/l) was added. The cells were separated from the incubation medium after 1 h by centrifugation through silicone oil and the radioactivity associated with the cells was measured by scintillation counting. Under these experimental conditions glucose uptake is mainly determined by the rate of transmembrane glucose transport (17). The cellular clearance of glucose from the medium was calculated according to the following formula and taken as an index of the rate of glucose uptake: cellular clearance of medium glucose = (c.p.m. cells  $\times$  volume)/(c.p.m. medium  $\times$  cell number  $\times$  time).

### **<sup>125</sup>I-insulin binding to adipocytes**

After the culture period the fat cells were washed four times and thus >90% of any bound insulin was removed (16). Thereafter, the cells (lipocrit 5–10%) were incubated with ADA (1 U/ml), PIA (1 µmol/l) and 2 mmol/l KCN for 5 min at 37°C to deplete the cells of ATP and stop receptor internalisation and recycling (16). Subsequently, cell surface binding of <sup>125</sup>I-insulin (0.2 ng/ml) was carried out for 60 min at 16°C. After the incubation period, cells and medium were separated by centrifugation through dinonyl phthalate and <sup>125</sup>I-insulin binding to cells was measured. Non-specific binding, defined as binding in the presence of 10<sup>5</sup> µU/ml unlabelled insulin, was subtracted.

### **Western analysis of total cellular lysates**

After the 24 h incubation period, cells were washed four times with PBS and lysed with 0.2–0.4 ml lysis buffer (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<sub>3</sub>VO<sub>4</sub>, 10 mmol/l NaF, 0.2 mmol/l leupeptin, 1 mmol/l benzamide and 0.1 mmol/l 4-(2-aminoethyl)-benzenesulphonyl fluoride hydrochloride) and rocked for 2 h at 4°C. The detergent-insoluble material was sedimented by centrifugation at 12 000 *g* for 15 min at 4°C and the supernatants collected. Protein determination was performed using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA) and BSA as the standard. Forty micrograms of total cellular protein were applied to each lane and were separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA) and blocked overnight at 4°C with 5% dry milk in 20 mmol/l Tris, 137 mmol/l NaCl, 0.5% Tween-20, pH 7.6. Immunological detection of IRS-1, IRS-2, p85α/β (PI3-K) and Akt1/2 (PKB) was carried out with specific polyclonal antibodies, and immunoreactive bands were visualised using the ECL Western blotting protocol and quantified by densitometry (Molecular Analyst; Bio-Rad Laboratories, Hercules, CA, USA). Films from each set of experiments were scanned at the same time and the background signal from each single film was subtracted.

### **Western analysis of PKB phosphorylation**

After washing four times, adipocytes (lipocrit ~15%) were incubated at 37°C in Medium 199 with 5.6 mmol/l glucose, 4% BSA, ADA (1 U/ml), PIA (1 µmol/l) with and without insulin as indicated. After 10 min incubation, cells and medium were transferred to pre-chilled tubes on ice and adipocytes were immediately separated from the medium by centrifugation through silicone oil. Cell lysis, protein determination and Western blotting procedures were

performed as described above with the pSer<sup>473</sup>-PKBα antibody to assess the amount of phosphorylated PKB (α isoform at Ser<sup>473</sup> and β isoform at Ser<sup>474</sup>). This is considered to reflect the PKB enzyme activation (18).

### **Western analysis of total cellular membrane GLUT4**

Total cellular membranes were prepared as previously described (19). After the 24 h incubation period, cells were washed twice with PBS and homogenised for 30 s with a Polytron PT-MR 3000 (Kinematica AG, Littau, Switzerland) in TES homogenisation buffer (20 mmol/l Tris-HCl, pH 7.4, 1 mmol/l EDTA, 255 mmol/l sucrose and 1 mmol/l polymethylsulphonyl fluoride). The homogenate was then centrifuged at 1000 *g* for 30 min at 4°C and the supernatant collected and centrifuged at 212 000 *g* for 1 h at 4°C. The resulting pelleted total membrane fraction was resuspended in TES and frozen at -70°C. Protein determination, based on the method of Bradford (20), was performed using the Bio-Rad protein assay and bovine gamma globulin as the standard. Two micrograms of total cellular membrane protein per lane were separated by SDS-PAGE and transferred to Immobilon-P membranes and blocked overnight at 4°C with 5% dry milk in 20 mmol/l Tris, 137 mmol/l NaCl, 0.5% Tween-20, pH 7.6. Immunological detection of GLUT4 was carried out with an anti-GLUT4 polyclonal antibody, and immunoreactive bands were visualised and quantified as above.

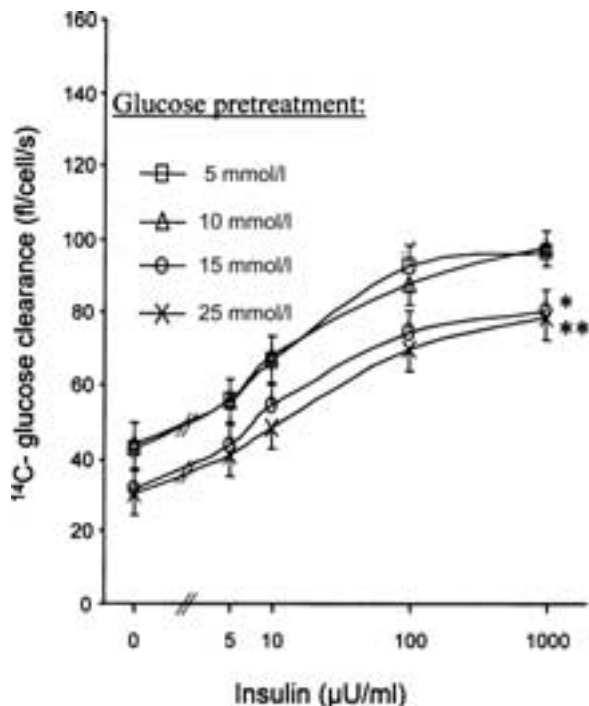
### **Statistics**

Statistical analyses were performed using the SPSS package (SPSS Inc., Chicago, IL, USA). Results are given as means ± S.E.M. and statistical significance was determined using two-way ANOVA for glucose uptake and the Dunnett's test as a post-hoc test. The Kruskal-Wallis non-parametric test with a Mann-Whitney test post-hoc was used when comparing protein concentrations and insulin binding. When appropriate, *P* values were adjusted for multiple comparisons and *P* < 0.05 was considered statistically significant.

## **Results**

### **Glucose uptake capacity**

Adipocytes cultured in medium containing 15 or 25 mmol/l glucose for 24 h exhibited a clear decrease in both basal (by ~30%) and insulin-stimulated (by ~20%) D-[U-<sup>14</sup>C]glucose uptake compared with cells incubated in 5 or 10 mmol/l glucose (Fig. 1). These actions of high glucose were not due to osmolarity effects since corresponding osmolarity changes using 20 mmol/l L-glucose+5 mmol/l D-glucose did not alter glucose uptake capacity (data not shown).



**Figure 1** Effect of long-term glucose treatment on glucose uptake capacity. Adipocytes were incubated with different glucose concentrations (5, 10, 15 and 25 mmol/l) for 24 h. After washing, insulin was added at the indicated concentrations (0–1000 µU/ml) for 15 min, and then glucose clearance was measured during 1 h. The data are expressed as means  $\pm$  S.E.M. of eight separate experiments. \* $P < 0.05$  for cells cultured in 15 mmol/l glucose, \*\* $P < 0.01$  for cells cultured in 25 mmol/l glucose vs 5 mmol/l glucose respectively.

Pretreatment with a high insulin concentration ( $10^4$  µU/ml) for 24 h of cells cultured at 5 mmol/l glucose did not alter subsequent basal or acutely insulin-stimulated glucose uptake capacity (Fig. 2A). In contrast, at a high glucose concentration (15 mmol/l), concomitant insulin pretreatment significantly impaired glucose uptake capacity, both basal and insulin-stimulated, and the additive reduction was approximately 30–50% (Fig. 2B). The  $EC_{50}$  for insulin's action to acutely stimulate glucose uptake was significantly increased in cells cultured at high insulin ( $52 \pm 7$  µU/ml vs  $18 \pm 3$  µU/ml for control,  $P < 0.01$ , inset Fig. 2A) at 5 mmol/l glucose. This was independent of the surrounding glucose concentration, and at 15 mmol/l,  $EC_{50}$  values were  $46 \pm 4$  and  $22 \pm 4$  µU/ml with and without insulin pretreatment respectively ( $P < 0.01$ , inset Fig. 2B).

In some experiments, cells were cultured with high glucose and insulin during the final 2 or 6 h of the 24 h incubation. These time-course experiments revealed that high insulin and glucose in combination had already induced a  $\sim 20\%$  decrease in glucose uptake capacity after 2 h (Fig. 2C). After 6 h of high insulin and glucose a further decrease in glucose

uptake was seen and it was similar to that with a 24 h treatment. Two and 6 h of high glucose in the absence of insulin did not impair glucose uptake capacity (not shown).

### Insulin binding

The glucose concentration of the culture medium did not *per se* affect cell surface  $^{125}$ I-insulin binding and it was similar following 5 and 15 mmol/l glucose ( $0.71 \pm 0.17$  vs  $0.69 \pm 0.15$  pg/ $10^5$  cells respectively,  $n = 4$ ,  $P =$  not significant). Pretreatment with insulin at a high concentration ( $10^4$  µU/ml) for 24 h induced a  $\sim 45\%$  decrease in  $^{125}$ I-insulin binding compared with the control situation without insulin ( $0.39 \pm 0.11$  and  $0.39 \pm 0.10$  pg/ $10^5$  cells for cells cultured in 5 and 15 mmol/l glucose respectively,  $n = 4$ ,  $P < 0.05$  vs no insulin), and, thus, the effect of insulin pretreatment was independent of the surrounding glucose concentration.

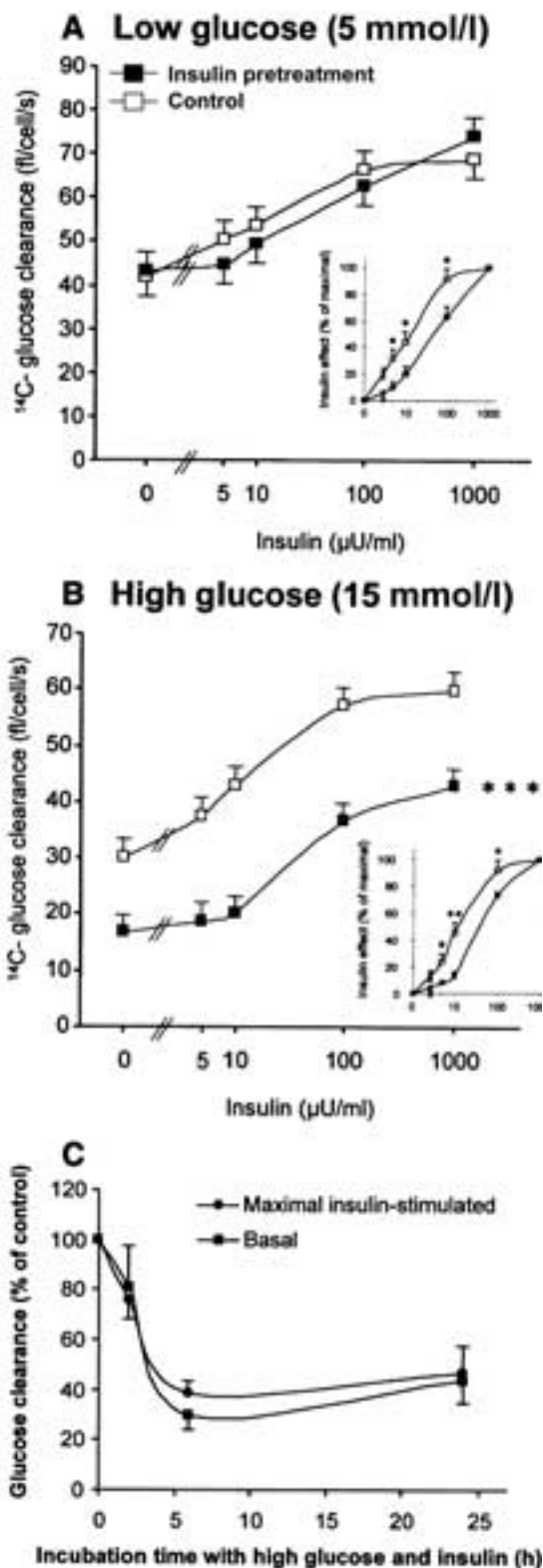
### Cellular content of insulin signalling proteins

High glucose levels in the surrounding medium exerted opposite effects on the expression of IRS-1 and -2. High glucose downregulated IRS-1 expression by up to  $\sim 50\%$  (Fig. 3A). In contrast, IRS-2 was markedly upregulated, up to 5-fold, in fat cells surrounded by glucose levels of 10 mmol/l or more as compared with 5 mmol/l glucose and this occurred in a concentration-dependent manner (Fig. 3B). High glucose in the medium exerted no significant changes in PI3-K and PKB levels (Fig. 3C and D).

High levels of insulin ( $10^4$  µU/ml) in the incubation medium markedly reduced IRS-2 expression by  $\sim 60$ – $95\%$ , and the most pronounced effect was seen when cells were concomitantly exposed to the highest glucose concentration, i.e. 25 mmol/l (Fig. 3B). High insulin ( $10^4$  µU/ml) produced no consistent effects on IRS-1, PI3-K and PKB content when the glucose level was physiological or near-physiological (5 or 10 mmol/l, Fig. 3A, C and D), but the concomitant presence of high insulin and high glucose in the medium exerted a further decrease in IRS-1 compared with high glucose alone. Thus, IRS-1 content was decreased by  $\sim 70\%$  in cells cultured in 15 mmol/l glucose and high insulin compared with 15 mmol/l glucose alone, and by  $\sim 80\%$  compared with the control situation, i.e. 5 mmol/l and no insulin (Fig. 3A). Also for IRS-2, there was an interaction effect for high insulin and high glucose in combination, and IRS-2 abundance was reduced by  $\sim 60$ – $70\%$  compared with high insulin alone (Fig. 3B). In fact, glucose exerted a concentration-dependent suppressing effect on the IRS-2 amount in the presence of high insulin, i.e. opposite to the glucose effect alone. The combination of insulin and high glucose in the incubation medium did not exert any significant alterations in the levels of PI3-K

(assessed by immunoblotting of the p85 $\alpha$ / $\beta$  subunit) or of PKB, although there was a tendency for increased PKB content following high insulin treatment when

the glucose level was high (Fig. 3C). Overall, there were in fact no consistent changes in the amount of PI3-K or PKB following any of the various incubation conditions (Fig. 3C and D).



**PKB phosphorylation**

Insulin pretreatment for 24 h caused an elevated basal phosphorylation of PKB, probably due to a sustained insulin-stimulated phosphorylation remaining after washing. However, the amount of Ser<sup>473</sup>-phosphorylated PKB following acute stimulation by insulin (1000  $\mu$ U/ml) for 10 min was not affected by prior pretreatment with high glucose or insulin, alone or in combination, for 24 h (Fig. 4A). This was true also when calculating the degree of phosphorylation, i.e. the amount of phosphorylated PKB per amount of total PKB protein (data not shown). In additional experiments we examined the dose-response relationship for insulin's ability to acutely phosphorylate PKB. Concomitant incubation with high insulin and glucose induced a significant rightward shift in the dose-response curve ( $EC_{50}$  92 $\pm$ 23 and 155 $\pm$ 42  $\mu$ U/ml for control cells and cells cultured in 15 mmol/l glucose plus insulin respectively,  $n = 4$ ,  $P < 0.05$ , Fig. 4B). In contrast, high glucose (15 mmol/l) or high insulin alone also tended to shift the dose-response curve for insulin-stimulated PKB phosphorylation to the right, but to a lesser extent ( $EC_{50}$  109 $\pm$ 31 and 122 $\pm$ 34  $\mu$ U/ml respectively,  $n = 4$ ,  $P = NS$ ).

**Cellular GLUT4 content**

Insulin in the medium did not affect the amount of cellular membrane-associated GLUT4 when the glucose level was low (Fig. 5). However, 15 mmol/l glucose in the medium for 24 h by itself induced an increase in total membrane GLUT4 by ~140% as compared with 5 mmol/l glucose. Concomitant incubation with high insulin completely prevented this glucose-induced upregulation and the GLUT4 content remained at similar levels to those in the control situation (Fig. 5).

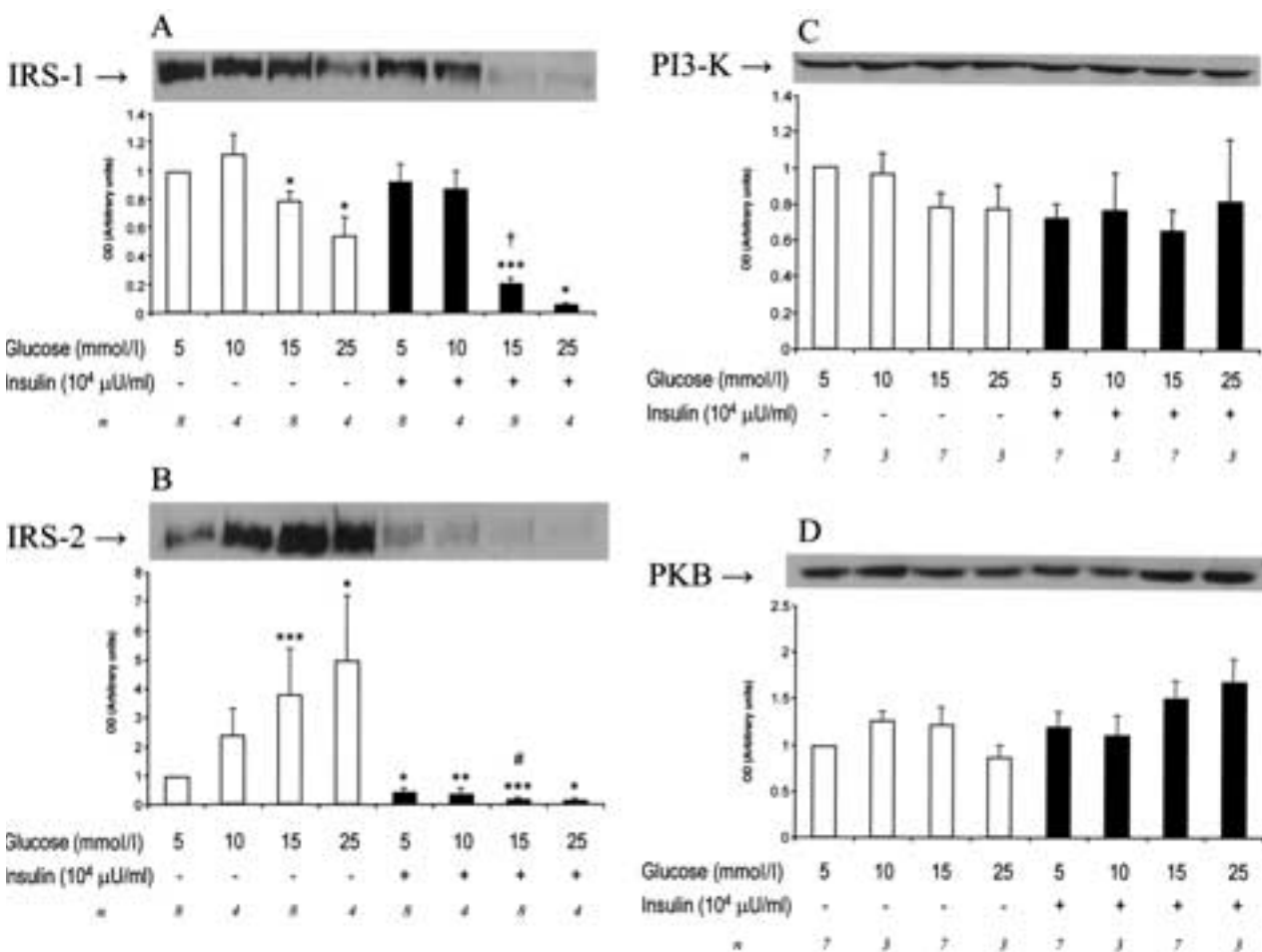
**Figure 2** Effect of insulin pretreatment at a high level on glucose uptake capacity. Adipocytes were incubated in 5 mmol/l (A) or 15 mmol/l (B) glucose with (■) or without (□) insulin (10<sup>4</sup>  $\mu$ U/ml) for 24 h. (C) Time course for the effects of pretreatment with high glucose and insulin to reduce basal and maximally insulin-stimulated glucose uptake. Adipocytes were exposed to high glucose (15 mmol/l) and insulin (10<sup>4</sup>  $\mu$ U/ml) for the final 2, 6 and 24 h of incubation (0 = control situation with 5 mmol/l glucose). After washing, insulin was added at the indicated concentrations (0–1000  $\mu$ U/ml) for 15 min, and then glucose clearance was measured in the presence of different insulin concentrations (0–1000  $\mu$ U/ml) during 1 h. Insets: data expressed as insulin effect (% of maximal) during 1 h. All data are means $\pm$ S.E.M. of eight or nine (A, B) or six (C) separate experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs control situation (no insulin).

## Discussion

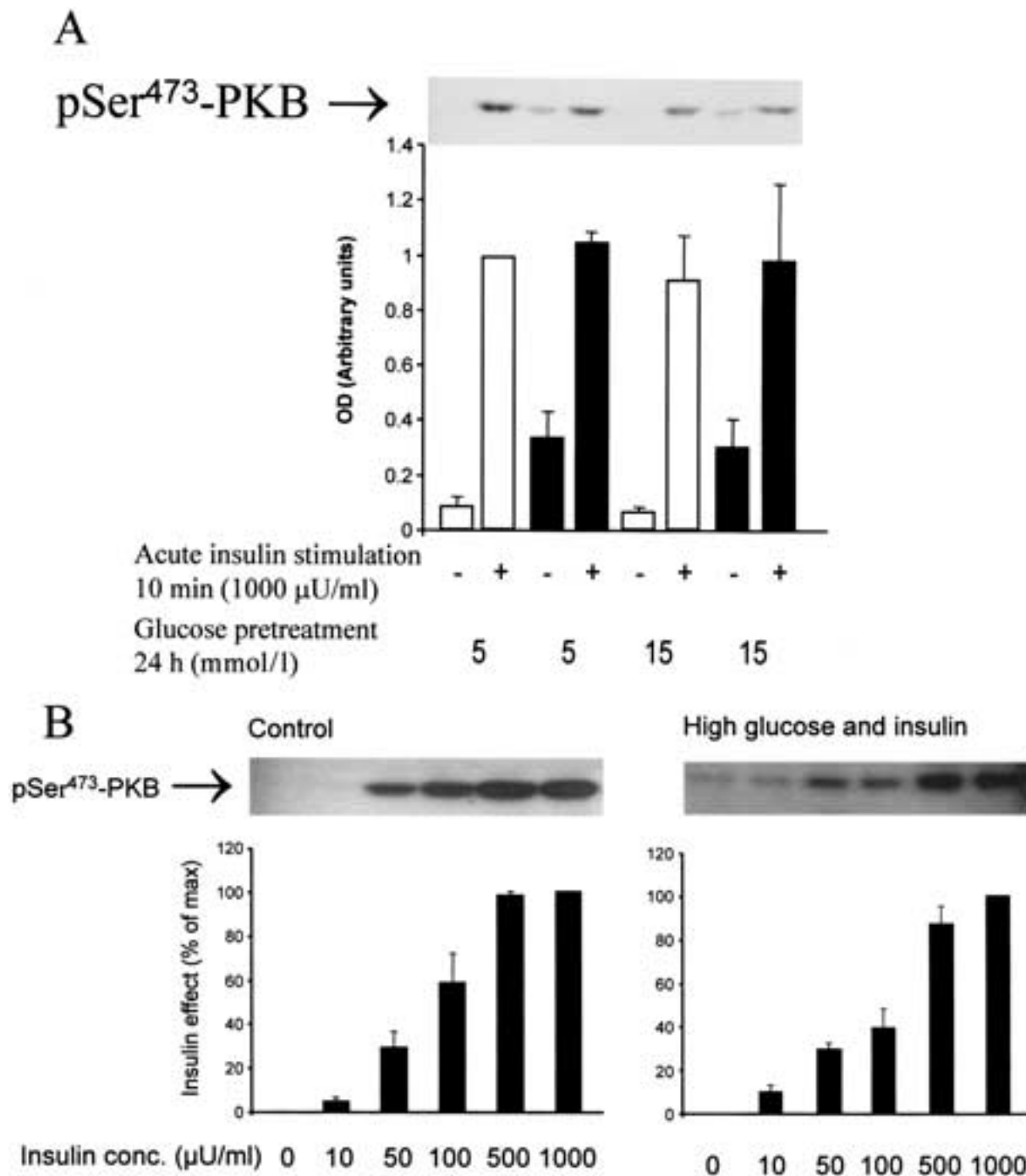
In the present study we have exposed primary cultured rat adipocytes to different glucose concentrations in the presence or absence of a high insulin level. This may reflect the natural course of the development of type 2 diabetes, i.e. hyperinsulinaemia alone that compensates for insulin resistance *in vivo*, hyperinsulinaemia together with hyperglycaemia and hyperglycaemia alone. Our results show that high levels of insulin and glucose in combination have deleterious effects on insulin sensitivity, glucose uptake capacity, the cellular content of IRS-1 and -2 and activation of PKB in rat fat cells.

We treated adipocytes for 24 h with insulin at a supraphysiological concentration, to ensure a saturating

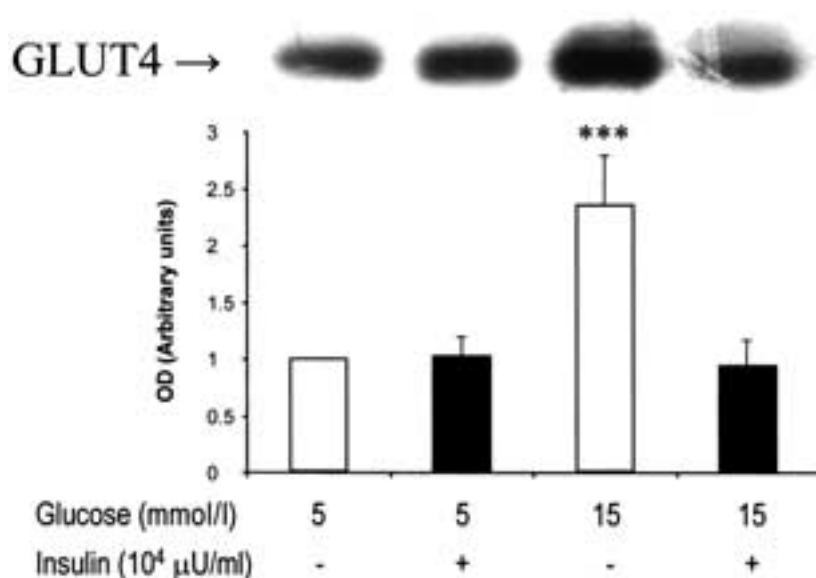
insulin effect throughout the incubation and this did not alter basal or insulin-stimulated glucose uptake capacity when the culture medium contained a low glucose level. However, high insulin and high glucose in combination suppressed both basal and insulin-stimulated glucose uptake and the maximal impairment seemed to occur within 6 h of incubation. Previously, Garvey *et al.* (21) reported that a high glucose level amplifies a long-term effect of insulin to impair glucose transport rates. In addition, the translocation of glucose transporters to the cell surface was impaired. We found that excess insulin, independent of glucose concentration, for 24 h induced a decrease in insulin sensitivity, as shown by a 2- to 3-fold rightward shift in the dose–response curve for glucose transport stimulation. This could, quantitatively, be



**Figure 3** Cellular content of insulin signalling proteins. Whole cell lysates were prepared from adipocytes incubated for 24 h in different glucose concentrations (5, 10, 15 and 25 mmol/l) in the absence (open bars) or presence (filled bars) of insulin (10<sup>4</sup> μU/ml). Whole cell lysates were separated on SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with antibodies against IRS-1 (A), IRS-2 (B), p85α/β (PI3-K) (C) and Akt1/2 (PKB) (D). The data are expressed as means ± s.e.m. of three to eight separate experiments as indicated. One representative blot is shown. The results of video densitometry analysis are shown and are expressed as arbitrary units of the amount of signalling protein in relation to the control situation (i.e. 5 mmol/l glucose, no insulin = 1 arbitrary unit). \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001 vs control; †*P* < 0.05 vs 15 mmol/l glucose without insulin and vs 5 mmol/l+insulin; #*P* < 0.05 vs 5 mmol/l glucose+insulin. For PI3-K and PKB no significant alterations were seen with any of the employed treatments (C, D).



**Figure 4** Effect of long-term high glucose and insulin on insulin-stimulated PKB phosphorylation. Isolated cells were cultured for 24 h in 5 or 15 mmol/l glucose without insulin (open bars) or with 10<sup>4</sup> μU/ml insulin (filled bars). After washing, cells were treated either with or without insulin (1000 μU/ml) for 10 min (A) or the dose–response relationship for insulin’s acute effect was examined with varying insulin concentrations as indicated (B). Then, total cellular lysates were prepared as described in Materials and methods and protein from total cellular lysates was separated on SDS-PAGE, followed by immunoblotting using an anti-phospho-Akt1 (Ser<sup>473</sup>) antibody. One representative blot is shown. The results of video densitometry analysis are shown and are expressed as arbitrary units in relation to the amount of Ser<sup>473</sup> phosphorylation in the control culture condition (i.e. 5 mmol/l glucose, no insulin = 1 arbitrary unit) after acute insulin stimulation. Data are means ± S.E.M. of four or five separate experiments.



**Figure 5** GLUT4 content in total cellular membranes. Adipocytes were incubated for 24 h in 5 or 15 mmol/l glucose with 10<sup>4</sup> μU/ml insulin (filled bars) or without insulin (open bars). After washing, total cellular membranes were prepared as described in Materials and methods. Protein from total cellular membranes was separated on SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with antibodies against GLUT4. The data are expressed as means ± s.e.m. of 13 separate experiments. One representative blot is shown. The results of video densitometry analysis are shown and data are expressed as arbitrary units of the amount of GLUT4 in relation to the control situation (i.e. 5 mmol/l glucose, no insulin = 1 arbitrary unit). \*\*\**P* < 0.001 vs control.

explained by the ~50% reduction in cell surface insulin binding capacity following insulin pretreatment as presently demonstrated, which is in accord with a downregulation of the number of insulin binding sites reported previously (21). However, in a recent study by Tang *et al.* (22) high glucose and insulin were reported not to affect total cellular insulin receptor content or basal insulin receptor phosphorylation, whereas maximal insulin stimulation of the insulin receptor was decreased in cells exposed to high glucose and insulin.

Our present work shows that long-term exposure to a high glucose level *per se* can impair the glucose transport system in adipocytes by reducing the rate of insulin-stimulated as well as basal glucose uptake. It appears that there is a threshold for this effect that occurs between 10 and 15 mmol/l. In harmony with our present data, it was previously reported that in muscle from human type 2 diabetes, insulin-stimulated glucose transport is impaired under hyperglycaemic conditions (23). However, the glucose transport capacity was restored *in vitro* during incubation at a physiological glucose level, supporting the proposition that glucose levels *per se* have regulatory effects on the glucose transport machinery (23). We recently found that insulin-resistant adipocytes from type 2 diabetes patients recover their insulin response after *in vitro* incubation at physiological glucose levels for 24 h, suggesting that cellular insulin resistance may be secondary to the hyperglycaemic state *in vivo* (24). Moreover, isolated fat cells from normoglycaemic individuals with a strong family history of type 2 diabetes displayed normal insulin action on glucose uptake and lipolysis, despite whole-body insulin resistance *in vivo* (15). These observations may suggest that cellular

insulin resistance is a consequence rather than a cause of hyperglycaemia in type 2 diabetes.

High insulin in the incubation medium markedly impaired cellular IRS-2 expression at both low and high glucose levels. To the best of our knowledge this has not been reported previously. Recent studies have revealed an important role for IRS-2 in the liver (25, 26).

Previous studies have found that long-term insulin treatment downregulates IRS-1 in 3T3-L1 adipocytes (27–29). However, 3T3-L1 cells are normally cultured in high glucose (25 mmol/l). We demonstrate that insulin does not reduce IRS-1 content at physiological glucose levels in primary cultured adipocytes, and that a prerequisite for insulin-induced downregulation of IRS-1 is high glucose (i.e. 15 mmol/l or more) surrounding the cells. We found that IRS-1 content in adipocytes is almost halved in the presence of 25 mmol/l glucose. In contrast, IRS-2 displayed a distinct upregulation that was closely linked to the prevailing glucose concentration in the incubation medium, and this is a novel finding. This is compatible with results of Rondinone *et al.* (30) showing that individuals with type 2 diabetes have a marked reduction in adipocyte IRS-1 protein expression and function, whereas IRS-2 is upregulated and could be the main docking protein binding to and activating PI3-K. It could be argued that the adipose tissue plays a minor role in overall insulin-mediated glucose turnover *in vivo*. However, similar alterations in insulin signalling may occur also in skeletal muscle in both human type 2 diabetes (31, 32) and animal models (33–35) of type 2 diabetes. For example in Zucker diabetic fatty rats (35) and *ob/ob* mice (36) displaying hyperglycaemia combined with hyperinsulinaemia, the expression of IRS-1 and -2 in muscle is reduced, similar to our present findings.



Our study shows that combined treatment for 24 h with high insulin and high glucose produced a massive reduction in the content of IRS-1 and -2 indicating synergistic interaction effects between insulin and glucose. These insulin- and glucose-induced aberrations in the amount of insulin signalling proteins might be of relevance for the development and progression of human type 2 diabetes, as also supported by data from mice with combined heterozygous null mutations suggesting that combined IRS-1 and -2 perturbations may contribute to the development of diabetes (37). In our study high insulin markedly reduced IRS-2 content. Moreover, insulin further decreased IRS-1 content compared with cells cultured in high glucose alone. It has been reported that low cellular IRS-1 expression in fat cells from healthy humans is associated with impaired downstream signalling and also insulin resistance *in vivo* (38, 39). Our present data also support the concept that low IRS-1 content is associated with a suppression of the glucose transport system, since the conditions displaying a reduced glucose uptake capacity, i.e. high glucose or high glucose+high insulin, also caused depletion of IRS-1. In contrast, we found no consistent alterations in the cellular content of PI3-K or PKB, and moreover, maximally insulin-stimulated PKB phosphorylation was not impaired by the high glucose+high insulin condition. Nevertheless, in our study pretreatment with high glucose and high insulin shifted the dose-response curve for insulin to phosphorylate PKB to the right and the effect of acutely phosphorylating PKB exerted by submaximal insulin concentrations was impaired. This is an indication of impaired upstream signalling that could be explained by reduced IRS-1 and -2 content and/or the demonstrated insulin-induced reduction in cell surface insulin binding capacity. However, in our high glucose+insulin model the maximal incremental response to insulin with respect to PKB phosphorylation as well as glucose transport was essentially preserved, although the amounts of IRS-1 and -2 were markedly decreased. This is compatible with recent findings suggesting that insulin can produce PKB phosphorylation in skeletal muscle of insulin-resistant humans despite a complete inability to stimulate IRS-1 phosphorylation (40). One possible explanation could be that small amounts of IRS-1 and -2 are enough for signal transduction to PKB and that there is normally a great redundancy in IRS levels. Furthermore, it cannot be excluded that other signalling pathways downstream of the insulin receptor could substitute for IRS-1 and -2. Nevertheless, in our insulin-resistant adipocyte model, the demonstrated reduction in PKB activation at submaximal insulin concentrations could be a consequence of the combined depletion of IRS-1 and -2 content. Of note, there are other studies that suggest that reduced amounts of IRS-1 are associated with a reduction in PKB activity in human (38) as well as rat (41) adipocytes.

In this study, we found no effect of high insulin for 24 h *per se* on the cellular GLUT4 content when glucose was at a low level, but when combined with a high glucose level (15 mmol/l), insulin exerted a suppressing effect, i.e. preventing the upregulation produced by high glucose alone. A previous study in 3T3-L1 adipocytes showed that prolonged insulin treatment decreases GLUT4 content by ~30% (42). Our group has recently reported that insulin instead exerts an upregulating effect on GLUT4 mRNA and protein levels in 3T3-F442A adipocytes at low (5.6 mmol/l) but not high (25 mmol/l) glucose levels (12). In comparison with the present results those data emphasise that one cannot directly extrapolate cell-line data to tissues in animals or humans.

Our present results show that long-term high glucose *per se* downregulated both basal and maximally stimulated glucose uptake capacity despite a clear concentration-dependent accumulation of GLUT4 protein in the total cellular membrane fraction, suggesting a deleterious effect on the translocation process or the intrinsic activity of GLUT4 (43). In rats with streptozotocin-induced diabetes, hyperglycaemia *per se* affects insulin-stimulated glucose transport by altering glucose transporter translocation in skeletal muscle (44). The impairment of glucose uptake in adipocytes does not seem to involve GLUT1 since it was unaffected by high glucose in diabetic rats (45, 46).

The mechanisms for glucose- and insulin-induced alterations in IRS-1 and -2 as well as GLUT4 abundance are not clarified. Preliminary data from our laboratory suggest that high insulin and high glucose do not alter IRS-1 gene transcription (F Renström, J Burén & J W Eriksson, unpublished observations). This is in accord with data from 3T3-L1 adipocytes where expression of IRS-1 mRNA was essentially unchanged following chronic exposure to insulin, but the rate of IRS-1 protein degradation seemed to be increased (27). Recent data from CHO cells and 3T3-L1 adipocytes suggest that insulin promotes IRS-1 degradation by the proteasome degradation pathway (47).

In summary, our data demonstrate that long-term high insulin and high glucose, particularly in combination, can produce, within 24 h of adipose cell culture, marked alterations in glucose uptake capacity, IRS-1/2 and GLUT4 content and in insulin's ability to phosphorylate PKB. Following insulin pretreatment, cell surface insulin binding is decreased and together with IRS depletion this may contribute to the demonstrated insensitivity to insulin's action on PKB activation and glucose transport. These alterations in the insulin signalling cascade and glucose transport system may potentially promote insulin resistance in the different stages of progression of human type 2 diabetes. Alterations in the insulin signalling pathway in type 2 diabetes, e.g. decreased cellular IRS-1 content, may be, at least partly, secondary to the diabetic state.

In conclusion, our data suggest that: (i) hyperinsulin-aemia and hyperglycaemia in combination severely compromise adipocyte insulin binding and IRS-1 and -2 content; (ii) this is accompanied by an impaired sensitivity for insulin with respect to PKB and glucose transport activation; (iii) there is a great redundancy in IRS-1 and -2 amounts in normal cells; and (iv) cellular insulin resistance in diabetes may be aggravated by alterations in glucose and insulin levels *in vivo*.

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