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Defective Akt activation is associated with glucose- but not glucosamine-induced insulin resistance

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Nelson, Bryce A., Katherine A. Robinson, and Maria G. Buse. Defective Akt activation is associated with glucose- but not glucosamine-induced insulin resistance. *Am J Physiol Endocrinol Metab* 282: E497–E506, 2002. First published October 30, 2001; 10.1152/ajpendo.00438.2001.—3T3-L1 adipocytes develop insulin-resistant glucose transport upon preincubation with high glucose or glucosamine, provided insulin (0.6 nM) is present during preincubation. Insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol (PI) 3-kinase activity is unaffected (30). Total cellular IRS-1, PI 3-kinase, or Akt concentrations were unchanged. Akt activation in subcellular fractions was assessed by immunoblotting with two phospho-Akt-specific antibodies. Upon acute 100 nM insulin stimulation, plasma membrane (PM)-associated phospho-Akt was highest in cells preincubated in low glucose with no insulin, less in high glucose with no insulin, even less in low glucose+insulin, and lowest in high glucose+insulin. Only high glucose+insulin caused insulin-resistant glucose transport. Acute insulin stimulation increased total PM-Akt about twofold after preincubation without insulin in low or high glucose. Preincubation with 0.6 nM insulin decreased Akt PM translocation by ~25% in low and ~50% in high glucose. Preincubation with glucosamine did not affect Akt phosphorylation or translocation. Conclusions: chronic exposure to high glucose or insulin downregulates acute insulin-stimulated Akt activation, acting synergistically distal to PI 3-kinase. Maximal insulin activates more Akt than required for maximal glucose transport stimulation. Insulin resistance may ensue when PM-associated phospho-Akt decreases below a threshold. High glucose and glucosamine cause insulin resistance by different mechanisms in 3T3-L1 adipocytes.

glucose toxicity; 3T3-L1 adipocytes; Akt activation; glucose transport

INSULIN'S INTERACTION with its receptor (IR) initiates a complex signaling cascade, which regulates cell growth and differentiation, protein synthesis, and glucose and lipid metabolism. The signaling cascades that mediate the transcriptional regulation of protein synthesis diverge early from those mediating short-term metabolic regulation. Insulin-mediated acceleration of glucose transport in skeletal and heart muscle and in adipocytes is a key example of the latter; the precise mechanism is still incompletely understood. Clinically, insulin resistance is defined as the reduced ability of insulin to lower plasma glucose, which reflects, in great part, impaired insulin stimulation of glucose transport and GLUT-4 translocation. It is a positive predictor and hallmark of the development of type 2 diabetes. Indeed, several defects in insulin signaling associated with insulin resistance and type 2 diabetes have been reported and are reviewed in Ref. 33.

Insulin binding to IR stimulates the autophosphorylation of specific tyrosine residues on the IR β-subunit, which increases its intrinsic tyrosine kinase activity. Substrates of the activated IR tyrosine kinase include the IR substrates (IRS) 1–4 (47). They are phosphorylated on specific tyrosine residues and act as docking motifs for Src homology 2-containing proteins, which include the p85 regulatory subunit of phosphatidylinositol (PI) 3-kinase (33). Binding of p85 to IRS activates the p110 catalytic subunit of PI 3-kinase, which in turn generates 3-phosphoinositides.

At least two downstream targets are activated by 3-phosphoinositides: protein kinase (PK) C-ζ/λ (5) and Akt/PKB (46). They are phosphorylated by phosphoinositide-dependent kinase 1 (PDK-1) and play a role in insulin-stimulated glucose transport and GLUT-4 translocation. It is still unclear whether either or both are important in regulating GLUT-4 translocation and glucose transport, but evidence suggests that PKC-ζ/λ and Akt function parallel to but not downstream of one another (24).

Three isoforms encoded by different genes have been cloned and designated as Akt-1/PKB-α (12), Akt-2/PKB-β (9), or Akt-3/PKB-γ (23). 3T3-L1 adipocytes express only Akt-1 and Akt-2 (2). PI-3 kinase-dependent phosphorylation of Akt-1 and Akt-2 on Thr^{308} and Ser^{473} and on Thr^{309} and Ser^{474}, respectively, is necessary for insulin-regulated activation of Akt. PDK-1 phosphorylates Akt-1 and Akt-2 on Thr^{308} and Thr^{309}, respectively (1, 38). The phosphorylation of Ser^{473} (4) of Akt is regulated by an as-yet-unidentified PDK-2, which may be Akt itself but is required for full activa-

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tion (43). Akt in the basal state, for the most part, resides in the cytosol. It is recruited to the inner leaflet of the plasma membrane after insulin stimulation (4), where 3-phosphoinositides bind with high affinity to its pleckstrin homology (PH) domain, inducing a conformational change that renders Thr^{308} in the activation loop accessible to the action of PDK-1. This phosphorylation, in turn, facilitates the phosphorylation of Ser^{473}.

Prolonged hyperglycemia causes insulin resistance in humans and in various animal models of diabetes (35, 48), which led to the concept of "glucose toxicity." Glucose-induced insulin resistance of glucose transport has been demonstrated in tissue culture systems (17, 27, 29, 41), and we have extensively characterized the model in 3T3-L1 adipocytes (30). Preexposure (18 h) to high (25 mM) glucose or to glucosamine (1–2.5 mM) decreased the subsequent response of glucose transport to maximal acute insulin stimulation compared with cells preincubated in low (5 mM) glucose. High glucose or glucosamine induced insulin resistance only if a relatively low dose (0.6 nM) of insulin was included during preincubation. Preexposure to low-dose insulin in the presence of low glucose did not affect the acute insulin stimulation of glucose transport. The expression of glucose transporters (GLUT-4 and GLUT-1) was not affected by the preincubation conditions, and robust acute insulin stimulation of IRS-1-associated PI 3-kinase was maintained under all conditions. However, although both high glucose and glucosamine decreased insulin-stimulated glucose transport to a similar degree, their effects on acute insulin-stimulated GLUT-4 translocation were different, as assessed by the plasma membrane lawn assay. Preincubation with glucosamine in the presence of 5 mM glucose and 0.6 nM insulin inhibited the translocation of GLUT-4 to the plasmalemma upon acute insulin stimulation. Preincubation in high glucose + 0.6 nM insulin did not inhibit GLUT-4 translocation, although both conditions inhibited acute insulin stimulation of glucose transport to a similar degree (~40%). We concluded that glucose-induced insulin resistance may involve impaired glucose transporter activation or impaired docking/fusion of GLUT-4-containing vesicles with the plasma membrane (30). In the present study, we assessed whether preincubation with low-dose insulin and/or high glucose affected the acute insulin-regulated serine/threonine phosphorylation of Akt and its translocation to the plasma membrane and whether preincubation with high glucose or glucosamine exerts different effects on the acute insulin regulation of Akt.

EXPERIMENTAL PROCEDURES

Materials. A polyclonal antibody specific for Akt-1/2 and a monoclonal antibody that recognizes the p85 subunit of PI 3-kinase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively. In addition, two phospho-specific polyclonal antibodies purchased from Cell Signaling (Beverly, MA) were used to detect the phosphorylation status of Akt on Ser^{473} and Thr^{308}. Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories (West Grove, PA), and enhanced chemiluminescence (ECL) reagents were purchased from Pierce (Rockford, IL). Crystalline recombinant human insulin was a gift from Eli Lilly Research Laboratories (Indianapolis, IN). Other reagents were purchased from suppliers, as described in previous publications (30), and were of the highest quality available.

Cell culture and general methods. 3T3-L1 fibroblasts were grown and differentiated into adipocytes in 35-mm culture dishes, as previously described (16, 30), and prepared for experiments as described (30). Briefly, after differentiation, adipocytes were placed into DMEM containing 25 mM glucose and 10% fetal bovine serum until they were used in experiments 10–14 days after initiation of the differentiation protocol, when 90–95% of the cells exhibited the adipocyte morphology.

In typical experiments, cells were preincubated for 18 h in DMEM containing 1% FBS and sugars, as indicated in Figs. 1–7, i.e., 5 or 25 mM glucose or 5 mM glucose + 2.5 mM glucosamine, each with or without 0.6 nM insulin. They were then transferred to identical media without FBS or insulin for 2 h and then acutely stimulated with or without 100 nM insulin for 15 min (30). After being washed twice with HES buffer [20 mM HEPES (pH 7.4), 1 mM EDTA, 255 mM sucrose, 1 mM sodium vanadate, 1 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 μM microcystin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aprotinin] without protease inhibitors, cells were lysed as we will describe in *Subcellular fractionation*.

Subcellular fractionation. Subcellular fractions were generated by differential centrifugation, as previously described (42). Cells prepared as described above were harvested in HES buffer. Cell lysates were prepared by shearing cells 10 times through a 22-gauge needle. An aliquot was saved for analyses of total cell lysates. The remainder was then centrifuged at 19,000 *g* for 20 min at 4°C. The resulting supernatant was centrifuged at 41,000 *g* for 20 min to yield a low-speed pellet containing high-density microsomes. That supernatant was centrifuged for 75 min at 180,000 *g* to generate a high-speed pellet containing low-density microsomes (LDM) and the cytosol in the supernatant. Crude

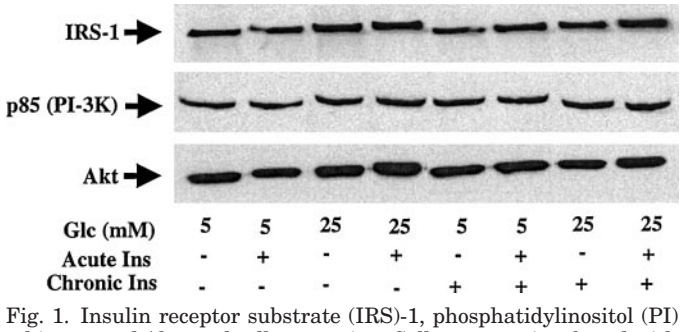

Fig. 1. Insulin receptor substrate (IRS)-1, phosphatidylinositol (PI) 3-kinase, and Akt total cell expression. Cells were preincubated with (+) or without (−) 0.6 nM insulin (Ins) with either 5 mM or 25 mM glucose (Glc). Cells were stimulated with (+) or without (−) acute insulin (100 nM) for 15 min. Total cell lysates were prepared as described in EXPERIMENTAL PROCEDURES, and 30 μg of protein were separated by SDS-PAGE and immunoblotted with antibodies specific to IRS-1, the p85 subunit of PI-3 kinase, or Akt. These immunoblots represent 3–5 individual experiments. Quantitative analyses showed no significant differences among treatment groups (data not shown).

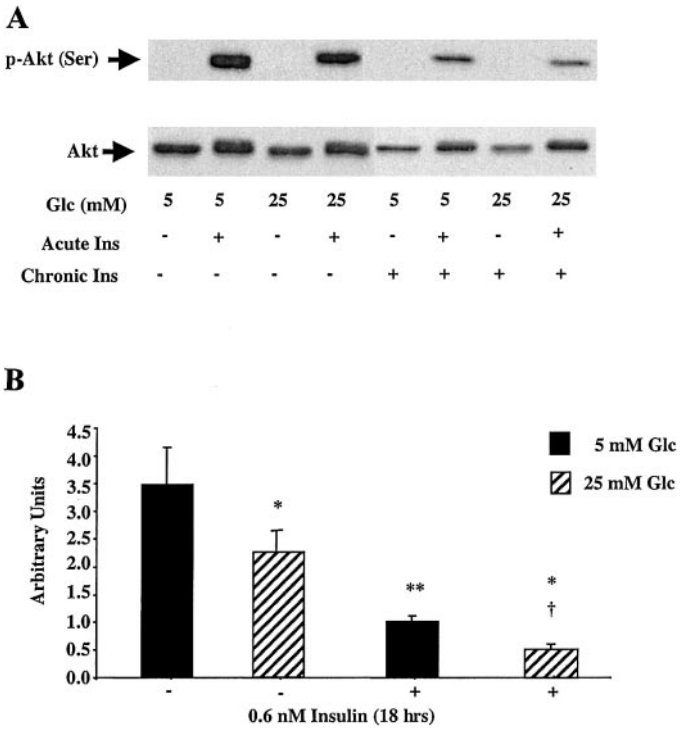

Fig. 2. Acute insulin-induced serine phosphorylation of plasma membrane (PM)-associated Akt. Effects of preincubation with insulin and high glucose. Cells were preincubated with (+) or without (−) 0.6 nM insulin with either 5 mM or 25 mM Glc. After washing and reequilibration, cells were stimulated with acute insulin (100 nM) for 15 min [phosphorylated Akt (P-Akt) was undetectable in the basal state]. Subcellular fractions were prepared as described in EXPERIMENTAL PROCEDURES, and 15 μg of protein from the Triton-soluble plasma membrane (TS-PM) were separated by SDS-PAGE and immunoblotted with an antibody specific to P-Akt (phosphorylated on Ser^{473} in Akt-1 and Ser^{474} in Akt-2) or with an antibody to total Akt (*A*). Graphs in *B* represent densitometric analysis of P-Akt levels from immunoblots of TS-PM. Data are quantitated as means ± SE from 6 independent experiments. In each experiment, data were normalized to the P-Akt levels measured in cells preincubated with 5 mM Glc + 0.6 nM insulin and acutely stimulated with 100 nM insulin. *$P < 0.05$ vs. 5 mM Glc ± 0.6 nM insulin; **$P < 0.02$ vs. 5 mM Glc − 0.6 nM insulin; †$P < 0.02$ vs. 25 mM Glc − 0.6 nM insulin.

plasma membranes (PM) were prepared by resuspending the pellet from the 19,000-*g* centrifugation and layering it onto a 1.12 M sucrose cushion, which was centrifuged at 100,000 *g* for 60 min. The PM layer was collected and centrifuged at 40,000 *g* for 20 min. Pellets of the PM and LDM were resuspended in HES buffer containing 1% Triton X-100 and solubilized for ≥1 h at 4°C. They were subsequently centrifuged for 75 min at 200,000 *g*. The Triton-soluble fractions (TS-PM, TS-LDM) were collected, and the Triton-insoluble pellets (TI-PM, TI-LDM) were resuspended in PBS containing 1% SDS and heated for 10 min at 95°C. Total cell extracts and cell fractions were analyzed by SDS-PAGE and immunoblotted with a polyclonal antibody specific for Akt-1/2. Insulin-stimulated phosphorylation of Akt was detected by immunoblot with two phospho-specific polyclonal antibodies recognizing Akt-1 phosphorylated on Ser^{473} and Thr^{308} and Akt-2 and -3 phosphorylated on the corresponding sites. The blots were developed using the ECL procedure, and the bands were then quantified using a Molecular Dynamics Personal Laser Densitometer (Amersham Pharmacia Biotech, Piscataway, NJ) equipped with ImageQuant version 3.3 software.

Other analyses. Protein concentrations in cell extracts were measured spectrophotometrically against BSA standards using Coomassie protein assay reagent purchased from Pierce (Rockford, IL).

Statistical analysis. Means ± SE are shown. Where error bars are not indicated in the figures, they are too small for graphical representation, i.e., they fall within the symbol of the mean. The significance of differences between means was evaluated by one-way or two-way ANOVA and Tukey's test for unbalanced design with the Statistica software or by two-tailed unpaired Student's *t*-test with Statview software. $P < 0.05$ was considered significant.

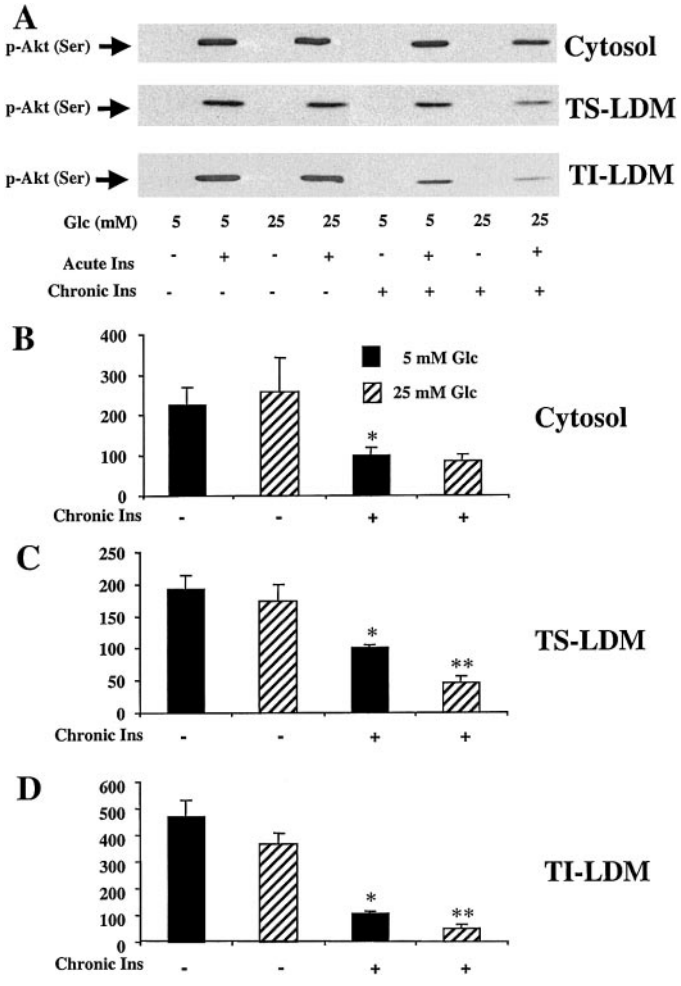

Fig. 3. P-Akt associated with cytosol, TS-low-density microsomes (LDM), and Triton-insoluble (TI)-LDM after acute insulin stimulation. Effects of preincubation with high glucose and low-dose insulin. Cells were preincubated and processed as described in Fig. 2. After 15 min of acute stimulation with or without 100 nM insulin, subcellular fractions were prepared, and 15 μg protein from TS-LDM or TI-LDM or 7.5 μg protein from the cytosolic fraction were separated on SDS-PAGE and immunoblotted with a specific antibody to serine-phosphorylated Akt (*A*). Graphs in *B*, *C*, and *D* represent densitometric analyses of P-Akt concentrations on immunoblots from cytosol (*B*), TS-LDM (*C*), and TI-LDM (*D*). Data are means ± SE from 6 independent experiments. In each experiment, data are normalized to P-Akt concentrations in the same fraction on the same gel in cells preincubated with 5 mM Glc + 0.6 nM insulin and are acutely stimulated with 100 nM insulin. *Different from preincubation in 5 mM Glc − 0.6 nM insulin, $P < 0.02$; **different from preincubation in 5 mM Glc + 0.6 nM insulin, $P < 0.02$.

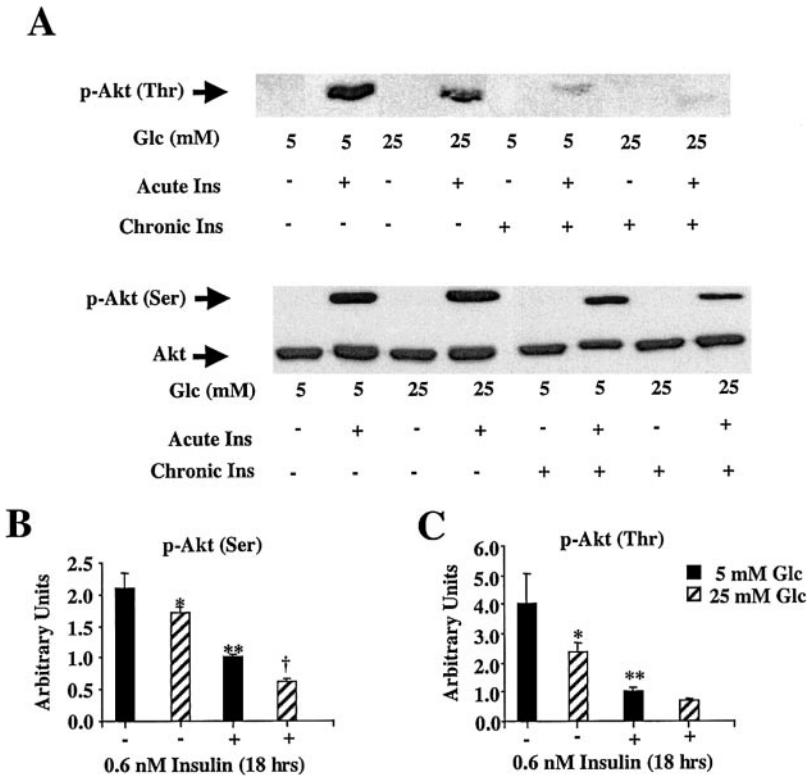

Fig. 4. Comparison of Akt phosphorylation on Ser and Thr upon acute insulin stimulation. Effects of preincubation with insulin and high glucose. Cells were preincubated with (+) or without (−) 0.6 nM insulin with either 5 mM (solid bars) or 25 mM Glc (hatched bars). Cells were stimulated with acute insulin (100 nM) for 15 min (P-Akt was undetectable in the basal state). Protein (30 μg) from total cell lysate was separated by SDS-PAGE and immunoblotted with either an antibody specific for P-Akt (serine-phosphorylated), P-Akt (threonine-phosphorylated), or total Akt-1/-2. *A*: representative immunoblots. Data are quantitated as means ± SE from 3 independent experiments for both *B* and *C*. In each experiment, data were normalized to the phospho-Akt levels measured in cells preincubated with 5 mM Glc + 0.6 nM insulin and acutely stimulated with 100 nM insulin (*$P < 0.05$ vs. 5 mM Glc + 0.6 nM insulin; **$P < 0.05$ vs. 5 mM Glc − 0.6 nM insulin; †$P < 0.05$ vs. 5 mM Glc + 0.6 nM insulin).

RESULTS

High glucose effects on Akt phosphorylation and localization. Because previous studies have implicated altered Akt activation in models of insulin resistance in muscle (26, 37), we tested whether our model of glucose-induced insulin resistance was associated with impaired activation of Akt. After preincubation in media containing either 5 mM or 25 mM glucose in either

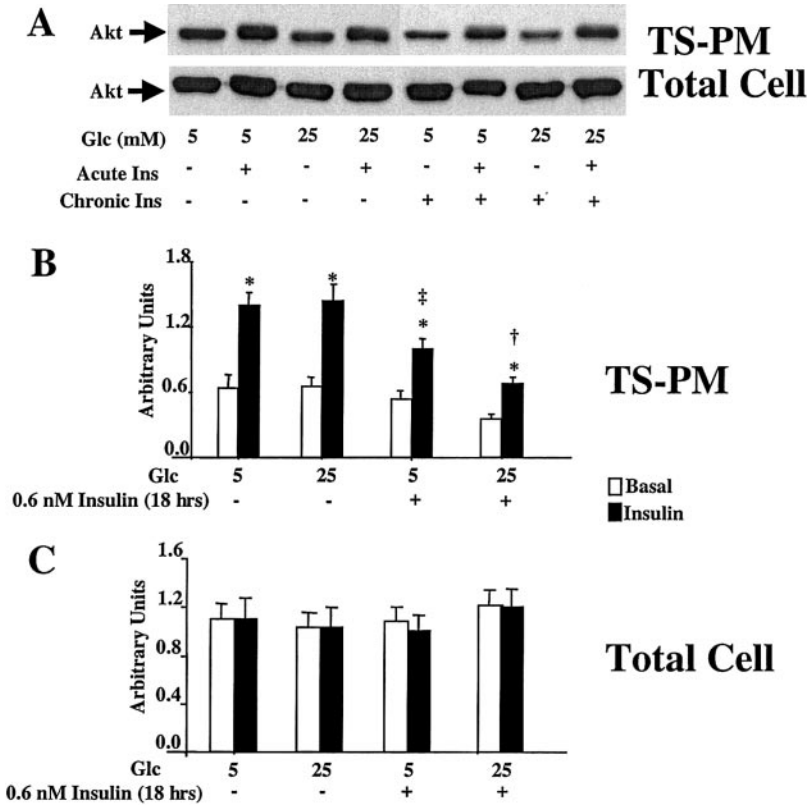

Fig. 5. Acute insulin-induced translocation of total Akt to the TS-PM. Effects of preincubation with insulin and high glucose. Cells were preincubated with (+) or without (−) 0.6 nM insulin with either 5 mM or 25 mM Glc. Cells were acutely stimulated with (black bars) or without (white bars) acute insulin (100 nM) for 15 min. Subcellular fractions or total cell lysates were prepared as described in EXPERIMENTAL PROCEDURES, and 15 or 30 μg of protein from TS-PM or total cell lysate, respectively, were separated by SDS-PAGE and immunoblotted with rabbit α-Akt. *A*: representative immunoblots of Akt from TS-PM and total cell lysate. Graphs represent densitometric analysis of Akt concentrations on immunoblots of TS-PM (*B*) or total cell lysate (*C*). Data are quantitated as means ± SE from 5 (*B*) or 4 (*C*) independent experiments. In each experiment, data were normalized to the Akt concentrations measured in the same fraction on the same gel in cells preincubated with 5 mM Glc + 0.6 nM insulin and then acutely stimulated with 100 nM insulin (*$P < 0.001$ vs. basal, ‡$P < 0.05$ vs. acute insulin stimulation after preincubation with 5 mM Glc − 0.6 nM insulin; †$P < 0.05$ vs. 25 mM Glc − 0.6 nM insulin or 5 mM Glc + 0.6 nM insulin, each after acute insulin stimulation).

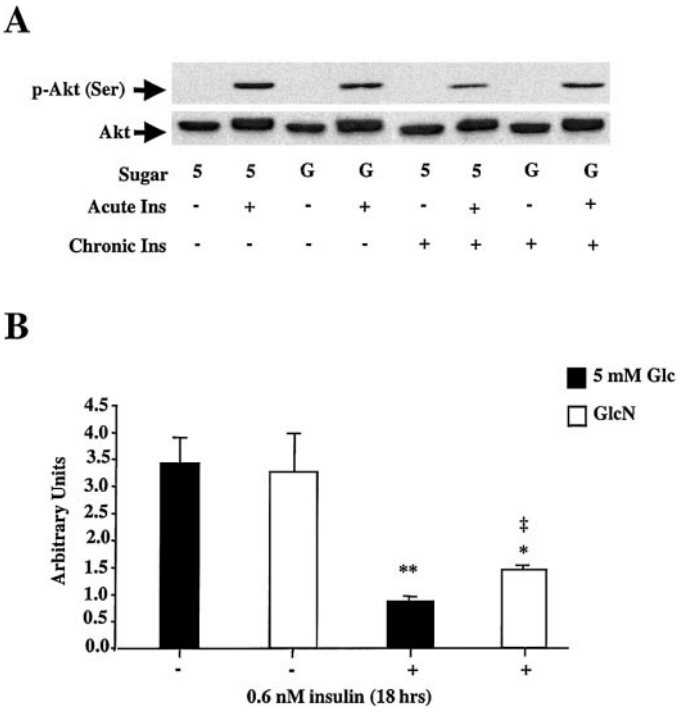

Fig. 6. Glucosamine (GlcN) effects on insulin-induced Akt phosphorylation in TS-PM. Cells were preincubated with (+) or without (−) 0.6 nM insulin with either 5 mM Glc or 5 mM Glc + 2.5 mM GlcN. Cells were stimulated with or without acute insulin (100 nM) for 15 min (P-Akt was undetectable in the basal state). Subcellular fractions were prepared as described in EXPERIMENTAL PROCEDURES, and 15 μg of protein from the TS-PM were separated by SDS-PAGE and immunoblotted with an antibody specific to serine-phosphorylated Akt or with an antibody to total Akt. Graphs represent densitometric analysis of P-Akt levels from immunoblots of TS-PM after acute insulin (100 nM) stimulation. Data are quantitated as means ± SE from 4 independent experiments for *B*. In each experiment, data were normalized to the P-Akt concentrations measured in cells preincubated with 5 mM Glc + 0.6 nM insulin and acutely stimulated with 100 nM insulin. (*$P < 0.05$ vs. 5 mM Glc + 0.6 nM insulin; **$P < 0.02$ vs. 5 mM Glc − 0.6 nM insulin, ‡$P < 0.05$ vs. GlcN − 0.6 nM insulin).

the absence or presence of 0.6 nM insulin, cells were deprived of insulin and serum for 2 h and then stimulated with or without a maximal dose of insulin (100 nM) for 15 min. Then, subcellular fractions of the adipocytes were prepared as described in EXPERIMENTAL PROCEDURES. Total expression of IRS-1, p85 subunit of PI 3-kinase, or Akt was not affected by any of the preincubation conditions (Fig. 1). Next, we immunoblotted for phosphorylated-Akt (P-Akt) with a phospho-specific antibody that recognizes Akt after it is phosphorylated on Ser^{473} (Akt-1) or Ser^{474} (Akt-2). As shown in Fig. 2*A*, P-Akt was detectable in the TS-PM only after acute insulin stimulation. This acute insulin-responsive phosphorylation of Akt decreased 70% (Fig. 2*B*, $P < 0.02$) after preincubation with 0.6 nM insulin in the presence of 5 mM glucose, a condition that did not induce insulin-resistant glucose transport. Compared with control (5 mM glucose without 0.6 nM insulin), P-Akt levels in the TS-PM decreased 30% (Fig. 2*B*, $P < 0.05$) and 85% (Fig. 2*B*, $P < 0.02$) in cells exposed to 25 mM glucose in the absence or presence of 0.6 nM insulin, respectively. Only the latter was associated with insulin-resistant glucose transport (30). Thus both chronic insulin exposure ($P < 0.001$) and high glucose concentration ($P < 0.05$) independently reduced the accumulation of acute insulin-induced P-Akt at the PM without affecting insulin-stimulated glucose transport. However, the effects of preincubation with 25 mM glucose and 0.6 nM insulin were additive, and only when present together did insulin-resistant glucose transport ensue.

In all fractions analyzed (Figs. 2 and 3), P-Akt was detected after acute insulin stimulation but not in the basal state. As reported by others, P-Akt was most abundant in the cytosol (Fig. 3) and was associated in significant amounts with TS-PM (Fig. 2) and the TS- and TI-LDM fractions (Fig. 3). P-Akt was not detected in the TI-PM fraction (not shown). Chronic exposure to low-dose insulin (0.6 nM) significantly decreased P-Akt abundance after acute insulin stimulation in the cytosol and in the TS- and TI-LDM, after preincubation in low or high glucose ($P < 0.02$, Fig. 3), similar to the effects seen in the TS-PM (Fig. 2). In the TS- and TI-LDM, the downregulation of acute insulin-mediated

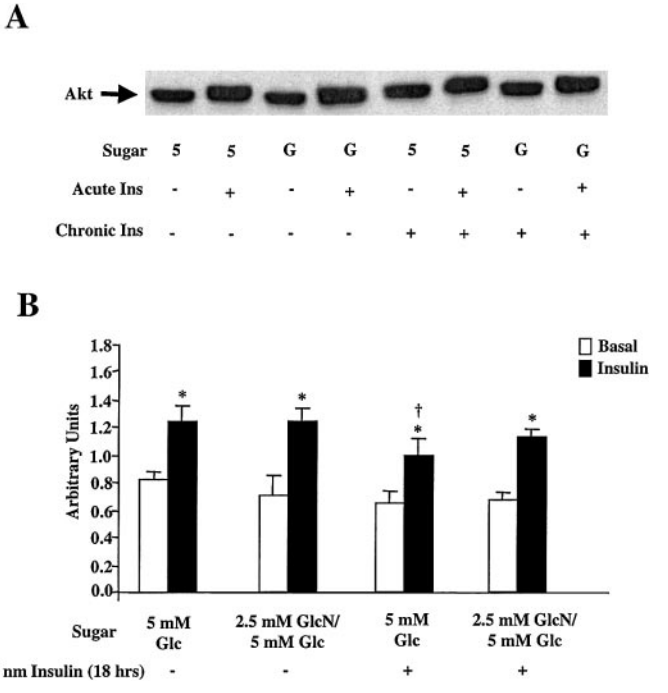

Fig. 7. Glucosamine effects on Akt PM translocation. Cells were preincubated for 18 h with (+) or without (−) 0.6 nM insulin with either 5 mM Glc or 5 mM Glc + 2.5 mM GlcN. Cells were then stimulated with (black bars) or without (white bars) acute insulin (100 nM) for 15 min. Subcellular fractions or total cell lysates were prepared as described in EXPERIMENTAL PROCEDURES, and 15 μg of protein were separated by SDS-PAGE and immunoblotted with rabbit α-Akt. Graphs represent densitometric analysis of Akt levels from immunoblots of TS-PM. Data are quantitated as means ± SE from 4 independent experiments. In each experiment, data were normalized to the Akt concentration in the same fraction measured in cells preincubated with 5 mM Glc + 0.6 nM insulin and acutely stimulated with 100 nM insulin (*$P < 0.05$ vs. basal; †$P < 0.05$ vs. preincubation in 5 mM glucose without 0.6 nM insulin and then acutely stimulated with 100 nM insulin, by paired Student's *t*-test).

Akt activation after chronic exposure to low-dose insulin was significantly enhanced in the presence of high glucose compared with low glucose ($P < 0.02$), but this effect did not reach statistical significance in the cytosol. Preincubation in high glucose in the absence of insulin decreased P-Akt abundance significantly only in the PM (compared with low glucose, $P < 0.05$, Fig. 2), but not in the cytosol or the TI- or TS-LDM fractions (Fig. 3).

Akt is first phosphorylated on Thr^{308} (Akt-1) or Thr^{309} (Akt-2) in response to insulin. In an effort to further characterize the phosphorylation defect associated with glucose-induced insulin resistance, total cell lysates, prepared as described in EXPERIMENTAL PROCEDURES, were separated by SDS-PAGE and immunoblotted with either a phospho-Ser-Akt or phospho-Thr-Akt antibody. As shown in Figs. 1 and 4*A*, total Akt expression was not altered by any preincubation condition. Similar to results shown in Fig. 2, neither Ser- nor Thr-phosphorylated Akt was detected in total cell lysates in the basal state. Acute insulin stimulation markedly increased the phosphorylation of Akt on both residues after preincubation with 5 mM glucose − 0.6 nM insulin (Fig. 4). Chronic 0.6 nM insulin preexposure decreased Ser phosphorylation by ~50% and Thr phosphorylation by ~75% after preincubation with 5 mM glucose (Fig. 4, *B* and *C*, $P < 0.05$). High glucose in the absence of 0.6 nM insulin decreased phosphorylation of Akt on Ser and Thr by ~20 and 37%, respectively ($P < 0.05$). Maximal inhibition of both Ser and Thr phosphorylation was observed after preincubation in high glucose + 0.6 nM insulin. Two-way ANOVA revealed a significant interaction between the effects of high glucose and the presence of 0.6 nM insulin during preincubation ($P < 0.05$). Thus acute insulin-induced phosphorylation of Akt on Ser and Thr in total cell lysate occurred in parallel, and chronic insulin or high glucose preexposure impaired phosphorylation of both sites similarly.

Acute insulin stimulation leads to activation and recruitment of Akt to the PM. The latter step has been implicated in insulin regulation of glucose transport and GLUT-4 translocation (13, 46). We assessed whether the observed impairment of Akt phosphorylation in the TS-PM resulted from decreased translocation of Akt to the PM in response to acute insulin. As shown in Figs. 2*A* and 5*A*, Akt is associated with the PM in the basal state, although P-Akt was only detectable in the PM after acute insulin stimulation (Fig. 2). Akt levels increased ~1.8- to 2-fold above basal in the TS-PM after acute insulin stimulation (Fig. 5*B*, $P < 0.001$). This acute insulin response was decreased 30% in cells preincubated with 5 mM glucose in the presence of 0.6 nM insulin compared with cells preincubated with 5 mM glucose without insulin ($P < 0.05$) and was independent of changes in total Akt expression (Fig. 5*C*). Whereas 25 mM glucose in the absence of insulin decreased insulin-stimulated P-Akt levels in the TS-PM (Fig. 2), this treatment was without effect on insulin-stimulated translocation of Akt to the TS-PM. A significant interaction existed between the presence of 0.6 nM insulin during preincubation and the acute insulin response of Akt translocation to the PM ($P < 0.01$), and also between glucose concentration and the presence of 0.6 nM insulin ($P < 0.05$) when assessed by three-way ANOVA. However, insulin resistance of glucose transport manifested only after preincubation in 25 mM glucose + 0.6 nM insulin, suggesting that the interaction between high glucose and chronic insulin stimulation is the predominant factor in the induction of insulin resistance.

Although it is clear that acute insulin stimulation increased Akt concentration in the PM without affecting its concentration in the whole cell, we were unable to determine a cell fraction from which Akt had translocated. There were no significant changes in Akt concentration after acute insulin stimulation in the TS-LDM or cytosol. The TI-LDM showed a trend toward decreased Akt after acute insulin stimulation that did not reach statistical significance (data not shown).

Glucosamine effects on Akt phosphorylation and localization. Because we previously observed different effects of high glucose or glucosamine preincubation on insulin-stimulated GLUT-4 translocation (30), we tested in a separate series of experiments (Fig. 6) whether preincubation with glucosamine affected Akt phosphorylation and whether the response was different from the effects of preexposure to high glucose. As in previous experiments, Akt phosphorylation was observed only after acute insulin stimulation, and this response was significantly decreased ($P < 0.02$) by including 0.6 nM insulin with 5 mM glucose during preincubation. In contrast to the effects of high glucose (Fig. 2), the addition of glucosamine during preincubation in the presence or absence of insulin had no effect on subsequent acute insulin-stimulated Akt phosphorylation. The presence of 0.6 nM insulin with glucosamine only decreased Akt phosphorylation by 55% ($P < 0.05$). This stands in contrast to the effects of high glucose + insulin that downregulated Akt phosphorylation by 85%. In contrast to high glucose preincubation, there was no evidence of a synergistic effect between chronic exposure to low-dose insulin and glucosamine.

Acute insulin stimulation increased Akt levels in the TS-PM ~1.8- to 2-fold (Fig. 7*B*, $P < 0.05$, similar to results in Fig. 5). This response was decreased ~25% after preincubation with 0.6 nM insulin and 5 mM glucose. Preincubation with glucosamine had no effect on insulin-stimulated Akt recruitment to TS-PM, regardless of the presence or absence of 0.6 nM insulin (Fig. 7). In contrast, 25 mM glucose and 0.6 nM insulin acted synergistically to downregulate acute insulin-responsive Akt translocation to the PM (Fig. 5, $P < 0.05$). No significant interactions were detected between preincubation with glucosamine and low-dose insulin on the acute insulin response of Akt translocation to the PM, which again is in contrast to the effects of high glucose (Fig. 5). Thus the ability of high glucose and glucosamine to induce insulin resistance likely represents different effects, at least at the level of acute insulin-mediated Akt activation.

DISCUSSION

In the model of glucose-induced insulin resistance used here, preincubation in high glucose impairs the subsequent acute insulin stimulation of glucose transport provided that low-dose insulin is also present during preincubation. Because the acute insulin stimulation of IRS-1-associated PI 3-kinase was unaffected, we searched for a defect in insulin's signaling cascade distal to PI 3-kinase. Our data clearly demonstrate that chronic exposure to high glucose (without insulin) or to insulin in low glucose impairs the subsequent acute activation of Akt by insulin by ~30 and ~70%, respectively. The two effects appear to be additive. Exposure to low-dose insulin in high glucose reduced PM-associated P-Akt to one-half of that observed after preincubation with insulin in low glucose. Under these conditions, acute insulin stimulation yielded only 15% of the P-Akt accumulation in the PM observed in controls preincubated in low glucose without insulin. Note that only the combined treatment resulted in insulin-resistant glucose transport in this model. Thus, if we assume that impaired Akt activation is causally related to the development of insulin resistance, these data suggest that, in 3T3-L1 adipocytes, a dose of insulin that maximally stimulates glucose transport results in Akt activation that is far in excess of that needed for maximal acute glucose transport stimulation. Thus a 70% reduction of P-Akt at the PM does not impair maximal insulin stimulation of glucose transport, but when P-Akt falls below a critical level (~20% of maximal), insulin-resistant glucose transport develops. The major role of fat cells is to store energy as lipids, when nutrients, e.g., glucose and insulin, are plentiful. The development of insulin-resistant glucose transport may serve as a "last resort" strategy in normal adipocytes, possibly to prevent excessive oxidative damage from chronically increased glucose flux. However, less drastic decreases in P-Akt may affect other insulin-regulated pathways.

In rat extensor digitorum longus muscles incubated for several hours under different conditions (26), high glucose caused insulin resistance of glucose transport only if insulin was present during preincubation. However, preincubation with high glucose without insulin did impair the acute insulin stimulation of glucose flux into glycogen and lipids. PI 3-kinase activation by insulin was unaffected under these conditions, but Akt activation was impaired. Two other recent papers support the notion that ambient glucose may regulate Akt activation by insulin at steps(s) distal to PI 3-kinase. One involves muscles of a rodent model of type 2 diabetes, the Goto-Kakizaki rat (37), and the other deals with primary adipocytes from patients with type 2 diabetes (7). In the latter model, adipocytes from diabetic patients demonstrated both impaired activation of Akt and diminished Akt translocation to the plasmalemma in response to insulin, similar to our findings in 3T3-L1 adipocytes after preincubation in high glucose in the presence of insulin.

Because in our model a 70% decrease of P-Akt at the PM did not diminish insulin-stimulated glucose transport, the question arises whether Akt activation is required for insulin-stimulated glucose transport. Akt-2 is most highly expressed in insulin-responsive cells and has been specifically implicated in insulin's stimulation of glucose transport (25). Recent work in Akt-2 knockout mice provides strong evidence for Akt-2's participation in insulin-regulated glucose homeostasis (11). Our observations suggesting that a fraction of insulin-activated Akt is sufficient for maximal glucose transport stimulation may explain previous conflicting results concerning Akt's role in insulin signaling by use of dominant-negative mutant technologies (13, 22). In Akt-2 knockout mice, insulin resistance of glucose transport was demonstrable in white (glycolytic) but not red (oxidative) muscles, and the impairment was mild in adipocytes (11). Insulin likely stimulates glucose transport via several parallel pathways, which may act synergistically and may compensate for one another until a critical threshold is reached. In our model, insulin resistance develops when activated Akt falls below ~20% of maximal stimulation.

Glucosamine has been extensively used as a model to elucidate the role of the hexosamine biosynthesis pathway in glucose-induced insulin resistance (6, 8, 18, 19, 21, 29, 30, 32, 34, 36, 44, 45). Previous studies suggested that high glucose and glucosamine may induce insulin resistance of glucose transport by different mechanisms (8, 19, 30, 45). In addition, preexposure to glucosamine caused several metabolic alterations in 3T3-L1 adipocytes that did not mimic the effects of high glucose (19, 30). The data presented here support these conclusions. Whereas preexposure to high glucose significantly inhibited Akt activation in the absence or presence of low-dose insulin during preincubation, glucosamine did not. In vivo, glucosamine coinfusion during a euglycemic insulin clamp causes insulin resistance as assessed by decreased peripheral glucose utilization (6, 21, 32, 36) and impaired translocation of GLUT-4 to the PM in skeletal muscle (6). However, insulin-mediated Akt activation in skeletal muscle was unimpaired, even under conditions in which diminished IRS-1-associated PI 3-kinase activation was detectable (21, 32). Akt activation was impaired after exposure to glucosamine + insulin in the absence of glucose in one study in 3T3-L1 adipocytes (19). The effect was attributed to ATP depletion and was accompanied by impaired activation of IR, IRS-1, and PI 3-kinase, as well as insulin resistance of glucose transport and GLUT-4 translocation. In our model, 3T3-L1 adipocytes were exposed to glucosamine in the presence of 5 mM glucose. Although a decrease in the ATP concentration was detectable, it was much more modest than in the work of Hresko et al. (19), and insulin-mediated IRS-1-associated PI 3-kinase activity was unimpaired (30). Glucosamine may cause insulin resistance of GLUT-4 translocation distal to the action of Akt. A possible mechanism may involve a decreased GTP-to-GDP ratio (30), which may impair the activa-

tion of small G proteins involved in the translocation of GLUT-4-containing vesicles (10, 14, 20).

In our experiments, preexposure to high glucose and/or to low-dose insulin decreased the acute insulin-stimulated phosphorylation of Akt on Thr and Ser in parallel. These observations are consistent with those obtained in primary adipocytes from patients with type 2 diabetes (7). Although we have no direct evidence to indicate that the decreased phosphorylation of Ser is secondary to that of Thr, previous observations support this hypothesis, because Thr phosphorylation is required for Ser phosphorylation (43).

Acute insulin stimulation increased PM-associated Akt approximately twofold compared with basal. This response was decreased by ~25–30% after preincubation with low-dose insulin in the presence of low glucose. High glucose + insulin further decreased Akt PM translocation (compared with low glucose + insulin), whereas glucosamine was ineffective. Thus chronic insulin preexposure downregulated the insulin-induced phosphorylation of Akt and its PM translocation. This effect was enhanced by the inclusion of high glucose but not of glucosamine.

The percent decrease in Akt phosphorylation was greater than that observed in PM translocation and was independent of changes in total Akt expression, similar to a study in adipocytes from patients with type 2 diabetes (7). Akt translocation to the PM may be required for its phosphorylation by PDK-1 (4), which is in part constitutively associated with the PM (3, 15). Therefore, the decreased phosphorylation of Akt could reflect, at least in part, its impaired translocation. Basal steady-state levels of Akt in the PM were not different among any of the preincubation conditions. Because the effects of chronic preexposure to high glucose and low-dose insulin appear to be synergistic, they may downregulate Akt activation by different mechanisms. Note that preincubation in high glucose without insulin significantly decreased acute insulin-stimulated Akt phosphorylation on both Thr and Ser, without a detectable impairment of Akt translocation to the PM. Thus the primary effect of sustained exposure to high glucose may be impairment of Akt phosphorylation, whereas chronic exposure to low-dose insulin affects both processes, with the possibility that impaired Akt translocation may be the primary event.

The mechanisms by which chronic exposure to high glucose or to low-dose insulin may downregulate insulin-stimulated Akt activation at a step downstream of PI 3-kinase activation are unknown. Decreased expression or decreased activity of PDK-1 is an obvious candidate. Alternatively, the dephosphorylation of Akt may be accelerated in our model by increased expression or activation of phosphoprotein phosphatases. A particularly attractive candidate may be the tumor suppressor protein PTEN, which acts both as a phosphoprotein phosphatase and a lipid phosphatase. The latter activity entails the specific dephosphorylation of the D3 position of inositol and PI substrates (28). Increased expression and/or activity of PTEN in cells preexposed to high glucose and low-dose insulin could explain the impaired translocation and phosphorylation of Akt. Both PTEN (28) and ceramide (39, 40) have been shown to independently and negatively regulate Akt activation.

The data presented support the concept that high glucose and glucosamine induce insulin resistance by different mechanisms in 3T3-L1 adipocytes. High glucose, but not glucosamine, impairs insulin stimulation of Akt activity. Chronic exposure to low-dose insulin impairs Akt translocation and phosphorylation and acts synergistically as a negative Akt modulator with high glucose but not with glucosamine. As previously discussed, the fact that glucosamine does not mimic the effect of high glucose does not negate the possible role of the hexosamine biosynthesis pathway in glucose-induced insulin resistance, but it suggests that glucosamine may not appropriately model the pathway (30). Preliminary evidence suggests that preexposure to high glucose in the presence of low-dose insulin may affect the appropriate docking/fusion of GLUT-4-containing vesicles with the PM (31). The data presented here raise the possibility that Akt may participate in the regulation of this distal step in insulin's stimulation of glucose transport.

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REFERENCES

1. **Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, and Cohen P.** Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B alpha. *Curr Biol* 7: 261–269, 1997.
2. **Altomare DA, Lyons GE, Mitsuuchi Y, Cheng JQ, and Testa JR.** Akt2 mRNA is highly expressed in embryonic brown fat and the AKT2 kinase is activated by insulin. *Oncogene* 16: 2407–2411, 1998.
3. **Anderson KE, Coadwell J, Stephens LR, and Hawkins PT.** Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. *Curr Biol* 8: 684–691, 1998.
4. **Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq JM, and Hemmings BA.** Role of translocation in the activation and function of protein kinase B. *J Biol Chem* 272: 31515–31524, 1997.
5. **Bandyopadhyay G, Standaert ML, Zhao L, Yu B, Avignon A, Galloway L, Karnam P, Moscat J, and Farese RV.** Activation of protein kinase C (alpha, beta, and zeta) by insulin in 3T3/L1 cells. Transfection studies suggest a role for PKC-zeta in glucose transport. *J Biol Chem* 272: 2551–2558, 1997.
6. **Baron AD, Zhu JS, Zhu JH, Weldon H, Maianu L, and Garvey WT.** Glucosamine induces insulin resistance in vivo by affecting GLUT 4 translocation in skeletal muscle. Implications for glucose toxicity. *J Clin Invest* 96: 2792–2801, 1995.
7. **Carvalho E, Eliasson B, Wesslau C, and Smith U.** Impaired phosphorylation and insulin-stimulated translocation to the

plasma membrane of protein kinase B/Akt in adipocytes from type II diabetic subjects. *Diabetologia* 43: 1107–1115, 2000.

8. **Chen H, Ing BL, Robinson KA, Feagin AC, Buse MG, and Quon MJ.** Effects of overexpression of glutamine:fructose-6-phosphate amidotransferase (GFAT) and glucosamine treatment on translocation of GLUT4 in rat adipose cells. *Mol Cell Endocrinol* 135: 67–77, 1997.
9. **Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN, and Testa JR.** AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc Natl Acad Sci USA* 89: 9267–9271, 1992.
10. **Chiang SH, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, Macara IG, Pessin JE, and Saltiel AR.** Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. *Nature* 410: 944–948, 2001.
11. **Cho H, Mu J, Kim JK, Thorvaldsen JL, Chu Q, Crenshaw EB III, Kaestner KH, Bartolomei MS, Shulman GI, and Birnbaum MJ.** Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* 292: 1728–1731, 2001.
12. **Coffer PJ and Woodgett JR.** Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur J Biochem* 201: 475–481, 1991.
13. **Cong LN, Chen H, Li Y, Zhou L, McGibbon MA, Taylor SI, and Quon MJ.** Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *Mol Endocrinol* 11: 1881–1890, 1997.
14. **Cormont M, Bortoluzzi MN, Gautier N, Mari M, van Obberghen E, and Le Marchand-Brustel Y.** Potential role of Rab4 in the regulation of subcellular localization of Glut4 in adipocytes. *Mol Cell Biol* 16: 6879–6886, 1996.
15. **Currie RA, Walker KS, Gray A, Deak M, Casamayor A, Downes CP, Cohen P, Alessi DR, and Lucocq J.** Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1. *Biochem J* 337: 575–583, 1999.
16. **Frost SC and Lane MD.** Evidence for the involvement of vicinal sulfhydryl groups in insulin-activated hexose transport by 3T3-L1 adipocytes. *J Biol Chem* 260: 2646–2652, 1985.
17. **Garvey WT, Olefsky JM, Matthaei S, and Marshall S.** Glucose and insulin co-regulate the glucose transport system in primary cultured adipocytes. A new mechanism of insulin resistance. *J Biol Chem* 262: 189–197, 1987.
18. **Heart E, Choi WS, and Sung CK.** Glucosamine-induced insulin resistance in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab* 278: E103–E112, 2000.
19. **Hresko RC, Heimberg H, Chi MM, and Mueckler M.** Glucosamine-induced insulin resistance in 3T3-L1 adipocytes is caused by depletion of intracellular ATP. *J Biol Chem* 273: 20658–20668, 1998.
20. **Kessler A, Tomas E, Immler D, Meyer HE, Zorzano A, and Eckel J.** Rab11 is associated with GLUT4-containing vesicles and redistributes in response to insulin. *Diabetologia* 43: 1518–1527, 2000.
21. **Kim YB, Zhu JS, Zierath JR, Shen HQ, Baron AD, and Kahn BB.** Glucosamine infusion in rats rapidly impairs insulin stimulation of phosphoinositide 3-kinase but does not alter activation of Akt/protein kinase B in skeletal muscle. *Diabetes* 48: 310–320, 1999.
22. **Kitamura T, Ogawa W, Sakaue H, Hino Y, Kuroda S, Takata M, Matsumoto M, Maeda T, Konishi H, Kikkawa U, and Kasuga M.** Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. *Mol Cell Biol* 18: 3708–3717, 1998.
23. **Konishi H, Kuroda S, Tanaka M, Matsuzaki H, Ono Y, Kameyama K, Haga T, and Kikkawa U.** Molecular cloning and characterization of a new member of the RAC protein kinase family: association of the pleckstrin homology domain of three types of RAC protein kinase with protein kinase C subspecies and beta gamma subunits of G proteins. *Biochem Biophys Res Commun* 216: 526–534, 1995.
24. **Kotani K, Ogawa W, Matsumoto M, Kitamura T, Sakaue H, Hino Y, Miyake K, Sano W, Akimoto K, Ohno S, and Kasuga M.** Requirement of atypical protein kinase c lambda for insulin stimulation of glucose uptake but not for Akt activation in 3T3-L1 adipocytes. *Mol Cell Biol* 18: 6971–6982, 1998.
25. **Kupriyanova TA and Kandror KV.** Akt-2 binds to Glut4-containing vesicles and phosphorylates their component proteins in response to insulin. *J Biol Chem* 274: 1458–1464, 1999.
26. **Kurowski TG, Lin Y, Luo Z, Tsichlis PN, Buse MG, Heydrick SJ, and Ruderman NB.** Hyperglycemia inhibits insulin activation of Akt/protein kinase B but not phosphatidylinositol 3-kinase in rat skeletal muscle. *Diabetes* 48: 658–663, 1999.
27. **Lima FB, Thies RS, and Garvey WT.** Glucose and insulin regulate insulin sensitivity in primary cultured adipocytes without affecting insulin receptor kinase activity. *Endocrinology* 128: 2415–2426, 1991.
28. **Maehama T and Dixon JE.** PTEN: a tumour suppressor that functions as a phospholipid phosphatase. *Trends Cell Biol* 9: 125–128, 1999.
29. **Marshall S, Bacote V, and Traxinger RR.** Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. *J Biol Chem* 266: 4706–4712, 1991.
30. **Nelson BA, Robinson KA, and Buse MG.** High glucose and glucosamine induce insulin resistance via different mechanisms in 3T3-L1 adipocytes. *Diabetes* 49: 981–991, 2000.
31. **Nelson BA, Robinson KA, and Buse MG.** Insulin-regulated Munc18-c trafficking is impaired in glucose-induced insulin resistance in 3T3-L1 adipocytes (Abstract). *Diabetes* 50: A405, 2001.
32. **Patti ME, Virkamaki A, Landaker EJ, Kahn CR, and Yki-Jarvinen H.** Activation of the hexosamine pathway by glucosamine in vivo induces insulin resistance of early postreceptor insulin signaling events in skeletal muscle. *Diabetes* 48: 1562–1571, 1999.
33. **Pessin JE and Saltiel AR.** Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest* 106: 165–169, 2000.
34. **Robinson KA, Sens DA, and Buse MG.** Pre-exposure to glucosamine induces insulin resistance of glucose transport and glycogen synthesis in isolated rat skeletal muscles. Study of mechanisms in muscle and in rat-1 fibroblasts overexpressing the human insulin receptor. *Diabetes* 42: 1333–1346, 1993.
35. **Rossetti L, Giaccari A, and DeFronzo RA.** Glucose toxicity. *Diabetes Care* 13: 610–630, 1990.
36. **Rossetti L, Hawkins M, Chen W, Gindi J, and Barzilai N.** In vivo glucosamine infusion induces insulin resistance in normoglycemic but not in hyperglycemic conscious rats. *J Clin Invest* 96: 132–140, 1995.
37. **Song XM, Kawano Y, Krook A, Ryder JW, Efendic S, Roth RA, Wallberg-Henriksson H, and Zierath JR.** Muscle fiber type-specific defects in insulin signal transduction to glucose transport in diabetic GK rats. *Diabetes* 48: 664–670, 1999.
38. **Stokoe D, Stephens LR, Copeland T, Gaffney PR, Reese CB, Painter GF, Holmes AB, McCormick F, and Hawkins PT.** Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* 277: 567–570, 1997.
39. **Stratford S, DeWald DB, and Summers SA.** Ceramide dissociates 3′-phosphoinositide production from pleckstrin homology domain translocation. *Biochem J* 354: 359–368, 2001.
40. **Summers SA, Garza LA, Zhou H, and Birnbaum MJ.** Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide. *Mol Cell Biol* 18: 5457–5464, 1998.
41. **Thomson MJ, Williams MG, and Frost SC.** Development of insulin resistance in 3T3-L1 adipocytes. *J Biol Chem* 272: 7759–7764, 1997.
42. **Thurmond DC, Ceresa BP, Okada S, Elmendorf JS, Coker K, and Pessin JE.** Regulation of insulin-stimulated GLUT4

translocation by Munc18c in 3T3-L1 adipocytes. *J Biol Chem* 273: 33876–33883, 1998.
43. **Toker A and Newton AC.** Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J Biol Chem* 275: 8271–8274, 2000.
44. **Virkamaki A, Daniels MC, Hamalainen S, Utriainen T, McClain D, and Yki-Jarvinen H.** Activation of the hexosamine pathway by glucosamine in vivo induces insulin resistance in multiple insulin sensitive tissues. *Endocrinology* 138: 2501–2507, 1997.
45. **Virkamaki A and Yki-Jarvinen H.** Allosteric regulation of glycogen synthase and hexokinase by glucosamine-6-phosphate during glucosamine-induced insulin resistance in skeletal muscle and heart. *Diabetes* 48: 1101–1107, 1999.
46. **Wang Q, Somwar R, Bilan PJ, Liu Z, Jin J, Woodgett JR, and Klip A.** Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol Cell Biol* 19: 4008–4018, 1999.
47. **White MF.** The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol Cell Biochem* 182: 3–11, 1998.
48. **Yki-Jarvinen H, Helve E, and Koivisto VA.** Hyperglycemia decreases glucose uptake in type I diabetes. *Diabetes* 36: 892–896, 1987.