Glucosamine-induced insulin resistance in 3T3-L1 adipocytes

EMMA HEART, WOO S. CHOI, AND CHIN K. SUNG
Department of Physiology and Biophysics, University of Southern California, School of Medicine, Los Angeles, California 90033

Heart, Emma, Woo S. Choi, and Chin K. Sung. Glucosamine-induced insulin resistance in 3T3-L1 adipocytes. Am. J. Physiol. Endocrinol. Metab. 278: E103–E112, 2000.—To study molecular mechanisms for glucosamine-induced insulin resistance, we induced complete and reversible insulin resistance in 3T3-L1 adipocytes with glucosamine in a dose- and time-dependent manner (maximal effects at 50 mM glucosamine after 6 h). In these cells, glucosamine impaired insulin-stimulated GLUT-4 translocation. Glucosamine (6 h) did not affect insulin-stimulated tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 and -2 and weakly, if at all, impaired insulin stimulation of phosphatidylinositol 3-kinase. Glucosamine, however, severely impaired insulin stimulation of Akt. Inhibition of insulin-stimulated glucose transport was correlated with that of Akt activity. In these cells, glucosamine also inhibited insulin stimulation of p70 S6 kinase. Glucosamine did not alter basal glucose transport and insulin stimulation of GLUT-1 translocation from an intracellular pool to the plasma membrane in skeletal muscles (2). The ability of this pathway to induce insulin resistance and that increased glucosamine flux through the hexosamine biosynthetic pathway in skeletal muscle may play an important role in hyperglycemia-induced insulin resistance in vivo.

The hexosamine biosynthetic pathway is a minor glucose metabolic pathway that metabolizes ~3% of glucose entering the cell (28). The end products of this pathway are UDP-N-acetylglucosamine and other nucleotide hexosamines that are used to glycosylate proteins and lipids (28). The involvement of this pathway in regulation of insulin action was originally discovered by Marshall et al. (27), who induced insulin resistance in isolated primary rat adipocytes with insulin, glucose, and glutamine. Glucosamine enters the hexosamine biosynthetic pathway distal to the first rate-limiting enzyme step, catalyzed by glutamine:fructose-6-phosphate amidotransferase, is metabolized to generate UDP-N-acetylglucosamine, and was proven to be more potent than glucose in induction of insulin resistance (27, 29). Overexpression of this rate limiting enzyme in transgenic mice was later reported to lead to insulin resistance (17).

Glucosamine-induced insulin resistance in skeletal muscle was reported to be due to impaired GLUT-4 translocation from an intracellular pool to the plasma membrane in skeletal muscles (2). The ability of glucosamine to impair GLUT-4 translocation could result from either defects in insulin signal transduction or defects intrinsic to the glucose transport effector system involving alteration in the trafficking/translocation of GLUT-4-containing vesicles (2). Presently, it is unclear whether insulin signal transduction is altered by glucosamine treatment of cells. In isolated muscles, glucosamine was reported not to alter insulin receptor number and activation of insulin receptor tyrosine kinase (29).

Numerous studies with inhibitors and mutants of phosphatidylinositol 3-kinase (PI 3-kinase) suggested that insulin stimulation of GLUT-4 translocation in insulin-sensitive tissues is dependent upon insulin-stimulated PI 3-kinase activity (6). One target of PI 3-kinase is the serine/threonine protein kinase Akt (5). Akt is activated by insulin and growth factors and is recently implicated in insulin-stimulated glucose transport in 3T3-L1 adipocytes and isolated rat primary adipocytes (8, 22, 23).
To study whether glucosamine affects insulin signal transduction, we employed 3T3-L1 adipocytes and successfully induced insulin resistance by treatment of cells with glucosamine in the absence of insulin. We chose this experimental condition because chronic insulin treatment per se was reported to induce insulin resistance (34). In these glucosamine-induced insulin-resistant 3T3-L1 adipocytes, we examined insulin-signaling molecules known to play a role in glucose transport after acute insulin stimulation of cells. Here, we report that glucosamine alone induced complete and reversible insulin resistance in 3T3-L1 adipocytes without affecting basal glucose transport. Glucosamine treatment of cells inhibited insulin stimulation of GLUT-4 translocation. In these cells, glucosamine severely impaired insulin stimulation of Akt, without significant impairment of signaling molecules upstream of Akt. Inhibitory effects of glucosamine on insulin-stimulated glucose transport and insulin-stimulated p70 S6 kinase appeared to be selective because other biological functions of insulin were not altered.

**EXPERIMENTAL PROCEDURES**

Materials. Monoclonal antibodies to phosphotyrosine (α-PY) and antiserum to the p85 regulatory subunit of PI 3-kinase (α-p85) were purchased from Upstate Biotechnology (Lake Placid, NY). Antiserum to the p110 catalytic subunit of PI 3-kinase (α-p110), α-Akt and α-mitogen-activated protein (MAP) kinase and α-p70 S6 kinase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). α-Akt reacted mainly with Akt1 and to a lesser extent Akt2. α-phosphoMAP kinase was from New England Biolabs (Beverly, MA). α-GLUT-4 was from Biogenesis (Sandown, NH) and α-GLUT-1 was a gift of Dr. Amira Klip at the Hospital for Sick Children (Toronto, Canada). 2-deoxy-D-[1,2-3H]glucose (2-DG) (26.2 Ci/mmol) and [γ-32P]ATP (26.2 Ci/mmol) were purchased from NEN Life Science Products. (Boston, MA). Other chemicals were from Sigma (St. Louis, MO), unless specified otherwise. Crototide is a synthetic peptide (GPRRTSSFAEG) that corresponds to the phosphorylation sequence in GSK3 (Upstate Biotechnology, Lake Placid, NY).

Cell culture and glucose uptake. 3T3-L1 fibroblasts obtained from Dr. Howard Green at Harvard University (Boston, MA) were grown and differentiated into 3T3-L1 adipocytes after the procedure of García de Herreros and Birnbaum (11) with some modification. Except for the first 5–7 days postdifferentiation during which cells were kept in DMEM containing 20 mM glucose, 3T3-L1 adipocytes were maintained in DMEM containing 5 mM glucose. For experiments, cells were used on 10–14 days postdifferentiation during which time more than 90% of the cells exhibited the fatty phenotype as judged by phase-contrast microscopy.

To study the effect of glucosamine on glucose uptake, we added varying concentrations of glucosamine to cells in 24-well plates and preincubated cells for indicated times. Mannitol was added to media to compensate for the total 50 mM sugar concentration. For the last 5 h of preincubation period, cells were starved in serum-free DMEM containing 5 mM glucose. Cells were washed, placed in buffer A (1.47 mM K$_2$HPO$_4$, pH 7.4, 140 mM NaCl, 1.7 mM KCl, 0.9 mM CaCl$_2$, 0.9 mM MgSO$_4$, and 0.1% BSA) and incubated with or without 100 nM insulin for 10 min at 37°C. Next, 2-DG (0.2 mM, 0.5 µCi) was added and incubation continued for 10 min. Nonspecific 2-DG uptake was measured in the presence of 10 µM cytochalasin B, accounted for ∼10% of basal 2-DG uptake, and was subtracted from all experimental data. Cells were washed in ice-cold phosphate-buffered saline and lysed for 30 min in 0.03% SDS. Aliquots were measured for radioactivity in a liquid scintillation β-counter and protein content was measured by the Bradford method (4).

For a glucosamine time-course study, we initially treated cells with 50 mM mannitol, replaced media with 50 mM glucosamine at various times and continued incubation for up to 24 h. Control cells received 50 mM mannitol for 24 h without media change and cells for 24-h glucosamine treatment received 50 mM glucosamine for 24 h without mannitol pretreatment. This method allowed cells to be exposed to the same osmolality throughout the incubation period, to be treated with glucosamine for various times (0, 3, 6, and 24 h) and to be processed at the same time (after 24 h of incubation).

Enzymatic assays for insulin receptor tyrosine kinase, PI 3-kinase, Akt, and p70 S6 kinase. 3T3-L1 adipocytes in 150-mm dishes were preincubated with 50 mM glucosamine for 0, 3, 6, and 24 h and were serum-starved during the last 5 h of preincubation period. Cells were next incubated for 5 min with or without 100 nM insulin and solubilized for 30 min in 1% NP-40 lysis buffer [20 mM Tris, pH 8.0, 1% NP-40, 137 mM NaCl, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM Na$_3$VO$_4$, 10 mM NaF, 10 mM Na$_2$P$_2$O$_7$, 1 µg/ml leupeptin, and 1 µg/ml aprotonin]. After microcentrifugation for 15 min at 15,000 g, the supernatant (cell lysates) was assayed for protein content and used for subsequent studies.

Wells of 96-well microtiter plate (Dynatech) were coated sequentially with 50 µl of rabbit α-mouse IgG (40 µg/ml), mouse α-insulin receptor antibody CT-1 (2 µg/ml), and normal mouse IgG (150 µg/ml). These components were prepared in 20 mM NaHCO$_3$, pH 9.6, and the plate was incubated with these for 12 h each at 4°C. Wells were washed four times with 100 µl of wash buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 0.1% Tween-20, and 0.1% BSA) before application of a next component. Next, wells were incubated with 50 µl of cell lysate (50 µg) for 12 h at 4°C. After the wells were washed, 50 µl of reaction mixture [50 mM HEPES pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 100 mM MgCl$_2$, 2 mM MnCl$_2$, 1 mg/ml poly(Glu:Tyr = 4:1), 10 µM ATP, and 2 µCi [γ-32P]ATP] were added and the reaction performed for 1 h at 20°C. Reaction was stopped by addition of 25 µl of stop buffer (75 mM EDTA, 7.5 mM ATP, and 0.36% BSA), and 25 µl of the reaction mixture were spotted on P81 phosphocellulose paper. After being thoroughly washed in 150 mM H$_3$PO$_4$ and rinsed briefly in methanol and acetone, P81 papers were counted in liquid scintillation counter. Insulin receptor tyrosine kinase activity was calculated as picomoles of ATP per milligrams of protein (poly(Glu:Tyr = 4:1) per milligrams cellular protein per hour). For PI 3-kinase assay, cell lysates (0.5 mg) were first immunoprecipitated for 3 h with α-PY (1 µg) followed by 2-h incubation with protein A-Sepharose. α-PY immunoprecipitates were then assayed for 10 min at 25°C in reaction mixture containing phosphatidylinositol (0.2 mg/ml) and [γ-32P]ATP (40 µM and 5 µCi) followed by thin-layer chromatography (25). Phosphatidylinositol 3-phosphate, a reaction product, was excised from thin layer plates and counted in a liquid scintillation β-counter. PI 3-kinase activity is presented as picomoles of ATP incorporated per milligram cellular protein per milligram phosphatidylinositol per minute. For Akt assay, cell lysates (0.5 mg) were immunoprecipitated for 2 h with α-Akt (1 µg) followed by 1-h incubation with protein A-Sepharose. α-Akt immunoprecipitates were then assayed for...
for 15 min at 25°C in reaction mixture containing Crosside (0.33 mg/ml) and [γ-32P]ATP (20 µM, 5 µCi). Aliquots of reaction mixture containing phosphorylated Crosside were spotted on P81 phosphocellulose paper followed by washing in 150 mM ice-cold H3PO4. Radioactivities in P81 papers were counted in liquid scintillation β-counter.

For p70 S6 kinase assay, cells were incubated for 30 min with or without 100 nM insulin and solubilized in S6 membrane lysis buffer (25). These cell lysates (100 µg) were immunoprecipitated with α-p70 S6 kinase (1 µg) for 2 h followed by 1-h incubation with protein A-Sepharose (30 µl). These immunoprecipitates were assayed for S6 kinase activity with Artemia salina shrimp egg 405 ribosomes as a substrate. S6 phosphorylation was then analyzed by SDS-PAGE followed by autoradiography (25).

Subcellular fractionation. Cells in 150-mm dishes were preincubated with 50 mM glucosamine for 0, 3, 6, and 24 h (2 dishes for each condition). During the last 5 h of preincubation, cells were serum-starved. Cells were next incubated for 30 min with and without 100 nM insulin. Cells were washed with ice-cold PBS and homogenized in homogenization buffer (20 mM HEPES pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM PMSF, 1 µg/ml aprotinin, and 1 µg/ml leupeptin). Subcellular fractionation procedure was carried out as previously described (32) with some modification. Cells were homogenized by 15 strokes of motor-driven Teflon pestle and total homogenate was subjected to centrifugation for 20 min at 19,000 g. The resultant pellet was resuspended in 200 µl of buffer B

To prepare low-density membrane fraction, the supernatant from 19,000 g spin was centrifuged for 20 min at 41,000 g. The pellet was suspended in 200 µl of buffer B (high-density membrane fraction), and the supernatant was centrifuged for 75 min at 180,000 g. The resultant pellet was rehomogenized in buffer B and subjected to centrifugation for 30 min at 50,000 g. The final pellet was suspended in 200 µl of buffer B and used as plasma membrane fraction.

To assess purity of the plasma membrane and low-density membranes, we performed assays for marker enzymes, 5′-nucleotidase and UDP-galactose: N-acetylgalactosamine galactosyltransferase, respectively (1, 10). In comparison with the total homogenate, 5′-nucleotidase activity in the plasma membrane was enriched 5.5 ± 0.3 fold (n = 13), whereas UDP-galactose: N-acetylgalactosamine galactosyltransferase activity in low-density membranes was enriched 9.2 ± 0.8 fold (n = 13).

Western blot analysis. To study tyrosine phosphorylation of cellular proteins, we incubated cells for 5 min with or without 100 nM insulin, solubilized in 1% NP-40 lysis buffer, and soluble cell lysates (25 µg) resolved by 7.5% SDS-PAGE followed by electrophoretic transfer of proteins onto Hybond-P membranes (Amersham, Buckinghamshire, UK). The membrane was then probed with α-PY, and signal was detected by the hydrogen peroxide-enhanced chemiluminescence method as previously described (25). These cell lysates were also studied by Western blot analysis with α-MAP kinase and α-phosphoMAP kinase. For Western blot analysis with α-p70 S6 kinase, cells were incubated for 30 min with or without 100 nM insulin, solubilized in 56 lysis buffer and soluble cell lysates (25 µg) used.

Plasma membrane and low-density membrane fractions (25 µg each) were resolved by 10% SDS-PAGE, and Western blot analysis was done with α-GLUT-4, α-GLUT-1, α-p85 and α-p110.

RESULTS

Glucosamine decreased insulin stimulation of 2-DG uptake in a dose- and a time-dependent manner in 3T3-L1 adipocytes. To study whether glucosamine could induce insulin resistance in 3T3-L1 adipocytes, we preincubated cells with 0–50 mM glucosamine for 24 h. In control cells not treated with insulin, insulin stimulated 2-DG uptake four- to eightfold compared with basal 2-DG uptake. Glucosamine decreased insulin-stimulated glucose uptake in a dose-dependent manner with an effective dose for a 50% change (ED50) of 25–30 mM (Fig. 1A). Maximal inhibition was achieved at 50 mM glucosamine. Glucosamine did not affect basal glucose uptake.

We next studied a time course for glucosamine-induced insulin resistance. Cells were preincubated with 50 mM glucosamine for 0–24 h. After incubation of cells for 10 min with 100 nM insulin, 2-DG uptake was measured. Glucosamine decreased insulin-stimulated 2-DG uptake in a time-dependent manner, reaching half-maximal inhibition at 2–4 h (Fig. 1B). The maximal inhibition was achieved at 6 h and maintained up to 24 h. These data clearly suggest that glucosamine alone, in the absence of insulin in media, could induce complete insulin resistance in 3T3-L1 adipocytes. Glucosamine-induced insulin resistance was completely reversible by removal of glucosamine from cells (data not shown).

Glucosamine did not affect insulin stimulation of tyrosine phosphorylation of the insulin receptor and insulin receptor substrate-1 and -2 (IRS-1/2). When insulin binds to the insulin receptor, insulin receptor is tyrosine phosphorylated and subsequently tyrosine phosphorylated and subsequently tyrosine phosphorylated and subsequently tyrosine phosphorylated...
phosphorylates cellular substrates such as IRS-1/2 (6). To study whether glucosamine affects tyrosine phosphorylation of these proteins, we preincubated cells with 50 mM glucosamine for 0–24 h and then incubated them with 100 nM insulin for 5 min. Cells were next solubilized in 1% NP-40 lysis buffer and soluble cell lysates were analyzed by Western blotting with α-PY as described in EXPERIMENTAL PROCEDURES. At basal state, there was little, if any, tyrosine phosphorylation of the insulin receptor β-subunit (Fig. 2A) and IRS-1/2 (Fig. 2B). As expected, insulin increased tyrosine phosphorylation of these proteins (Fig. 2, A and B). Glucosamine treatment of cells for 6–24 h did not significantly affect tyrosine phosphorylation of these proteins. We have also measured insulin receptor tyrosine kinase activity toward the synthetic peptide poly(Glu:Tyr = 4:1) and detected no change in its activity after glucosamine treatment (Fig. 2C).

Glucosamine weakly, if at all, impaired insulin stimulation of PI 3-kinase activity. Tyrosine-phosphorylated IRS-1/2 binds to PI 3-kinase via the p85 regulatory subunit of PI 3-kinase and activates PI 3-kinase (6). PI 3-kinase has been strongly implicated in insulin-stimulated GLUT-4 translocation from an intracellular pool to the plasma membrane and subsequent increase in glucose transport (18). To study whether glucosamine affects insulin stimulation of PI 3-kinase activity, we preincubated cells with 50 mM glucosamine for 0–24 h and incubated them with 100 nM insulin for 5 min. The cells were solubilized and immunoprecipitated with α-PY. These α-PY immunoprecipitates were next assayed for PI 3-kinase activity in reaction mixture containing phosphatidylinositol and [γ-32P]ATP followed by thin-layer chromatography. Spots containing phosphatidylinositol-3-phosphate were excised and counted in a liquid scintillation β-counter as described in EXPERIMENTAL PROCEDURES.

At basal state, there was little PI 3-kinase activity immunoprecipitated with α-PY (i.e., little PI 3-kinase associated with tyrosine-phosphorylated proteins, namely IRS-1/2; Fig. 3). Insulin at 100 nM stimulated PI 3-kinase activity ~40-fold. When cells were preincubated with 50 mM glucosamine for 6 h, there was a small, if any, decrease in insulin-stimulated PI 3-kinase activity. It should be noted that at 6 h, there was complete inhibition of insulin-stimulated 2-DG uptake (Fig. 1B), suggesting that a small decrease in PI 3-kinase activity after 6 h of glucosamine treatment might not account for complete inhibition of 2-DG uptake. After 24 h, insulin-stimulated PI 3-kinase activity was decreased by 34% (Fig. 3).
We have also prepared α-IRS-1 immunoprecipitates, assayed for PI 3-kinase activity, and obtained similar results as with α-PY immunoprecipitates (data not shown). Glucosamine did not alter PI 3-kinase protein contents as assessed by Western blot analysis of total cell lysates with antibodies to the p110 catalytic subunit and p85 regulatory subunit (data not shown).

Glucosamine severely impaired insulin stimulation of Akt activity. Akt is a serine-threonine kinase, which is the cellular homologue of the viral oncogene v-Akt (3) and reported to be downstream of PI 3-kinase (5). Kohn et al. (23) first reported that expression of a constitutively active Akt construct in 3T3-L1 adipocytes induced glucose transport in the absence of insulin by GLUT-4 translocation to the plasma membrane. To study whether glucosamine affects insulin stimulation of Akt activity, we prepared α-Akt immunoprecipitates from the same cell lysates used for the PI 3-kinase study and assayed for Akt enzymatic activity in reaction mixture containing Crosstide and [γ-32P]ATP as described in EXPERIMENTAL PROCEDURES. Insulin at 100 nM stimulated Akt activity approximately fourfold. We obtained a similar fold stimulation of Akt activity when we used histone H2b as a substrate (data not shown). When cells were preincubated with 50 mM glucosamine for 3 and 6 h, insulin-stimulated Akt activity was decreased by ∼15 and ∼50%, respectively (Fig. 4). Glucosamine also decreased a basal Akt activity by 45%. These data were confirmed by Western blot analysis of cell lysates with antibodies to phosphorylated (thus active) Akt (data not shown). Under these experimental conditions, glucosamine did not alter Akt protein content as assessed by Western blot analysis of total cell lysates with α-Akt (data not shown).

To study correlation between inhibition of insulin stimulation of 2-DG uptake and that of Akt activity, we performed linear regression analysis on individual paired data (2-DG uptake vs. Akt activity). When these data were plotted against each other, there was a correlation between 2-DG uptake and Akt activity ($r^2 = 0.84$, $P < 0.05$). It should be noted, however, that 50 mM glucosamine (3 h) greatly inhibited 2-DG uptake with only a small decrease in Akt activity. In addition, there was still a significant insulin-stimulated Akt activity remaining after glucosamine treatment for 6–24 h. Employing control cells (i.e., no glucosamine treatment), we observed an excellent correlation between insulin stimulation of 2-DG uptake and that of Akt activity (data not shown).

Glucosamine impaired insulin stimulation of GLUT-4 translocation to the plasma membrane. To study whether glucosamine affects GLUT-4 translocation in 3T3-L1 adipocytes, we preincubated cells with 50 mM glucosamine for 0–24 h and incubated them with 100 nM insulin for 30 min. These cells were next subjected to subcellular fractionation to prepare plasma membrane and low-density membrane fractions as described in EXPERIMENTAL PROCEDURES. Each fraction (25 µg each) was resolved by 10% SDS-PAGE and subsequent Western blot analysis with α-GLUT-4. At basal state, most GLUT-4 was present in the low-density membranes and little GLUT-4 amount was detected in the plasma membrane (Fig. 5A). When cells were stimulated with insulin, there was an increase in GLUT-4 in the plasma membrane and a decrease in GLUT-4 in the low-density membranes as expected. Treatment of cells with glucosamine for 3 and 6 h inhibited insulin-stimulated increase in GLUT-4 content in the plasma membrane (Fig. 5A). Interestingly, glucosamine did not affect the ability of insulin to decrease GLUT-4 content in the low-density membranes. These data may be interpreted to mean that, in glucosamine-treated cells, GLUT-4 left the low-density membrane pool in response to insulin but did not reach the plasma membrane. When the total cell homogenates were tested for total GLUT-4 content, there was no change in total GLUT-4 amount after 3 and 6 h of glucosamine treatment compared with that in mannitol-treated control cells (data not shown).

PI 3-kinase has been previously demonstrated to move to the low-density membranes in response to insulin (15, 18). To study whether glucosamine affects insulin-stimulated movement of PI 3-kinase to the low-density membranes, we analyzed low-density membranes by Western blotting with α-p85. As expected, insulin treatment of cells increased p85 PI 3-kinase content in the low-density membranes (Fig. 5B). After 3–6 h of glucosamine treatment, there was little, if any, decrease in movement of p85 PI 3-kinase content by insulin in the low-density membranes. Western blot analysis with α-p110 PI 3-kinase yielded the same pattern as in Fig. 5B (data not shown). These data suggest that glucosamine impaired insulin-stimulated GLUT-4 translocation without affecting insulin-stimulated PI 3-kinase translocation to the low-density membranes. It should be noted, however, that these data cannot rule out the possibility that PI 3-kinase activa-

![Fig. 4. Effect of glucosamine on insulin-stimulated Akt activity in 3T3-L1 adipocytes. Cell lysates prepared as in Fig. 3 were immunoprecipitated with α-Akt and these α-Akt immunoprecipitates were assayed for Akt activity in reaction mixture containing Crosstide (0.33 mg/ml) and [γ-32P]ATP (20 µM, 5 µCi). Aliquots of reaction mixture containing phosphorylated Crosstide were spotted on P81 phosphocellulose paper followed by thorough washing in 150 mM H3PO4 as described in EXPERIMENTAL PROCEDURES. Radioactivity in P81 paper was counted in liquid scintillation β-counter. Data are presented as pmoles of ATP incorporated·mg cell lysates·min$^{-1}$ and are means ± SD of 2 independent measurements. A representative experiment is shown.](http://ajpendo.physiology.org/Downloadedfrom: http://ajpendo.physiology.org/Downloadedfrom)
tion by insulin in the low-density membranes was not impaired by glucosamine.

After 24 h of glucosamine treatment of cells, however, a different picture emerged. The total GLUT-4 content was significantly decreased (data not shown), and there was less of GLUT-4 in both plasma membrane and low-density membrane fractions (Fig. 5A). Interestingly, after 24 h there was a significant decrease in insulin-induced increase in p85 PI 3-kinase content in the low-density membranes (Fig. 5B). This decrease may or may not be associated with a large decrease in insulin-stimulated PI 3-kinase activity (~30%, Fig. 3). Taken together, there appears to be at least two mechanisms operative for glucosamine-induced insulin resistance in 3T3-L1 adipocytes. First, there is a development of impaired insulin stimulation of Akt activity and GLUT-4 translocation. Second, there is a subsequent development of impaired PI 3-kinase activity and a decrease in GLUT-4 content.

Next, we studied insulin stimulation of GLUT-1 translocation. Unlike GLUT-4, GLUT-1 is present in most cells and has been reported to play a role mainly in basal glucose transport (18). In 3T3-L1 adipocytes, we observed that insulin stimulated GLUT-1 translocation from the low-density membranes to the plasma membrane (Fig. 5C). In glucosamine-treated cells, this insulin function was not altered. Interestingly, after 24 h of glucosamine treatment, there was rather an increase in basal GLUT-1 content in the plasma membrane.

Glucosamine affected insulin stimulation of p70 S6 kinase but not that of MAP kinase. To study whether glucosamine could affect other functions of insulin, we studied insulin stimulation of MAP kinase and p70 S6 kinase. To study insulin stimulation of MAP kinase activity, we incubated cells for 5 min with 100 nM insulin and solubilized. These cell lysates were next assessed by Western blot analysis with α-MAP kinase and α-phosphoMAP kinase for total MAP kinase content and active MAP kinase content, respectively. PhosphoMAP kinase content was then normalized to MAP kinase protein content (Fig. 6A). These data demon-
For p70 S6 kinase activity, 32P-labeled S6 protein bands were excised from gels and counted in liquid scintillation counter. P70 S6 kinase activities were next normalized to the protein content of p70 S6 kinase as assessed by Western blot analysis with α-p70 S6 kinase (0.1 µg/ml) and α-phosphoMAP kinase (0.05 µg/ml) for total MAP kinase content and MAP kinase activity, respectively (A). Data are expressed as relative MAP kinase activities by calculating the ratio of phosphoMAP kinase to MAP kinase content scanned by a laser scanner. Insulin-stimulated MAP kinase in control cells (0 h GLN) was considered as 100%. Data are means ± SE of 4 separate experiments. For p70 S6 kinase assays, cells treated with glucosamine for 0, 6, and 24 h were incubated for 30 min with or without 100 nM insulin, solubilized and immunoprecipitated with α-p70 S6 kinase. These immunoprecipitates were next assayed in reaction mixture containing Artemia salina egg 40S ribosome as a substrate followed by SDS-PAGE and autoradiography (B). For p70 S6 kinase activity, 32P-labeled S6 protein bands were excised from gels and counted in liquid scintillation counter. P70 S6 kinase activities were next normalized to the protein content of p70 S6 kinase as assessed by Western blot analysis of soluble cell lysates with α-p70 S6 kinase (0.1 µg/ml). Data are units where 1 nmol of ATP incorporated·mg 40S ribosome·min and are means ± SD of 2 independent measurements. A representative experiment is shown.

**DISCUSSION**

In this study, we demonstrated that glucosamine could induce complete and reversible insulin resistance in 3T3-L1 adipocytes. Under our experimental conditions, ED50 for glucosamine-induced insulin resistance was 25–30 mM, which was higher than that reported in isolated cells treated with both insulin and glucosamine (27, 29). Insulin accelerates glucosamine transport into the cell via the glucose transport system and has been used to induce insulin resistance with glucosamine. However, chronic insulin treatment per se can induce insulin resistance, raise basal glucose transport, and render data analysis complex (34). Thus we attempted to induce insulin resistance in 3T3-L1 adipocytes without insulin in the media to unequivocally study mechanisms for glucosamine-induced insulin resistance. Although not studied in detail, Marshall et al. (27) could also induce insulin resistance with glucosamine without insulin in media in rat primary adipocytes.

Glucosamine treatment of cells (at 50 mM for 6 h) induced complete inhibition of insulin-stimulated glucose transport. Under these conditions, glucosamine did not affect immediate proximal effects of insulin including insulin receptor tyrosine autophosphorylation, IRS-1/2 tyrosine phosphorylation, and insulin receptor tyrosine kinase activity. Glucosamine weakly, if at all, inhibited insulin stimulation of PI 3-kinase activity but severely inhibited insulin stimulation of Akt activity. In glucosamine-induced insulin-resistant 3T3-L1 adipocytes, inhibition of insulin stimulation of Akt activity was correlated with inhibition of insulin stimulation of 2-DG uptake. It should be noted, however, that glucosamine (at 50 mM for 2–4 h) greatly inhibited 2-DG uptake with little inhibition of Akt activity, suggesting the presence of another more rapidly occurring mechanism operative in glucosamine-induced insulin resistance.

Krook et al. (24) reported that in glucosamine-infused rat skeletal muscle there was inhibition of Akt activity in response to maximal insulin concentration. Lately, Kurowski et al. (26) studied hyperglycemia-induced insulin resistance in rat skeletal muscle and reported similar findings to ours (i.e., impaired Akt activation without impaired PI 3-kinase activation by insulin). In these muscles, MAP kinase activation by insulin was not affected (26) as in the case of glucosamine-induced insulin-resistant 3T3-L1 adipocytes (Fig. 6A). Glucosamine-induced insulin resistance has been used as an experimental model for hyperglycemia-induced insulin resistance, both being reported to be mediated by a common mechanism, (i.e., hexosamine biosynthetic pathway) (30). In light of these reports, it is not surprising to obtain similar data on insulin
resistance induced by either glucosamine or hyperglycemia. Our study and the study of Kurowski et al. are consistent with the concept that Akt plays a role in insulin stimulation of glucose transport (8, 22, 23).

Insulin stimulates glucose transport by GLUT-4 translocation from the intracellular low-density membranes to the plasma membrane (18). In glucosamine-treated 3T3-L1 adipocytes, GLUT-4 translocation was impaired, resulting in a decrease in GLUT-4 content in the plasma membrane after acute insulin stimulation. Total GLUT-4 content was normal up to 6 h as assessed by Western blot analysis of total cell homogenates. Interestingly, in these glucosamine-treated cells, insulin could decrease GLUT-4 content from the low-density membranes. These data may be interpreted to mean that in glucosamine-treated 3T3-L1 adipocytes, GLUT-4 left the low-density membranes normally but might have been lost on its way to the plasma membrane. In search of this missing GLUT-4 pool, we studied high-density membranes but failed to detect GLUT-4 (data not shown). Further studies are required to identify the missing GLUT-4 pool in glucosamine-treated cells. After prolonged glucosamine treatment of cells (24 h), total GLUT-4 content was decreased. A decrease in GLUT-4 content may also account for insulin resistance in these cells after prolonged glucosamine treatment. These data are consistent with those of Chen et al. (7).

It should be noted that a fold increase in GLUT-4 content in the plasma membrane did not fully explain the multiples of increase in 2-DG uptake in response to insulin. In addition, there was no significant difference in GLUT-4 content in the plasma membrane after 3 vs. 6 h of glucosamine treatment although a different degree of insulin-stimulated 2-DG uptake was obtained at these times. These data suggest that GLUT-4 content in the plasma membrane did not directly correlate with 2-DG uptake and an additional step might be required for full stimulation of 2-DG uptake such as stimulation of intrinsic activity of GLUT-4 (18). It should also be noted that subcellular fractionation does not detect changes in fusion of GLUT-4 vesicles with the plasma membrane, which is a critical step for functional GLUT-4.

In glucosamine-induced insulin-resistant 3T3-L1 adipocytes, we identified a defect in insulin signaling (i.e., impaired Akt activation) and a defect in insulin stimulation of GLUT-4 translocation. Accumulating studies strongly support the idea that insulin stimulation of Akt may be directly linked to that of GLUT-4 translocation (and that of glucose transport) (8, 22, 23). The defect in insulin stimulation of GLUT-4 translocation could be either a consequence of or, alternatively, independent of the defect in insulin signaling. The crucial role of Akt in insulin-stimulated glucose uptake has been recently challenged by Kitamura et al. (21) who claimed no effect of dominant inhibitory Akt overexpression in glucose transport in 3T3-L1 adipocytes. This report is in direct contrast with that of Cong et al. (8) who reported that expression of dominant inhibitory Akt inhibited insulin-stimulated glucose transport in rat primary adipose cells. Growth hormone and hyperosmolality, which stimulate glucose transport, also stimulate Akt activity, suggesting the importance of Akt activity in glucose transport (31). The role of Akt in insulin action on glucose transport may be tested unequivocally with a specific inhibitor of Akt, which is not available at the present time.

In contrast to glucosamine effects on insulin stimulation of GLUT-4 translocation and glucose transport, glucosamine did not affect basal glucose transport. Unlike primary adipocytes, 3T3-L1 adipocytes contain more GLUT-1 (19) and insulin-stimulated GLUT-1 translocation from the low-density membranes to the plasma membrane. Glucosamine treatment of cells did not alter this insulin function. These data are consistent with the report that GLUT-1 does not play a major role in insulin stimulation of glucose transport (18). Also, in glucosamine-treated cells, insulin stimulation of MAP kinase was normal. Insulin stimulation of p70 S6 kinase was impaired, presumably as a result of defective Akt activation. These data are consistent with the concept that P70 S6 kinase, but not MAP kinase, is downstream of the PI 3-kinase/Akt pathway in 3T3-L1 adipocytes (22). Alternatively, glucosamine may directly affect the p70 S6 kinase pathway independent of Akt activation. Interestingly, after 24 h of glucosamine treatment of cells, p70 S6 kinase activity was increased at basal state and the GLUT-1 content in the plasma membrane was also increased at basal state. It is unclear whether these two are related. Recently, Somwar et al. (33) reported in L6 myotubes two different mechanisms for insulin stimulation of P70 S6 kinase: one dependent and the other independent of the PI 3-kinase/Akt pathway. In these L6 myotubes, PI 3-kinase/Akt-independent activation of P70 S6 kinase has been reported to be associated with GLUT-1 protein biosynthesis (33).

Molecular mechanisms underlying impaired Akt activation and impaired GLUT-4 movement are unclear at the present time. Because concentrations of UDP-N-acetylglucosamine and other nucleotide hexosamines increase in glucosamine-treated cells and these nucleotide hexosamines serve as substrates for glycosylation of proteins and lipids, it is conceivable that Akt, GLUT-4 and/or other proteins involved in insulin stimulation of GLUT-4 translocation might be affected by changes in their glycosylation. Hawkins et al. (14) reported an increase in N-acetylglucosamine content in GLUT-4 vesicles from skeletal muscle of glucosamine-infused rats. Yki-Jarvinen et al. (37) also reported that insulin and glucosamine infusion increases O-linked N-acetylglucosamine in skeletal muscle proteins in vivo. Currently, we are carrying out studies to determine which cellular proteins are glycosylated resulting in alteration of their functions after glucosamine treatment of cells.

Recently, Hresko et al. (19) reported that glucosamine-induced insulin resistance in 3T3-L1 adipocytes was caused by depletion of intracellular ATP concentration. In their study, cells were incubated with glucosamine in glucose-free media in the presence of 6 nM insulin. Within 1 h, glucosamine (20 mM) induced severe
insulin resistance, accompanied by an 80% decrease in intracellular ATP concentration. Under these conditions, all insulin functions were inhibited, including insulin receptor tyrosine autophosphorylation, IRS-1 tyrosine phosphorylation, PI 3-kinase activation, Akt activation, and translocation of both GLUT-1 and GLUT-4.

In our present study, we used different experimental conditions to induce insulin resistance, i.e., inclusion of 5 mM glucose and exclusion of insulin in all media. Under our experimental conditions (at 50 mM glucosamine for 6 h), we observed a modest (15–20%) decrease in cellular ATP content. To study whether this small decrease in ATP affects hexokinase and in turn inhibits 2-DG uptake, we investigated transport of 3-O-methyl-D-glucose that is not subject to phosphorylation by hexokinase and ATP. As in the case of 2-DG action, the 20 mM inosine that was used to replenish cellular ATP (to the extent that impairs insulin receptor function) is the major mechanism for glucosamine-induced insulin resistance in 3T3-L1 adipocytes.

In conclusion, glucosamine was able to induce complete and reversible insulin resistance in 3T3-L1 adipocytes. This insulin resistance was accompanied by impaired insulin stimulation of Akt activity and GLUT-4 translocation without significant alterations of the insulin receptor tyrosine kinase. In these cells, glucosamine did not significantly alter insulin stimulation of GLUT-1 translocation and MAP kinase activity, supporting selective inhibitory effects of glucosamine on certain insulin action.

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Address for reprint requests and other correspondence: C. K. Sung, Dept. of Physiology and Biophysics, School of Medicine, Univ. of Southern California, 1333 San Pablo St., MMR 626, Los Angeles, California 90033 (E-mail: csung@usc.edu).

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