Glucosamine-induced Insulin Resistance in 3T3-L1 Adipocytes Is Caused by Depletion of Intracellular ATP

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Glucosamine, which enters the hexosamine pathway downstream of the rate-limiting step, has been routinely used to mimic the insulin resistance caused by high glucose and insulin. We investigated the effect of glucosamine on insulin-stimulated glucose transport in 3T3-L1 adipocytes. The Δ-insulin (insulin-stimulated minus basal) value for 2-deoxyglucose uptake was dramatically inhibited with increasing concentrations of glucosamine with an EC50 of 1.95 mM. Subcellular fractionation experiments demonstrated that reduction in insulin-stimulated 2-deoxyglucose uptake by glucosamine was due to an inhibition of translocation of both Glut 1 and Glut 4 from the low density microsomes (LDM) to the plasma membrane. Analysis of the insulin signaling cascade revealed that glucosamine impaired insulin receptor autophosphorylation, insulin receptor substrate (IRS-1) phosphorylation, IRS-1-associated PI 3-kinase activity in the LDM, and AKT-1 activation by insulin. Measurement of intracellular ATP demonstrated that the effects of glucosamine were highly correlated with its ability to reduce ATP levels. Reduction of intracellular ATP using azide inhibited Glut 1 and Glut 4 translocation from the LDM to the plasma membrane, insulin receptor autophosphorylation, and IRS-1 tyrosine phosphorylation. Additionally, both the reduction in intracellular ATP and the effects on insulin action caused by glucosamine could be prevented by the addition of inosine, which served as an alternative energy source in the medium. We conclude that direct administration of glucosamine can rapidly lower cellular ATP levels and affect insulin action in fat cells by mechanisms independent of increased intracellular UDP-N-acetylhexosamines and that increased metabolism of glucose via the hexosamine pathway may not represent the mechanism of glucose toxicity in fat cells.

Insulin resistance is a major contributing factor in the pathogenesis of long term complications of non-insulin-dependent diabetes mellitus (1). Hyperglycemia is known to cause insulin resistance. High concentrations of glucose and insulin have been shown to impair insulin-stimulated glucose transport in primary rat adipocytes (2, 3) and rat hind limb muscles (4). Previous studies have suggested that an increased flux through the hexosamine biosynthetic pathway may be the mechanism by which hyperglycemia leads to insulin resistance (5, 6). Only 2–3% of the total glucose taken up by the cell is metabolized by this pathway that ultimately produces UDP-N-acetylglycosamine, which serves as a substrate in the formation of glycoproteins, glycolipids, and proteoglycans. Three lines of evidence led Marshall et al. (5) to conclude that hexosamine biosynthesis is involved in insulin resistance. First, glucose and insulin per se were not sufficient to establish insulin resistance of glucose transport in primary adipocytes, but the presence of glutamine was essential (7, 8). Second, desensitization could be prevented by inhibitors of the rate-limiting enzyme of the pathway, glutamine-fructose-6-phosphate aminotransferase (GFAT) (5). Third, the strongest evidence was that glucosamine, entering the pathway downstream of the rate-limiting step, was 40 times more effective than glucose in mediating desensitization of glucose transport (5). The effects of glucosamine were confirmed by many other studies, in vitro, including studies in primary rat adipocytes (9), 3T3-L1 adipocytes (10), and isolated skeletal muscle (11) and in vivo by measuring whole body glucose disposal after glucosamine clamping (12–14).

More direct evidence that the hexosamine pathway is involved in insulin resistance is through the overexpression of GFAT. Insulin sensitivity of glycogen synthase was decreased in fibroblasts stably transfected with GFAT (15–17). Glucose disposal was decreased by half (18) in transgenic mice overexpressing GFAT specifically in muscle and fat. In addition, mice that overexpress Glut 1 in muscle were insulin-resistant (19, 20) and had elevated GFAT expression compared with wild-type mice or insulin-sensitive Glut 4 overexpressors (21). Interestingly, however, a recent study that examined the effect of GFAT overexpression on insulin-stimulated translocation of Glut 4 by co-transfecting primary rat adipose cells with GFAT and an epitope-tagged Glut 4 transporter found that overexpression of GFAT had no effect on the insulin dose-response curve for Glut 4 translocation compared with control cells transfected with only tagged Glut 4 (9). The authors did observe an inhibition of Glut 4 translocation when adipose cells were treated with glucosamine.

Glucosamine has become routinely used to mimic the insulin resistance caused by high glucose and insulin. It is naturally assumed that higher intracellular concentrations of glucosa-
mine will have the same metabolic effect as increasing the flux through the hexosamine biosynthetic pathway. It is not clear, however, what cellular process is specifically affected by glucosamine to confer insulin resistance and whether it is by the same mechanism as high glucose and insulin or even the over-expression of GFAT. To address this question, we investigated the effects of glucosamine on insulin-stimulated glucose transport in 3T3-L1 adipocytes. Our results revealed that direct administration of glucosamine can rapidly lower intracellular ATP levels and affect insulin action in fat cells by mechanisms independent of increased intracellular UDP-N-acetylhexosamines.

**Experimental Procedures**

**Treatment of 3T3-L1 Adipocytes—**3T3-L1 fibroblasts were grown to confluence and 48 h later subjected to differentiation as described previously (22). 3T3-L1 adipocytes were used 10–14 days after differentiation. Cells treated with glucosamine were first washed three times with PBS and then incubated for 1 h at 37 °C in glucose-free DMEM supplemented with 1 mM sodium pyruvate, 6 mM insulin, and various concentrations of glucosamine (Sigma). L-Glucose was added to adjust the osmolality of the sugars to 250 mM. Following three washes with PBS, cells were incubated for 1.5 h in the same media but without insulin. At the end of this period, proteins were also pretreated with insulin in the same manner but were incubated in either normal DMEM (25 mM glucose) or in glucose-free DMEM supplemented with 25 mM L-glucose. Media containing more than 5 mM glucosamine were adjusted to pH 7.4.

Cells used in the sodium azide studies were fed with DMEM supplemented with 10% fetal bovine serum the day prior to the experiment. Fully differentiated 3T3-L1 adipocytes were washed three times with PBS and then incubated for 2.5 h at 37 °C in glucose-free DMEM containing 6 or 7 mM sodium azide.

**2-Deoxyglucose Uptake Measurements—**[3H]2-Deoxyglucose uptake was measured as described previously (22). After the 2.5-h preincubation described above, cells were washed three times with Krebs-Ringer phosphate and then treated or not treated for 20 min at 37 °C with 1 mM insulin. Non-transport-mediated uptake was measured in the presence of 20 mM cytochalasin B. Protein content was measured using the bicinchoninic acid method (Pierce).

**Subcellular Fractionation—**Three culture dishes (10 cm) of 3T3-L1 adipocytes were pretreated as described above, incubated for 20 min at 37 °C with or without 1 mM insulin, and then washed three times with ice-cold PBS containing 1 mM sodium vanadate. Cells were scraped into 12 ml of ice-cold HES buffer (255 mM sucrose, 20 mM HEPES, pH 7.4, and 1 mM EDTA) supplemented with 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and protease inhibitors and then homogenized by passing the cells 11 times through a Yamato LSC homogenizer at a speed of 1200 rotations/min at 4 °C, total membranes were prepared, and then scraped in 12 ml of ice-cold buffer (50 mM HEPES, pH 7.5, 1 mM EDTA) supplemented with protease inhibitors. Cells were homogenized by passing them 11 times through a Yamato LSC homogenizer at a speed of 1200 rotations/min at 4 °C, total membranes were prepared, and insulin receptors were semi-perfused with wheat germ agglutinin-Sepharose as described previously (25). L-Glucose was added or not added for 20 min to an aliquot of insulin receptor (10 μg of total protein in 0.1% Triton X-100). Receptors were autophosphorylated for 5 min at room temperature in 50 mM HEPES (pH 6.9), 0.1% Triton X-100, 5 mM manganous acetate, and 20 μM [γ-32P]ATP (25–50 cpm/nmol). RCM-lysozyme (Sigma), was added for 1 min at a final concentration of 100 μM before the reactions were quenched with the addition of EDTA (20 mM final) and analyzed by SDS-PAGE.

**ATP Analysis—**3T3-L1 adipocytes were pretreated as described above. Culture plates (3.5 cm) were washed four times with ice-cold PBS and then solubilized with 0.4 ml of ice-cold 0.05 N NaOH. The cell extracts were sheared using a 25-gauge syringe. 120 μl were heated to 80 °C for 20 min and then neutralized with 60 μl of 0.1 M Tris-HCl, pH 8.1, 0.02% bovine serum albumin, and 0.5 mM leupeptin, 1 μg/ml aprotinin, 100 mM glucosamine for 2.5 h in which the first hour was in the presence of 6 mM insulin and the remaining 1.5 h was without insulin. Cells were washed three times and then scraped in 12 ml of ice-cold buffer (50 mM HEPES, pH 7.5, 1 mM EDTA) supplemented with protease inhibitors. Cells were homogenized by passing them 11 times through a Yamato LSC homogenizer at a speed of 1200 rotations/min at 4 °C, total membranes were prepared, and insulin receptors were semi-perfused with wheat germ agglutinin-Sepharose as described previously (25). L-Glucose was added or not added for 20 min to an aliquot of insulin receptor (10 μg of total protein in 0.1% Triton X-100). Receptors were autophosphorylated for 5 min at 37 °C in glucose-free DMEM (pH 6.9), 0.1% Triton X-100, 5 mM manganous acetate, and 20 μM [γ-32P]ATP (25–50 cpm/nmol). RCM-lysozyme (Sigma), was added for 1 min at a final concentration of 100 μM before the reactions were quenched with the addition of EDTA (20 mM final) and analyzed by SDS-PAGE.

**RESULTS**

**Effect of Glucosamine on Glucose Transport in 3T3-L1 Adipocytes—**To determine the effects of increasing flux through the hexosamine pathway on glucose transport, 3T3-L1 adipocytes were incubated with increasing concentrations of glucosamine in glucose-free DMEM for 1 h in the presence of 6 mM insulin. The cells were then washed, incubated in the same media without insulin for 1.5 h, then acutely stimulated or not stimulated with 1 mM insulin prior to measuring 2-deoxyglucose uptake (Fig. 1A). Glucose-free media were used, since glucosamine competes poorly with glucose for transport into cells. The 1-h incubation with insulin was done to increase the number of glucose transporters on the plasma membrane and thus facilitate glucosamine uptake. Insulin was removed after 1 h to restore acute insulin sensitivity. In the absence of glucose and glucosamine under these experimental conditions, 2-deoxyglucose uptake increased 2.75-fold with acute insulin from 0.363 ± 0.03 to 1.02 ± 0.03 on a normalized scale. The rather low-fold increase in insulin-stimulated uptake is due to a glucose starvation effect. Incubation of 3T3-L1 adipocytes in glucose-free DMEM results in a higher basal and insulin-stimulated 2-DOG uptake, which is likely due largely by an increase in the number of Glut 1 transporters at the plasma membrane (27). When 2-deoxyglucose uptake experiments were performed under identical conditions as described above but in the presence of 25 mM D-
glucose and no glucosamine, uptake increased from 0.06 ± 0.008 to 0.77 ± 0.027, which is a 12.8-fold increase (data not shown).

If cells are treated with increasing concentrations of glucosamine (glucose-free DMEM), 2-DG uptake in the presence of insulin decreased dramatically (Fig. 1A). Basal uptake also decreased but at higher glucosamine concentrations (>5 mM). The change in 2-DG uptake with insulin (Δ-insulin) was plotted as a function of glucosamine concentration (Fig. 1B). The ED50 was 1.95 mM, which is between the values reported by Marshall et al. (5) for glucosamine treatment (5 h) of primary rat adipocytes (28) and 3T3-L1 adipocytes (23). Subcellular fractionation of 3T3-L1 adipocytes was carried out (see “Experimental Procedures”) after treating cells with D-glucose-free DMEM containing L-glucose alone or in the presence of 2, 5, and 20 mM glucosamine. The actual value of 2-DG uptake for insulin-stimulated glucose-free control cells was 794 ± 13.97 pmol of [3H]2-DOG/min/mg of cellular protein normalized to insulin-stimulated control cells (glucose-free DMEM, 0 mM glucosamine). The actual value of 2-DG uptake for insulin-stimulated glucose-free control cells was 794 ± 13.97 pmol of [3H]2-DOG/min/mg of protein. Data represent the mean ± S.E. of at least three independent experiments. S.E. values not shown are hidden by the symbols. B, Δ-insulin values (insulin-stimulated minus basal 2-DG uptake) were calculated for each experiment followed by mean and S.E. determination.

**Effect of Glucose Transport on PM Translocation**—Glut 1 and Glut 4 transporters have been shown to translocate to the plasma membrane (PM) with insulin from a low density microsomal fraction (LDM) in both primary adipose cells (28) and 3T3-L1 adipocytes (23). Subcellular fractionation of 3T3-L1 adipocytes was carried out (see “Experimental Procedures”) after treating cells with D-glucose-free DMEM containing L-glucose alone or in the presence of 2, 5, and 20 mM glucosamine. The amounts of Glut 1 (Fig. 2A) and Glut 4 (Fig. 2B) in the plasma membrane for both basal and insulin-treated cells were determined by Western blot analysis. For control cells incubated in L-glucose DMEM (no glucosamine), the amount of Glut 1 transporter at the PM tended to increase with insulin (from 0.7 to 1.0) although it was not statistically significant. If cells were incubated in DMEM containing 25 mM D-glucose, Glut 1 content in the PM increased from 0.47 to 1.08 with insulin (data not shown). The observed increase in Glut 1 at the PM in the basal state under glucose starvation conditions is consistent with previously published data (27) and is also in agreement with the 2-DG uptake results described above. Glut 4 increased in the PM approximately 2-fold (from ~0.5 to 1.0) with insulin in cells incubated with glucose-free DMEM containing either L-glucose (Fig. 2B) or D-glucose (data not shown). Glucosamine decreased insulin-stimulated translocation of both Glut 1 and Glut 4 to the plasma membrane. At 2 mM glucosamine, translocation of Glut 1 and Glut 4 with insulin was inhibited 80 and 70%, respectively, and translocation of both transporters was completely abolished at 20 mM glucosamine. The severity of the inhibition especially at 2 mM glucosamine was higher than one would have predicted based on the Δ-insulin uptake results (ED50 = 1.95 mM). The apparent inconsistency is probably due to the fact that the PM fractions were contaminated with other subcellular compartments that also contain each of the transporters, which masked the degree of insulin-activated translocation to the PM. Therefore, any translocation that we did observe in the presence of glucosamine was not statistically significant.

Insulin-stimulated depletion of Glut 1 and Glut 4 from the LDM is shown in Fig. 2, C and D, respectively. In control cells (t-glucose DMEM) the amount of Glut 1 and Glut 4 in the LDM decreased with insulin from 1.0 to 0.43 and from 1.0 to 0.67, respectively. Glucosamine inhibited insulin-induced translocation from the LDM for both Glut 1 and Glut 4 in a concentration-dependent manner. At 2 mM glucosamine, only Glut 1 was statistically depleted from the LDM with insulin treatment. Interestingly, the absolute amount of Glut 4 in the LDM under basal conditions was significantly higher with glucosamine treatment compared with the control (t-glucose), suggesting that glucosamine caused a redistribution of Glut 4 to the LDM under basal conditions. A similar glucosamine-induced redistribution of Glut 4 to the LDM in the basal state was observed in primary rat adipocytes (29).

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control cells (Fig. 3A). Glucosamine inhibited insulin-activated autophosphorylation dramatically in a concentration-dependent manner. Δ-Insulin autophosphorylation was reduced 52, 64, and 72% at 2, 5, and 20 mM glucosamine, respectively, compared with L-glucose controls. LDM fractions were then analyzed for tyrosine phosphorylation to examine the phosphorylation of IRS-1 by the insulin receptor. Insulin stimulated IRS-1 tyrosine phosphorylation approximately 12-fold in control cells (Fig. 3B). Glucosamine inhibited Δ-insulin tyrosine phosphorylation of IRS-1 to approximately 50% of that observed in L-glucose controls when cells were treated with 2, 5, or 20 mM glucosamine. On the whole, insulin receptor autophosphorylation was more sensitive to glucosamine treatment than IRS-1 tyrosine phosphorylation. This is consistent with the idea of a spare insulin receptor population.

The next step in the cascade, IRS-1-associated PI 3-kinase activity, was analyzed using LDM fractions from control and glucosamine-treated cells. LDM were solubilized with Nonidet P-40 and then immunoprecipitated with IRS-1 antibody. PI 3-kinase activity was measured in the immune complexes bound to protein A-Sepharose beads as described under “Experimental Procedures.” The IRS-1-associated PI 3-kinase activity in control cells increased during the first 5 min after insulin addition to 8.4-fold basal activity and then dropped to 5-fold (Fig. 4A). In glucosamine cells, the IRS-1-complexed PI 3-kinase activity was 30% of control after 2 min of insulin, 35% after 5 min of insulin, and 50% after 15 min of insulin.

AKT activation by insulin was analyzed from AKT-1 immunoblots of cytosol from control and glucosamine-treated cells. Insulin causes an increase in serine and threonine phosphorylation of AKT, which shifts the migration of the protein to a higher molecular mass on a SDS-polyacrylamide gel (37). This shift was observed using control LDM but was absent with LDM from glucosamine-treated cells (Fig. 4B).

The most likely interpretation of our signaling data is that glucosamine impaired insulin-stimulated translocation by inhibiting insulin receptor autophosphorylation, which would then progressively affect each subsequent step in the cascade. To determine whether glucosamine treatment modified the receptor directly, we performed in vitro phosphorylation reactions using semipurified insulin receptors isolated from glucosamine-treated and control cells. Cells were incubated in DMEM containing d-glucose, l-glucose, or 20 mM glucosamine for 2.5 h using our standard protocol. Insulin receptors were then semipurified from isolated membranes using wheat agglutinin-Sepharose. In vitro phosphorylation of RCM lysozyme (38), a synthetic substrate of the insulin receptor tyrosine kinase, was carried out with receptors that were first preactivated or not preactivated with 1 μM insulin for 20 min and then autophosphorylated for 5 min with [γ-32P]ATP. After 1 min of

FIG. 2. The effect of glucosamine on the translocation of Glut 1 (G1) and Glut 4 (G4) from the low density microsomes to the plasma membrane. 3T3-L1 adipocytes were incubated for 1 h in the presence of 6 nM insulin in glucose-free DMEM containing 0, 2, 5, 10, or 20 mM glucosamine, adjusted to equal osmolarity with l-glucose. Following three washes with PBS, cells were incubated in the same media without insulin for 1.5 h. Cells were then treated (hatched bars) or not treated (black bars) with 1 μM insulin for 20 min, washed with ice-cold buffer containing phosphatase and protease inhibitors, and then homogenized. Subcellular fractionation was carried out by differential centrifugation (see “Experimental Procedures”). PM and LDM fractions were separated on SDS-PAGE (50 μg of protein), immunoblotted, and incubated with antibodies raised against the carboxyl terminus of either Glut 1 or Glut 4. Signal intensities from 125I-labeled secondary antibodies were quantitated by PhosphorImager analysis. Data represent the mean ± S.E. of three independent experiments. #, relative abundance differed significantly (p < 0.05) in the stimulated state compared with the basal state. *, relative abundance in the basal glucosamine samples differed significantly compared with the basal control samples. An unpaired Student’s t test was used in all statistical analyses. A, amount of Glut 1 transporter at the PM plotted relative to insulin-stimulated control cells. B, amount of Glut 4 transporter at the PM plotted relative to insulin-stimulated control cells. C, amount of Glut 1 transporter in the LDM plotted relative to basal control cells. D, amount of Glut 4 transporter in the LDM plotted relative to basal control cells.
substrate phosphorylation, the reactions were quenched and analyzed by SDS-PAGE. The results, shown in Fig. 5, indicate that insulin stimulated autophosphorylation of the insulin receptor $\beta$-subunit approximately 2-fold and phosphorylation of RCM-lysozyme about 5-fold regardless of whether the receptors were prepared from control or glucosamine-treated cells. These results demonstrate that the intrinsic tyrosine kinase activity of the insulin receptor was not directly affected by glucosamine treatment.

**Effect of Glucosamine on Intracellular ATP Levels in 3T3-L1 Adipocytes**—Since the insulin receptor itself was not directly altered by glucosamine treatment, the observed decrease in in vivo insulin receptor autophosphorylation and substrate phosphorylation may be caused by a reduction in intracellular ATP levels by glucosamine. Glucosamine is taken up and is rapidly phosphorylated by hexokinase at the expense of ATP. Since glucose was absent from the media, only the exogenously added pyruvate or the endogeneous proteins, polysaccharides, or fat could be utilized to replenish ATP. Therefore, if ATP were used faster than it was made, ATP levels would fall. The effect of increasing concentrations of glucosamine on intracellular ATP levels is shown in Fig. 6A. The ATP concentration of cells incubated in glucose-free DMEM (5.17 mM $\pm$ 0.18) was normalized to 1.0. Interestingly, the ATP concentration in cells incubated in 25 mM $\nu$-glucose was slightly lower (4.86 $\pm$ 0.15 mM). Therefore, in the absence of glucosamine, glucose-starved cells can adequately maintain energy levels. However, intracellular ATP levels rapidly decreased with increasing concentrations of glucosamine (ED$_{50}$ = 1.3 mM). The decline in ATP values closely followed the decrease in $\Delta$-insulin 2-DOG uptake (Fig. 1B). This is more dramatically illustrated in Fig. 6B as a plot of relative ATP concentration versus $\Delta$-insulin 2-DOG uptake ($r = 0.934$). To rule out the possibility that glucosamine or glucosamine 6-phosphate in the cellular extracts interfered with the ATP analysis, we assayed extracts from $\nu$-glucose control cells or cells treated with either 2 or 20 mM glucosamine in which we added a known quantity of ATP. The amount of ATP we added to the extracts would have in theory increased the intracellular ATP concentration by 3.5 mM. The actual concentrations of additional ATP we measured in the control, 2 mM glucosamine-treated, and 20 mM glucosamine-treated extracts were 3.49 $\pm$ 0.05, 3.52 $\pm$ 0.02, and 3.32 $\pm$ 0.02 mM, respectively. Since we...
could, respectively, recover 99.7, 100.6, and 94.6% of the ATP we added to the extracts, we conclude that nothing in the glucosamine extracts interfered with the ATP assay.

**Effect of Sodium Azide on Glucose Transporter Translocation and Insulin Signaling**—It is possible that in our system glucosamine may inhibit insulin action in ways other than by lowering ATP concentrations and that the effect on ATP may not be the principal cause of the insulin resistance. To strengthen the correlation between decreases in intracellular ATP levels and effects on glucose transporter translocation and insulin signaling, 3T3-L1 adipocytes were treated with sodium azide for 2.5 h in order to lower ATP levels by an independent mechanism (inhibition of mitochondrial electron transport) and determine whether decreased energy levels per se could produce the same effects seen with glucosamine treatment. The ability of azide to reduce intracellular ATP levels is shown in Fig. 8C. 6 and 7 μM azide for 2.5 h decreased cellular ATP to 0.35 and 0.18, respectively, compared with control cells (L-glucose DMEM), which is equivalent to treating cells with approximately 3.5 and 20 mM glucosamine, respectively (see Fig. 6A). Insulin-stimulated translocation of both Glut 1 and Glut 4 to the plasma membrane was reduced with azide in a concentration-dependent manner as shown in Fig. 7, A and B, respectively. In the basal state, the amount of Glut 1 and Glut 4 on the cell surface was largely unaffected by azide except for a slight but not statistically significant reduction of Glut 4 with 7 mM azide. Similarly, translocation from the LDM with insulin of Glut 1 (Fig. 7C) and Glut 4 (Fig. 7D) was also decreased with increasing azide. Effects on insulin signaling are shown in Fig. 8. Insulin-stimulated tyrosine autophosphorylation of the insulin receptor at the plasma membrane was dramatically inhibited with increasing azide concentrations (Fig. 8A); however, tyrosine phosphorylation of IRS-1 in the LDM by insulin was reduced only at the highest azide concentration of 7 mM (Fig. 8B). This same differential reduction in tyrosine phosphorylation between the insulin receptor and IRS-1 was also observed with glucosamine treatment (Fig. 3).

**Inosine Prevents Glucosamine-induced Effects in 3T3-L1 Adipocytes**—Next we attempted to prevent the glucosamine-induced reduction in ATP levels by supplementing the media with an additional energy source to determine whether glucosamine can still affect insulin-stimulated glucose uptake and translocation without depleting ATP. Glucose was not used, since it competes very effectively for uptake with glucosamine, and therefore a negative result would be more difficult to interpret. Pyruvate and lactate (10 mM each) failed to prevent ATP declines with glucosamine (data not shown). Inosine has been shown to increase ATP concentrations in red blood cells (39), and at a concentration of 20 mM it did inhibit the reduction in ATP with low glucosamine concentrations (<2 mM) in 3T3-L1 adipocytes (Fig. 9A). Insosine is taken up by the cell and phosphorylated to IMP at the expense of one ATP. IMP is broken down to hypoxanthine and ribose 5-phosphate. Ribose 5-phosphate is converted to 3-phosphoglyceraldehyde, which then enters the glycolytic pathway to generate pyruvate and two molecules of ATP. Although highly inefficient, each inosine molecule taken up generates one ATP molecule. Therefore, it was not surprising that inosine had little effect on maintaining ATP levels at higher glucosamine concentrations (data not shown). The effect of inosine on preventing glucosamine-induced decreases in Δ-insulin 2-DOG uptake is shown in Fig. 9A. The progressive decrease in Δ-insulin uptake by low concentrations of glucosamine was eliminated with 20 mM inosine. The uptake results correlated quite well with ATP values (Fig. 9B). Glucosamine decreased intracellular ATP levels in a concentration-dependent manner, and 20 mM inosine prevented the ATP reduction.

Next we studied the effect of inosine on glucosamine-induced
inhibition of Glut 1 and Glut 4 translocation with insulin. 3T3-L1 adipocytes were treated for 2.5 h in glucose-free DMEM containing 0, 6, or 7 mM sodium azide. Cells were treated (hatched bars) or not treated (black bars) with 1 μM insulin for 20 min prior to subcellular fractionation (see "Experimental Procedures"). PM and LDM fractions were separated on SDS-PAGE (50 μg of protein), immunoblotted, and incubated with antibodies raised against the carboxyl terminus either of Glut 1 or Glut 4. Signal intensities from 125I-labeled secondary antibodies were quantitated by PhosphorImager analysis. Data represent the mean ± S.E. of three independent experiments. *, relative abundance in the insulin-stimulated azide samples differed significantly compared with the stimulated control samples. A, amount of Glut 1 transporter at the PM plotted relative to insulin-stimulated control cells. B, amount of Glut 4 transporter at the PM plotted relative to insulin-stimulated control cells. C, amount of Glut 1 transporter in the LDM plotted relative to basal control cells. D, amount of Glut 4 transporter in the LDM plotted relative to basal control cells.

Glucosamine and ATP Depletion
both autophosphorylation and substrate tyrosine phosphorylation was almost completely restored with inosine. ATP concentrations for the translocation and signaling experiments with inosine are shown in Fig. 11C. The 60% reduction in ATP by 2 mM glucosamine was largely but not completely eliminated with 20 mM inosine (80% of control), and this correlated well with the ability of inosine to increase but not fully restore transporter translocation and insulin signaling.

**Effect of Overnight Glucosamine Treatment Carried Out in the Presence of 5 mM d-Glucose on Cellular ATP Levels**—In several published glucosamine studies (9, 11), 5 mM d-glucose was added to the media to ensure cell viability, especially for longer glucosamine incubation times. Therefore we decided to examine the effects of glucosamine treatment under these conditions on intracellular ATP concentrations. In the first experiment, cells were treated with DMEM containing varying concentrations of glucosamine, 5 mM d-glucose, and 6 nM insulin for 1 h. The cells were subsequently washed three times with PBS and incubated for another 16 h in the same medium but without insulin, and then cellular ATP levels were determined.

**FIG. 9. Prevention of glucosamine-induced insulin resistance of 2-deoxyglucose uptake by inosine.** 3T3-L1 adipocytes were incubated for 1 h in the presence of 6 nM insulin in glucose-free DMEM containing 0, 0.5, 1, or 2 mM glucosamine, adjusted to equal osmolarity with L-glucose. In one set of culture plates nothing else was added (black bars), and in the other set 20 mM inosine was supplemented (hatched bars). Following three washes with PBS, cells were incubated in the same media without insulin for 1.5 h. A, ([3H]2-deoxyglucose uptake was measured for 6 min under basal or acute insulin stimulation (20 min at 37 °C) with 1 μM insulin. Uptake was quantitated as pmol of [3H]2-deoxyglucose/min/mg of cellular protein. Δ-insulin values were calculated and normalized to control cells (0 mM glucosamine with or without inosine). Data represent the mean ± S.E. of at least three independent experiments. *, relative Δ-insulin 2-DOG uptake in the glucosamine cells differed significantly (p < 0.05) from that in the control cells. B, ATP concentrations were determined as described (see “Experimental Procedures”) and normalized to control values (0 mM glucosamine with or without inosine). Data represent the mean ± S.E. of at least three independent experiments. *, relative ATP concentrations in the glucosamine cells differed significantly (p < 0.05) from that in the control cells.

**FIG. 10. Prevention of glucosamine-induced inhibition of Glut 1 (G1) and Glut 4 (G4) translocation by inosine.** 3T3-L1 adipocytes were incubated for 1 h in the presence of 6 nM insulin in glucose-free DMEM containing nothing (C), 2 mM glucosamine (GLN), or 2 mM glucosamine supplemented with 20 mM inosine (GLN + INOSINE). Following three washes with PBS, cells were incubated in the same media without insulin for 1.5 h. Cells were then treated (hatched bars) or not treated (black bars) with 1 μM insulin for 20 min prior to subcellular fractionation (see “Experimental Procedures”). Data represent the mean ± S.E. of three independent experiments. #, relative abundance differed significantly (p < 0.05) in the stimulated state compared with the basal state. *, relative abundance in the basal glucosamine samples differed significantly compared with the basal control samples. A, amount of Glut 1 transporter at the PM plotted relative to insulin-stimulated control cells. B, amount of Glut 4 transporter at the PM plotted relative to insulin-stimulated control cells. C, amount of Glut 1 transporter in the low density microsomes plotted relative to basal control cells. D, amount of Glut 4 transporter in the low density microsomes plotted relative to basal control cells.
The results shown in Fig. 12A indicated that 5 mM d-glucose protected the cells completely from ATP depletion only at very low glucosamine concentrations (0.5–1 mM glucosamine). At 2 mM glucosamine and higher, ATP levels decreased in a glucosamine concentration-dependent manner with an ED50 of 6.08 mM. This ED50 was considerably higher than the value we observed in the absence of d-glucose (ED50 = 1.3 mM), and the absolute cellular ATP levels at all glucosamine concentrations tested were also higher when the media contained 5 mM d-glucose (Fig. 12A) compared with the levels we observed originally in the absence of d-glucose (see Fig. 6A for comparison).

In the second experiment, cells were treated with DMEM containing varying concentrations of glucosamine, 5 mM d-glucose, and 1 mM insulin for 16 h prior to the ATP measurements. Insulin supplementation is often used in many glucosamine experiments, since it has been reported that insulin is a necessary cofactor for glucosamine-induced insulin resistance (8–10). Interestingly, 5 mM d-glucose failed to protect intracellular ATP levels even at 0.5 mM glucosamine (Fig. 12B). Intracellular ATP decreased in the presence of 0–2 mM glucosamine in an almost linear fashion and then reached a lower limit of 0.4 relative to control cells (0 mM glucosamine). The same lower limit was observed in the first experiment shown in Fig. 12A. These results indicate that overnight insulin treatment shifted the glucosamine dose-response curve to the left from an ED50 of 6.08 mM to an ED50 of 0.95 mM, thus making 3T3-L1 adipocytes more sensitive to glucosamine with regard to ATP depletion. As a control, cells were treated for 16 h with DMEM containing 25 mM d-glucose and 1 mM insulin, and we observed no change in ATP levels when compared with cells treated for 16 h with 5 mM d-glucose, 0 mM glucosamine, and 1 mM insulin (data not shown).

**DISCUSSION**

High glucose and insulin are known to cause insulin resistance in the two target tissues of insulin, skeletal muscle and fat, that are primarily responsible for glucose disposal (2, 4). The underlying mechanism responsible for the defect is not well understood, but recent studies have suggested that the hexosamine pathway may play an important role (6). Glucosamine, which enters the hexosamine pathway downstream from the rate-limiting step, has been routinely used to study insulin resistance, and it is much more effective in eliciting insulin desensitization than high glucose. In this study, the effects of glucosamine on insulin-stimulated glucose transport were investigated in 3T3-L1 adipocytes, one of the best characterized cell lines used to study insulin action. Δ-insulin of 2-deoxyglucose uptake was inhibited by glucosamine in a dose-dependent manner with an ED50 that was similar to that reported by Marshall et al. (5) for primary rat adipocytes. Our subcellular fractionation results indicate that glucosamine inhibited insulin-stimulated glucose uptake in 3T3-L1 adipocytes by blocking Glut 4 and Glut 1 translocation from the LDM to the PM. Effects on Glut 4 translocation by glucosamine have been previously observed in vitro in primary rat adipocytes (9) and in vivo in skeletal muscle (14). Our analysis of the signaling cascade showed that glucosamine impaired insulin receptor autophosphorylation, IRS-1 phosphorylation, PI 3-kinase activity, and AKT activation by insulin. In vitro phosphorylation experiments using semipurified insulin receptor isolated from glucosamine-treated cells revealed that the receptor itself was not directly affected. In one set of insulin receptor preparations, we included tyrosine and serine phosphatase inhibitors to preserve the in vivo phosphorylation state of the receptor. In vitro phosphorylation results were the same for receptors purified from control or glucosamine-treated cells but activated in vitro with insulin (data not shown). Therefore, reductions in autophosphorylation and IRS-1 phosphorylation observed in vivo could not be due to a glucosamine-induced increase in serine phosphorylation of the insulin receptor itself. We cannot rule out, however, that glucosamine may modulate receptor activity indirectly through another protein that is lost in the receptor isolation. These results are in apparent disagreement with the only published report on the effects of glucosamine per se on early insulin signaling. Robinson et al. (11) found no effect of glucosamine on in vitro substrate phosphorylation of the synthetic substrate polyglutyr using semipurified insulin receptor isolated from muscle stimulated in vivo with acute insulin. However, insulin-stimulated tyrosine phosphorylation of the insulin receptor, IRS-1 tyrosine phosphorylation, or any downstream signaling step in the translocation cascade were not directly investigated, nor were intracellular ATP concentrations reported. One simple explanation for the discrepancy is that glucosamine affects muscle and fat differently. Another possibility is that glucosamine does affect early steps in insulin signaling in muscle such as IRS phosphorylation, PI 3-kinase activity, or AKT activation and that phosphorylation of the
synthetic peptide substrate polyglutyr is not an appropriate assay. Insulin receptors phosphorylate IRS-1 on multiple tyrosine residues (40). Failure to phosphorylate a few residues may not result in a significant reduction in the overall tyrosine phosphorylation but may have a dramatic effect on the signaling cascade.

Based upon the high correlation between decreases in ATP levels and inhibition of insulin-stimulated 2-DG uptake, the azide experiments, and the prevention of glucosamine-induced insulin resistance by inosine, our data strongly indicate that any desensitization of the insulin-stimulated glucose transport effector system in 3T3-L1 adipocytes by glucosamine treatment is due entirely to effects on ATP and not to increases in intracellular UDP-N-acetylglucosamine levels. What was most surprising was how tight the correlation was between declines in ATP by glucosamine and induction of insulin resistance. Even what may be considered minor reductions in ATP (10–20%) resulted in insulin desensitization. Incubation of cells with high glucose and insulin overnight, however, had no effect on ATP concentrations. We suspect that the decrease in ATP by glucosamine is a result of rapid phosphorylation by hexokinase of newly transported glucosamine. ATP is used but not quickly replenished, since the cells are incubated in glucose-free media. This phenomenon is quite similar to that of a recent study reported by Wang and Iynedjian (41) in which they overexpressed glucokinase 20-fold in insulinoma cells. The addition of D-glucose above 6 mM resulted in a large accumulation of glucose 6-phosphate followed by a rapid decrease in cellular ATP and eventual cell death. To the best of our knowledge, our report is the first glucosamine study to report intracellular ATP levels. It should also be considered that a 20% decline in ATP levels measured from whole cell extracts may represent a much more significant drop locally where the various phosphorylation reactions occur. Since almost every step in the signaling cascade involves ATP utilization, small effects early on will be amplified down the cascade. This was apparent in our results, where IRS-1 phosphorylation was reduced 50% but later events such as AKT activation and Glut 4 translocation were completely inhibited. Even treating 3T3-L1 adipocytes overnight with 0.5 mM glucosamine, 5 mM D-glucose, and insulin resulted in significant ATP depletions. Almost every previously published glucosamine study could be reinterpreted in terms of ATP depletion by us it was specifically ruled out. For example, a recent study by Chen et al. (9) found that incubation of primary fat cells with 2.5 mM glucosamine and 5 mM insulin for 4 h decreased 2-DG transport by 50% but had no effect on Glut 4 translocation. At longer glucosamine incubation times (16 h), Glut 4 translocation was significantly reduced. The authors speculate that glucosamine may affect the intrinsic activity of the transporter at shorter incubation times. An alternative explanation is that glucosamine causes a reduction in cellular ATP, which could be initially localized near the site of phosphorylation by hexokinase and then propagated with time throughout the entire cell. Therefore, at short incubation times with glucosamine, local reductions in ATP could cause the hexokinase reaction to become rate-limiting for transport and reduce 2-DG uptake but have no effect on Glut 4 translocation. Glut 4 translocation could be inhibited at longer glucosamine incubation times when ATP levels were decreased in other regions of the cell. Insulin resistance has also been induced by in vivo infusion of glucosamine into rats. Glucosamine infusions were carried out for 7 h with 7 mM D-glucose, 1.2 mM glucosamine, and 2.5 mM insulin (12, 13). Uridine administration also induced insulin resistance in these animals, and its effects were additive with glucosamine. The authors concluded that reductions in insulin action by glucosamine and uridine were mediated by the production of UDP-N-acetylhexosamines. Since ATP levels were not reported, an alternative explanation is that both glucosamine and uridine could decrease intracellular ATP levels and cause the insulin resistance. We observed significant declines in ATP when 3T3-L1 adipocytes were treated under similar glucosamine conditions (1 mM glucosamine, 5 mM D-glucose, and insulin), and although we never tested uridine directly, three high energy phosphates are needed to generate a UDP-sugar from uridine. A recent study by Miles et al. (42) reported that troglitazone treatment can reverse hyperglycemia-induced insulin resistance but not glucosamine-induced insulin resistance in rats. These authors raised the possibility that glucose may cause insulin resistance by a mechanism independent of its entry into the hexosamine pathway. The strongest evidence offered by Marshall et al. (5) that increased flux through the hexosamine pathway is responsible for glucose-induced insulin resistance was the observation that glucosamine is 40 times more potent at inhibiting insulin-stimulated glucose transport in fat cells than glucose. Our data suggest that glucosamine may have acted by a different mechanism than high glucose altogether, i.e., ATP depletion, and thus call into question the role of increased flux through GFAT in the induction of insulin resistance.

Overexpression of GFAT, the enzyme catalyzing the rate-limiting step in hexosamine biosynthesis, may be a more relevant approach to investigate hexosamine involvement in insulin resistance if the effects induced by glucosamine are found to be routinely correlated to ATP depletion. Interestingly, however, when Chen et al. (9) overexpressed GFAT in primary fat cells, they found it had little effect and therefore they used glucosamine instead. On the other hand, GFAT overexpression in fibroblasts by stable transfection does decrease insulin sensitivity of glycogen synthase (15–17). In addition, the transgenic study of Hebert et al. (18) showed that overexpression of
GFAT in muscle and fat did reduce glucose disposal. Similarly, transgenic mice that overexpress Glut 1 in muscle are insulin-resistant and have normal ATP levels but have increased GFAT activity and UDP hexosamine levels (19–21).

Our results may have some profound mechanistic implications for the pathogenesis of insulin resistance in human disease states such as type 2 diabetes mellitus. If our results with 3T3-L1 adipocytes are representative of insulin-sensitive tissues 

in vivo, such as liver, fat, and muscle, then relatively small reductions in intracellular ATP may translate into significant decreases in insulin responsiveness. The implication is that any cellular defect that results in decreased steady-state levels of ATP may induce a state of insulin resistance.

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