Sustained hyperglycemia induces insulin resistance, but the mechanism is still incompletely understood. Glucosamine (GlcN) has been extensively used to model the role of the hexosamine synthesis pathway (HSP) in glucose-induced insulin resistance. 3T3-L1 adipocytes were preincubated for 18 h in media ± 0.6 nmol/l insulin containing either low glucose (5 mmol/l), low glucose plus GlcN (0.3–2.5 mmol/l), or high glucose (25 mmol/l). Basal and acute insulin-stimulated (100 nmol/l) glucose transport was measured after re-equilibration in serum and insulin-free media. Preincubation with high glucose or GlcN (1–2.5 mmol/l) inhibited basal and acute insulin-stimulated glucose transport only if insulin was present during preincubation. However, only preincubation with GlcN plus insulin inhibited insulin-stimulated GLUT4 translocation. GLUT4 and GLUT1 protein expression were not affected. GlcN (2.5 mmol/l) increased cellular UDP-N-acetylglycosamines (UDP-HexNAc) by 400 and 900% without or with insulin, respectively. High glucose plus insulin increased UDP-HexNAc by 30%. GlcN depleted UDP-hexoses, whereas high glucose plus insulin increased them. Preincubation with 0.5 mmol/l GlcN plus insulin maximally increased UDP-HexNAc without affecting insulin-stimulated or basal glucose transport. GlcN plus insulin (but not high glucose plus insulin) caused marked GlcN dose-dependent accumulation of GlcN-6-phosphate, which correlated with insulin resistance of glucose transport (r = 0.935). GlcN plus insulin (but not high glucose plus insulin) decreased ATP (10–30%) and UDP (>50%). GDP was not measured, but GDP increased. Neither high glucose plus insulin nor GlcN plus insulin prevented acute insulin stimulation (~20-fold) of insulin receptor substrate 1–associated phosphatidylinositol (PI)-3 kinase. We have come to the following conclusions. 1) Chronic exposure to high glucose or GlcN in the presence of low insulin caused insulin resistance of glucose transport by different mechanisms. 2) GlcN inhibited GLUT4 translocation, whereas high glucose impaired GLUT4 “intrinsic activity” or membrane intercalation. 3) Both agents may act distally to PI-3 kinase. 4) GlcN has metabolic effects not shared by high glucose. GlcN may not model HSP appropriately, at least in 3T3-L1 adipocytes. Diabetes 49:981–991, 2000

Sustained hyperglycemia impairs insulin-stimulated glucose utilization by peripheral tissues (i.e., muscle and fat) in animal models and humans and decreases the ability of pancreatic β-cells to respond to hyperglycemia with acute insulin release. These observations gave rise to the “glucose toxicity” hypothesis, which is thought to account for the insulin resistance associated with uncontrolled type 1 diabetes and to contribute to insulin resistance in type 2 diabetes (1,2). Glucose transport is the rate-limiting step for glucose utilization by skeletal muscle and adipocytes under most conditions (1,3,4). In isolated rat adipocytes, chronic exposure to high glucose in the presence of insulin downregulates subsequent basal and acutely insulin-stimulated glucose transport; the effects of glucose and insulin during pre-exposure are synergistic (5) and appear to be associated with a post–insulin receptor defect (6).

Although the concept of glucose-induced insulin resistance is well documented, the underlying mechanisms are still not well understood. Marshall et al. (7) proposed that glucose-induced desensitization of glucose transport may be mediated by products of the hexosamine biosynthesis pathway (HSP). Glutamine:fructose-6-phosphate amidotransferase (GFAT) regulates the entry of glucose into this pathway by catalyzing the conversion of fructose-6-phosphate and glutamine to glucosamine-6-phosphate (GlcN-6-P) and glutamate. The major products of this pathway are UDP-N-acetylglucosamines (UDP-HexNAc), i.e., UDP-N-acetylgalactosamine and UDP-N-acetylfromalactosamine in an ~3:1 ratio (7). UDP-HexNAc are obligatory precursors for the synthesis of glycosyl side chains of proteins and lipids. Glucosamine (GlcN) enters cells on the same carrier as glucose, although its affinity for glucose transporters is less than that of glucose. On entry, GlcN is rapidly phosphorylated to GlcN-6-P. GlcN has been widely used as a model to assess the role of HSP products in insulin resistance (7–14). Because glucose-induced desensi-

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2-DG, 2-deoxy-D-glucose; 2-DG-6-P, 2-deoxy-D-glucose-6-phosphate; 3-OMG, 3-O-methyl-D-glucose; ANOVA, analysis of variance; BSA, bovine serum albumin; DMEM, Dulbecco’s minimal essential medium; FBS, fetal bovine serum; FITC-PE, fluorescein isothiocyanate–labeled phosphatidyethanolamine; GCV, GLUT4-containing vesicles; GFAT, glutamine:fructose-6-phosphate amidotransferase; GlcN, glucosamine; GlcN-6-P, glucosamine-6-phosphate; HPLC, high-pressure liquid chromatography; HSP, hexosamine biosynthetic pathway; IRS, insulin receptor substrate; KRBH, Krebs-Ringer bicarbonate/HEPES buffer; LDM, low-density microsomal; PBS, phosphate-buffered saline; PCA, perchloric acid; PI, phosphatidylinositol; PKB, protein kinase B; UDP-Hex, UDP-hexoses; UDP-HexNAc, UDP-N-acetylglycosamines.
GLUCOSE-INDUCED INSULIN RESISTANCE

Materials. Site-specific polyclonal rabbit antibodies against GLUT4 and GLUT1, respectively, were gifts from Dr. Mike Mueckler (Washington University, St. Louis, MO). A polyclonal antibody generated against the 14 COOH-terminal amino acids of rat liver insulin receptor substrate (IRS)-1 was purchased from Upstate Biotech (Lake Placid, NY). Horseradish peroxidase-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-rabbit IgG were gifts from Dr. Mike Mueckler (Washington University, St. Louis, MO). 3T3-L1 adipocytes were isolated from wild-type C57/BL6 mice by collagenase digestion, grown on 35-mm culture dishes, as described by Farese and Lane (19). Cells were grown to confluence in Dulbecco’s minimal essential medium (DMEM) containing 25 mmol/l glucose and 10% calf serum at 37°C in a humidified atmosphere containing 5% CO2. Two days after confluence, cells were placed in DMEM containing 25 mmol/l glucose, 0.5 mmol/l isobutylmethylxanthine, 10 µmol/l insulin, and 10% FBS for 3 days and then for 2 days in DMEM containing 25 mmol/l glucose, 10 µmol/l insulin, and 10% FBS. Thereafter, cells were maintained in and refed every 2 days with DMEM, 25 mmol/l glucose, and 10% FBS until used in experiments 10-14 days after the start of treatment, when between 90 and 95% of the cells exhibited adipocyte phenotypes.

In typical experiments, 3T3-L1 adipocytes were preincubated for 18 h at 37°C with DMEM (1% FBS) containing 5 mmol/l glucose with or without 0.6 mmol/l insulin, 5 mmol/l glucose plus 2.5 mmol/l GlcN with or without 0.6 mmol/l insulin, or 25 mmol/l glucose with or without 0.6 mmol/l insulin. The insulin concentration in the media was determined by radioimmunoassay at the start of preincubation. There was 1 mmol/l insulin added to the media, but ~40% was lost during filter sterilization and by adsorption to plastic ware.

After the end of the preincubation, insulin concentrations had decreased to ~0.3 nmol/l, reflecting insulin degradation by the cells (20).

Media glucose concentration decreased minimally when cells were preincubated in either medium in the absence of insulin. When insulin was included in the media, glucose concentrations decreased significantly by 86% (low glucose, 5.17 ± 0.12 vs. 0.72 ± 0.02, P < 0.001), 85% (low glucose plus GlcN, 5.62 ± 0.07 vs. 0.845 ± 0.07, P < 0.005), and 43% (high glucose, 26.31 ± 0.99 vs. 14.98 ± 1.12, P < 0.001) after 18 h.

Before preincubation, adipocytes were washed 3 times with phosphate-buffered saline (PBS) containing 0.1% BSA at 37°C and incubated for 2 h in serum- and insulin-free DMEM containing the same sugar concentrations that were added to the preincubation media with 0.5% BSA and 25 mmol/l HEPES at 37°C in a 5% CO2 atmosphere. Cells were then rapidly washed 3 times and equilibrated for 10 min at 37°C with 0.1% BSA in Krebs-Ringer bicarbonate/HEPES buffer (KRBH), pH 7.4 (KRBH = 25 mmol/l HEPES, 120 mmol/l NaCl, 4.6 mmol/l KCl, 1.9 mmol/l CaCl2, 1 mmol/l MgSO4, and 1.2 mmol/l KH2PO4).

Glucose transport. Cells prepared as described above were incubated in glucose-free KRBH at 37°C with or without an acute insulin dose (100 nmol/l) for 15 min. Glucose transport was initiated by the addition of 2-deoxy-D-glucose (2-DOG) (0.05 mmol/l and 0.5 µCi/ml). [3H]Glucose (0.05 mmol/l and 0.05 µCi/ml) was also added as an extracellular space marker. After 5 min at 37°C, 2-DOG transport was terminated by the addition of phlorizin (48 µmol/l). The cells were immediately placed on ice and washed 3 times with ice-cold PBS and sonicated for 10 min at 3°C with 1% Triton X-100 in 1% TCA. Triton X-100 and 1% TCA concentrations were determined by liquid scintillation spectrometry. The intracellular concentration of 2-DOG was calculated by correcting for the label present in the extracellular space and normalized to the protein concentration in the extract.

In additional experiments, 3-O-methyl-D-glucose (3-OMG) transport was measured as described by Sweeney et al. (21). Briefly, cells were washed and equilibrated for 10 min at 37°C with HEPES-buffered saline (140 mmol/l NaCl, 20 mmol/l HEPES, pH 7.4, 2.5 mmol/l MgSO4, 1 mmol/l CaCl2, and 5 mmol/l KCl) and then acutely stimulated with or without insulin (100 nmol/l) for 15 min. The buffer was aspirated, and cells were exposed for 30 s to HEPES-buffered saline containing 50 µmol/l 3-O-[14C]methylglucose (4 µCi/ml) and 50 µmol/l [14C]glucose (0.4 µCi/ml). The cells were then immediately placed on ice, washed 3 times with 1 mmol/l HgCl2 in saline, and lysed with 0.05% NaOH, and ¹⁴C and ³²P concentrations were determined by liquid scintillation spectrometry.

Glucose transporter expression. For GLUT4 detection by Western blot, 30 µg total cell protein from the lysates prepared during the 2-DOG transport assay was separated by Laemmli’s SDS-PAGE. To detect GLUT1, in separate experiments, total membrane fractions were prepared by homogenizing cells in buffer containing 0.25 mol/l sucrose, 10 mmol/l Tris, pH 7.4, 2 mmol/l EDTA, and protease inhibitors. The homogenate was centrifuged for 5 min at 1,000 rpm in a tabletop centriuge, and the pellet was removed. The acidified supernatant was then centrifuged at 4°C for 2 h at 200,000 x g. The resulting pellet was resuspended in the same buffer supplemented with 1% Triton X-100. After 30 min at 4°C, the suspension was centrifuged for 10 min at 10,000 x g, and 10 µg protein from the supernatant was analyzed by Laemmli’s SDS-PAGE. After separation, proteins were transferred to nitrocellulose membranes, blocked with 5% dry nonfat milk in Tris-buffered saline for 1 h, and incubated overnight with rabbit-a-GLUT4 (1:500) or rabbit-a-GLUT1 (1:500) IgG, detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) and enhanced chemiluminescence, and scanned by laser densitometry as previously described (18).

Bands were quantitated using NIH Image 1.61 software (www.nih.gov).

GLUT4 translocation. Cells prepared as above were stimulated with or without insulin (100 nmol/l) for 15 min. Dish-adherent plasma membrane fractions were prepared by modification of published protocols (22). Briefly, cells were placed on ice and washed twice in ice-cold buffer A (50 mmol/l HEPES, pH 7.2, and 100 mmol/l NaCl) and once in ice-cold buffer B (20 mmol/l HEPES, pH 7.2, 100 mmol/l KCl, 2 mmol/l CaCl2, 1 mmol/l MgCl2, 2 µg/ml trypsin inhibitor, 10 µmol/l leupeptin, and 0.5 mmol/l benzamidine). The cells were then ruptured by mechanical shearing with a controlled stream of buffer B, washed twice with buffer B, and fixed for 15 min with 3.7% paraformaldehyde/PBS. Membrane sheets were then washed 3 times with PBS and stained with 10 µg/ml FITC-PE for 15 min, washed 3 times with PBS, blocked for 10 min with 0.2% crystalline BSA/PBS, and after 3 more PBS washes, immunostained with rabbit-a-GLUT4 IgG (1:100 in 0.2% crystalline BSA/PBS) for 30 min. After 3 PBS washes and blocking, plates were incubated for 30 min with rhodamine-conjugated goat anti-rabbit IgG (1:400), and after further washing, fixed for 30 min with rhodamine-conjugated rabbit anti-goat IgG. After 3 washes with PBS, plates were post-fixed with 3.7% formaldehyde and coverslipped.

To quantitate plasma membrane-associated GLUT4, plates were coded before immunostaining to blind the investigator to the treatment conditions and to prevent bias during quantitation and image acquisition. Three digital images per plate were acquired using a Zeiss Axioplan microscope (Zeiss, Jena, Germany) equipped with a DAGE 100 integrating charge-coupled device camera and NIH Image software. Fields were picked based solely on their FITC-PE labeling (green fluorescence) with no data on their corresponding red fluorescence (rhodamine labeling). Eight membrane sheets per image were quantitated as mean pixel brightness using NIH Image 1.61 software. Three measurements of background mean pixel brightness were taken in areas lacking membrane remnants and subtracted. In some experiments, plates were stained only with the rhodamine-conjugated antibodies and not incubated with a-GLUT4 to quantitate nonspecific binding of the secondary and tertiary antibodies. This value typically represented 8-10% of the total mean pixel brightness and was subtracted from each data point. As an additional control for the specificity of immunostaining, some plates were initially immunostained with rabbit-a-GLUT4 IgG in the presence of the 15-mer COOH-terminal peptide (10 µg/ml) to which the antibody was generated (23).

Analysis of nucleotide-linked hexoses and hexosamines. This procedure and its validation have been described in detail (15-17). After 18 h of preincubation in conditions described in CELL CULTURE AND GENERAL METHODS, 35-mm plates of 3T3-L1 adipocytes were washed once with ice-cold PBS and quick-frozen in liquid N2. Cells were homogenized in 0.5 ml of 0.3 mol/l perchloric acid (PCA) with a Potter-Elvejem homogenizer. The precipitates were pelleted
by centrifugation (5 min, 10,000g, 4°C), and PCA was extracted from the supernatants with 2 volumes of 1:4 trictyloamine:1.2 trichloro-fluoroethane (Freon). The aqueous phase was stored at −80°C for 3 days before HPLC analysis. Nucleotides were separated on a Beckman Ultraphase ODS 5 µm C18 reverse-phase HPLC column (Beckman, Fullerton, CA) eluted isocratically with 100 mmol/l triethylamine phosphate, pH 5.8/1% acetonitrile, as previously described (17). ATP, ADP, GDP, and UTP were quantitated by ultraviolet absorption and compared with external standards. Nucleotide-linked hexoses and hexosamines were separated and measured by anion exchange high-pressure liquid chromatography (HPLC). UDP-HEPES, UDP-hexoses (UDP-Hex), GDP mannose, and UDP were quantified by ultraviolet absorption and compared with external standards.

**Results**

**Glucose transport.** As expected after differentiation, 3T3-L1 adipocytes exhibited a marked response to an acute stimulation (100 nmol/l) induced an ~4-fold increase in glucose transport (Fig. 1A). The addition of 0.6 mmol/l insulin during preincubation did not significantly affect either basal or insulin-stimulated 2-DOG transport. Also, when cells were preincubated without insulin in either 5 mmol/l glucose plus 2.5 mmol/l GlcN or 25 mmol/l glucose, the acute maximal insulin-stimulated glucose transport did not significantly differ when compared with cells preincubated in 5 mmol/l glucose. On the other hand, 18 h of preincubation in media containing GlcN high glucose in the presence of 0.6 mmol/l insulin decreased acute maximal insulin-stimulated glucose transport by 43 and 42% respectively (Fig. 1A, P < 0.001), when compared with cells preincubated with 5 mmol/l glucose in the presence of 0.6 mmol/l insulin. Basal or insulin-independent glucose transport also decreased by 36 and 46%

![Diagram](image-url)
respectively, after preincubation with GlcN or high glucose in the presence of 0.6 nmol/l insulin for 18 h, although differences did not reach statistical significance. Acute insulin-stimulated 2-DOG transport above basal (Δ insulin) also decreased by ~40% after preincubation with GlcN or high glucose in the presence of 0.6 nmol/l insulin as compared with the control (Fig. 1B, P < 0.006). Thus, the decrease in the acute maximal insulin response is not accounted for solely by a decrease in basal 2-DOG transport. The induction of this desensitization required at least 6 h and was maximal by 18 h (data not shown).

2-DOG is transported into cells in the same way as glucose; on entry, it is phosphorylated to 2-deoxy-D-glucose-6-phosphate (2-DOG-6-P), which is not further metabolized and is trapped intracellularly. To assess whether the downregulation of insulin-stimulated 2-DOG uptake after pre-exposure to high glucose or GlcN in the presence of insulin reflected effects on sugar transport or phosphorylation, in some experiments, we measured 3-OMG transport. The latter is transported like glucose into cells but is not phosphorylated. The data are similar to those obtained in 2-DOG uptake studies (Fig. 1C). Preincubation in the presence of 0.6 nmol/l insulin decreased subsequent acute insulin-stimulated 3-OMG transport (vs. cells preincubated without insulin) if high glucose (P < 0.001) or GlcN (P < 0.05) was present during preincubation but not if low glucose was present. We concluded that insulin is required for glucose- and GlcN-induced desensitization of acute insulin-stimulated glucose transport and that both high-glucose and GlcN pretreatment affect glucose transport per se.

**Glucose transporter expression.** The decrease in insulin-stimulated glucose transport could be explained by decreased expression of the glucose transporter isoforms GLUT1 and/or GLUT4. It has been previously suggested that in this cell type, insulin resistance is associated with decreased GLUT4 expression (20). A representative immunoblot is shown in Fig. 2A, and the densitometric quantitation of the means from 5 independent experiments are shown in Fig. 2B. Total GLUT4 protein levels were determined in parallel with glucose transport using the same cell lysates. After an 18-h preincubation in media containing either high glucose (25 mmol/l glucose) or GlcN (5 mmol/l glucose plus 2.5 mmol/l GlcN), total GLUT4 protein levels were not significantly different from cells preincubated in media containing 5 mmol/l glucose. Further, the addition of insulin during preincubation did not significantly alter total GLUT4 expression. Similar results were obtained when GLUT4 expression was quantitated in total membrane preparations (data not shown). The latter preparation was used to quantify GLUT1 expression, and again, no significant differences were detected between the treatment conditions (Fig. 2C and D).

**Fig. 2. GLUT4 and GLUT1 protein expression.** Total GLUT4 protein levels were determined in parallel with glucose (Glc) transport from 1% Triton X-100 solubilized total cell extracts. A: Representative immunoblot of 30 µg total cell protein separated by Laemmli’s SDS-PAGE and immunoblotted with polyclonal rabbit α-GLUT4. B: Densitometric analysis of GLUT4 immunoblots from 5 independent experiments. Data are normalized to GLUT4 levels after preincubation with 5 mmol/l glucose plus 0.6 nmol/l insulin, each analyzed in duplicate or quadruplicate. C and D: Representative immunoblot for GLUT1 using 10 µg total membrane protein prepared as described in RESEARCH DESIGN AND METHODS and the subsequent densitometric analysis of 3 independent experiments.
GLUT4 translocation. We next assessed whether the decrease in insulin-stimulated glucose transport was associated with a defect in insulin-induced recruitment of GLUT4 to the plasma membrane. Plasma membrane lawns were prepared as described in RESEARCH DESIGN AND METHODS after a 15-min stimulation with or without 100 nmol/l insulin. An acute maximal insulin dose markedly increased plasma membrane-associated GLUT4 immunostaining after all preincubation conditions (Fig. 3A). However, insulin-stimulated GLUT4 translocation to the plasma membrane was impaired by ~45% (P < 0.0007) only in those cells preincubated with GlcN plus insulin but not in the cells preincubated with high glucose plus insulin (Fig. 3B). Neither high glucose nor GlcN affected GLUT4 translocation in the absence of insulin during preincubation. Basal association of GLUT4 with the plasma membrane was not affected significantly by any of the preincubation conditions. These data suggest that in the presence of insulin, high glucose and GlcN act via different mechanisms in downregulating glucose transport.

Nucleotide sugars. To assess the contribution of HSP to glucose transport desensitization, nucleotide sugar concentrations in the cells were measured. UDP-HexNAc (which represents both UDP-N-acetylgalactosamine and UDP-N-acetylgalactosamine in an ~3:1 ratio) is the major product of HSP. Preincubation with low glucose plus insulin had no significant effect on UDP-HexNAc concentration (100% = 6.76 ± 0.659 nmol UDP-HexNAc/mg protein) as compared with its concentration after preincubation with low glucose in the absence of insulin (Fig. 4A).

As expected, preincubation with GlcN markedly increased UDP-HexNAc concentration as compared with control (low glucose plus insulin) by ~400 and ~900% in the absence and presence of insulin, respectively (Fig. 4A, P < 0.0005). Preincubation with high glucose, on the other hand, increased UDP-HexNAc concentration only modestly by ~30%. This increase required the presence of insulin and was significant (P < 0.001) compared with low glucose plus insulin.
The measurement of UDP-Hex (Fig. 4B) represents the concentrations of UDP-glucose plus UDP-galactose in an ~3:1 ratio. UDP-glucose is the obligatory substrate of glycogen synthase. Preincubation with GlcN or high glucose exerted opposing effects on cellular UDP-Hex concentrations. High glucose in the presence of insulin, but not in its absence, increased cellular UDP-Hex concentrations by ~60% (P < 0.01, compared with preincubation in 5 mmol/l glucose). In contrast, preincubation with 2.5 mmol/l GlcN in the presence of 5 mmol/l glucose depleted UDP-Hex concentrations by ~60% in the presence or absence of insulin (P < 0.01).

The data in Figs. 1A and 4A suggest that there is no simple correlation between cellular concentrations of UDP-HexNAc and the development of insulin-resistant glucose transport. Preincubation in 2.5 mmol/l GlcN in the absence of insulin increased intracellular UDP-HexNAc ~4-fold but failed to cause insulin resistance. Preincubation with low-dose insulin in the presence of high glucose or GlcN caused similar (~45%) inhibition of the insulin response, yet UDP-HexNAc increased by 90% in the latter condition and by only 30% in the former condition.

To further analyze the relationship between the accumulation of UDP-HexNAc and the impairment of basal and maximal insulin-stimulated 2-DOG transport, cells were preincubated for 18 h in 5 mmol/l glucose plus 0.6 nmol/l insulin with or without varying concentrations of added GlcN (0.1–2.5 mmol/l, Fig. 5). In the presence of insulin, cellular UDP-HexNAc concentrations rose steeply and dose dependently between 0.1 and 0.5 mmol/l GlcN and were increased ~10-fold at 0.5 mmol/l GlcN. No further increase in UDP-HexNAc was observed between 0.5 and 2.5 mmol/l GlcN, and levels tended to fall (Fig. 5A). Under the same conditions, maximal insulin-stimulated 2-DOG transport or basal 2-DOG transport were not inhibited after preincubation with 0.5 mmol/l GlcN but decreased between 1 and 2.5 mmol/l GlcN. Maximal inhibition at 2.5 mmol/l GlcN was ~50% for insulin-stimulated transport (P < 0.01) and ~30% for basal transport (Fig. 5B). Insulin-mediated GLUT4 translocation also decreased in parallel to 2-DOG transport after preincubation with increasing concentrations of GlcN in the presence of insulin (Fig. 5C). These data suggest that in the presence of insulin, the maximal capacity of these cells to metabolize GlcN to UDP-HexNAc is reached at ~0.5 mmol/l GlcN in the medium. Furthermore, there appears to be no correlation between cellular UDP-HexNAc accumulation and the development of insulin-resistant glucose transport in this model.

**Nucleotide concentrations.** The data suggested that UTP availability may limit the formation of UDP-Hex (Fig. 4B) and UDP-HexNAc (Fig. 5A) in cells preincubated with increasing doses of GlcN both in the presence or absence of insulin. Furthermore, Hresko et al. (27) recently reported that ATP depletion may account for the development of GlcN-induced insulin resistance in 3T3-L1 adipocytes. We therefore measured the concentration of several nucleotides in 3T3-L1 adipocytes incubated for 18 h in media with or without 0.6 nmol/l insulin and containing 5 mmol/l glucose, 5 mmol/l glucose supplemented with increasing concentrations of GlcN (0.1–2.5 mmol/l), or 25 mmol/l glucose. As shown in Fig. 6, ATP did not change significantly in the presence of increasing concentrations of GlcN without insulin. In the presence of insulin, ATP tended to decline 10–20% at low GlcN concentrations (0.1–0.5 mmol/l) and 25–30% after incubation with 1–2.5 mmol/l GlcN (P < 0.0002). ADP tended to increase with increasing concentrations of GlcN, but the effect did not reach statistical significance. GDP concentrations were elevated with increasing concentrations of GlcN by more than ~50% the GlcN effect was statistically significant only in the presence of insulin (P < 0.0002). UTP concentrations decreased significantly during incubation with GlcN with or without insulin (P < 0.0001). The lowest concentration of GlcN tested (0.1 mmol/l) decreased UTP by ~30% and depletion was near maximal at 1 mmol/l GlcN.

The effects on nucleotide concentrations of incubation in low glucose versus high glucose in the absence and presence of insulin are shown in Fig. 7. Insulin did not affect ATP or ADP concentrations during incubation in low glucose. High glucose tended to increase the concentration of ATP (vs. low glucose), but the effect was only significant in the absence of insulin (P < 0.05) by 1-way analysis of variance (ANOVA). Changes in ADP concentration paralleled those in ATP (P < 0.05). The ratio of ATP to ADP was unchanged (~10) in each condition. By 2-way ANOVA, high glucose but not insulin increased both ATP.
and ADP ($P < 0.01$) concentrations, whereas both high glucose and insulin appeared to independently increase the concentrations of GDP ($P < 0.01$) and UTP ($P < 0.006$).

**GlcN-6-P.** If the rate-limiting step in the metabolism of GlcN to UDP-HexNAc is distal to hexokinase, intracellular GlcN-6-P may accumulate with increasing GlcN concentrations in the medium. The latter may explain the observed depletion of ATP. GlcN-6-P was undetectable in the cells (the lower limit of detection using this method is ~40 pmol) after preincubation with low glucose or high glucose with or without insulin (data not shown). GlcN-6-P was only minimally elevated with increasing concentrations of GlcN (0.1–2.5 mmol/l) in the absence of insulin increasing to 0.44 ± 0.05 nmol/mg protein with 2.5 mmol/l GlcN (Fig. 8A). In the presence of insulin, however, GlcN-6-P was detected in the cells with 0.5 mmol/l GlcN (2.26 ± 0.11 nmol/mg protein) and increased by as much as an order of magnitude between 0.5 and 2.5 mmol/l GlcN (Fig. 8B; note that the difference in the scale of the ordinates is 40× between Fig. 8A and B). The dose-dependent increase in GlcN-6-P concentration after preincubation with GlcN in the presence of insulin correlated positively with the observed decrease in acute insulin-stimulated glucose transport ($r = 0.94, P < 0.05$, Fig. 8C).

**PI-3 kinase activity.** In an effort to determine if glucose or GlcN-induced insulin resistance was associated with impaired insulin signal transduction, we assayed PI-3 kinase activity as described in CELL CULTURE AND GENERAL METHODS.

**DISCUSSION**

Our model of glucose-induced insulin resistance in 3T3-L1 adipocytes is consistent with data from several laboratories, i.e., glucose and insulin act synergistically to downregulate basal and insulin-stimulated glucose transport, and the effect of high glucose is mimicked by GlcN at lower concentrations (Fig. 1). This downregulation is time dependent, requiring several hours. The mechanism, however, is controversial.

In most experiments, we measured 2-DOG uptake as an indicator of glucose transport. The cells were deprived of insulin and FBS for 2 h and then pre-equilibrated for 25 min in glucose-free media before starting the 5-min 2-DOG uptake assay to minimize differences in intracellular concentrations of glucose, glucose-6-P, and GlcN-6-P between treatment groups. Glucose-6-P is a potent inhibitor of hexokinase (28), whereas 2-DOG-6-P (28) and GlcN-6-P (14) are relatively weak inhibitors. Therefore, the decreased acute insulin response of 2-DOG uptake observed in cells that had been preincubated for 18 h with 0.6–0.3 mmol/l insulin in the presence of high glucose or...
GlcN likely represents mainly downregulation of transport rather than inhibition of hexokinase activity. This conclusion is supported by the fact that insulin-stimulated 3-OMG transport was inhibited under the same conditions as 2-DOG uptake. The inhibition of the former was slightly less than that of the latter (compare Fig. 1A and C), which may reflect technical difficulties associated with measuring initial rates of 3-OMG transport, particularly in dish-adherent cells that cannot be as rapidly separated from the medium as cells in suspension. Whereas we cannot rule out the possibility that hexokinase inhibition by glucose-6-P and/or GlcN-6-P contributed to the observed downregulation of 2-DOG uptake, based on the 3-OMG transport data, it was likely a minor contribution.

In our studies, glucose/GlcN-induced glucose transport downregulation occurred without detectable changes in total expression of GLUT4 or GLUT1. Chronic exposure to pharmacological doses of insulin (>100 nmol/l) have been shown by several laboratories to decrease GLUT4 and increase GLUT1 expression in 3T3-L1 adipocytes (20,29–32). Decreased GLUT4 expression reflects in part insulin-mediated repression of the GLUT4 promoter (33) and in part accelerated degradation of the GLUT4 protein (30). The insulin-mediated stimulation of GLUT1 gene expression appears to be mediated, at least in part, by activation of protein kinase B (PKB)/Akt (34). However, even when using high insulin doses, several laboratories concluded that exposure of 3T3-L1 adipocytes to insulin for 10–24 h caused only mild or no decrease in total GLUT4 expression, which did not account for the marked downregulation of insulin-stimulated glucose transport and GLUT4 translocation (32,35,36). Prolonged exposure to 100–500 nmol/l insulin markedly accelerates the degradation of IRS-1 protein and strongly inhibits the insulin-mediated tyrosine phosphorylation of IRS-1 and activation of PI-3 kinase (32,37). In contrast to the above articles, we used physiological doses of insulin during preincubation, which can occur in plasma postprandially in vivo and are commonly attained in insulin-resistant conditions (4). However, Thomson et al. (20) reported insulin dose-dependent decreased GLUT4 protein expression after exposing 3T3-L1 adipocytes to low insulin concentrations similar to those used here. They proposed that the insulin-mediated desensitization of glucose transport directly reflected decreased GLUT4 expression, which was controlled posttranscriptionally (i.e., GLUT4 degradation) and required the presence of glucose or GlcN (20). There are several differences in experimental design that may have contributed to the variation in results. The concentration of FBS during preincubation was 10% in the study by Thomson et al. and 1% in our experiments; the media were changed every 2 h in the study by Thomas et al. to maintain glucose and insulin concentrations constant over 12 h, whereas cells were...
responsive cells is the induction of GLUT4 translocation studies in which cells were exposed to low insulin concentrations (0.05–5 nmol/l) for 12 h, GLUT4 was quantitated in the low-density microsomal (LDM) fraction versus the total cell extracts or unfractionated total membrane preparations in this article. Thus, it is possible that in the study by Thomson et al. (20), GLUT4 was segregated, in part, in a different cellular compartment after chronic insulin treatment. Finally, because 3T3-L1 fibroblasts have been propagated and differentiated in different laboratories for many years, clonal differences may have arisen. The present study demonstrates that marked insulin resistance of glucose transport can develop in cells after pre-exposure to low-dose insulin in the presence of high glucose (but not low glucose) without detectable changes in glucose transporter expression, suggesting the involvement of alternate mechanisms in the development of glucose toxicity.

Insulin’s major effect in stimulating glucose transport in responsive cells is the induction of GLUT4 translocation from an LDM-associated intracellular compartment to the cell membrane (3). We were surprised to find that insulin was equally effective in acutely increasing plasma membrane–associated GLUT4 in cells preincubated with or without insulin in the presence of low or high glucose, as assessed by the “lawn assay.” Inhibition of insulin-stimulated glucose transport distal to the translocation of GLUT4-containing vesicles (GCV) may reflect impaired fusion of GCV with the cell membrane, inappropriate intercalation, or decreased GLUT4 intrinsic activity. The insulin resistance of glucose transport after stimulation of certain G-protein-coupled receptors may reflect impaired fusion of translocated GCV (38). Although GLUT4 expression is unchanged, muscles overexpressing GLUT1 exhibit markedly increased basal glucose flux and are resistant to insulin stimulation of glucose transport in vitro (39). The translocation and intercalation of GLUT4 into the cell membrane (as assessed by exofacial photo affinity labeling after insulin stimulation) is normal, suggesting that chronically increased glucose flux into the cell impairs the ability of plasma membrane–associated GLUT4 to transport glucose (40). In 3T3-L1 adipocytes, pretreatment with low doses of the PI-3 kinase inhibitor wortmannin downregulated insulin-stimulated glucose transport without affecting GLUT4 translocation or membrane insertion, whereas higher doses inhibited all of the above processes (41). Thus, as in our model of glucose toxicity, activation of glucose transport can be dissociated from GLUT4 translocation. In both 3T3-L1 adipocytes and L-6 myocytes, insulin-mediated activation of p38 MAP kinase may be involved in activating GLUT4 at the cell membrane (21).

Chronic infusion of glucose into rats caused marked insulin resistance without affecting GLUT4 expression or translocation in skeletal muscle (42). The molecular mechanism(s) by which chronically increased glucose flux down-regulates glucose transport at step(s) distal to GLUT4 translocation is unknown. Our data indicate that insulin-stimulated PI-3 kinase activation was not inhibited by preincubation in high glucose in the presence of 0.6 nmol/l insulin. However, we cannot rule out the possibility that the subcellular distribution of IRS-1–associated activated PI-3 kinase may have been altered (43). Two recent articles suggest that glucose-induced insulin resistance of glucose transport in muscle occurs at step(s) distal to PI-3 kinase and may involve, at least in part, impaired activation of Akt/PKB (44,45). The complex machinery that regulates docking and fusion of GCV is under intense investigation (46,47) and may include proteins that regulate GLUT4 activity.

In contrast to high glucose, preincubation with GlcN in the presence of insulin did inhibit GLUT4 translocation in response to subsequent acute insulin stimulation. The inhibition was GlcN dose-dependent and was observed at the lowest dose of GlcN (1 nmol/l) that elicited glucose transport insulin resistance in our system. In vivo infusion of GlcN has been reported to inhibit insulin-stimulated GLUT4 translocation in muscle (10). The discrepancy between the effects of high glucose and GlcN in our system was the first indication that the 2 agents may desensitize glucose transport via different mechanisms. The lack of correlation between the accumulation of UDP-HexNAc and the development of insulin-resistant glucose transport further suggested that GlcN may not mimic the effects of high glucose. In the absence of insulin, preincubation with 2.5 mmol/l GlcN increased UDP-HexNAc 4-fold in cells without affecting the acute insulin response of glucose transport. On the other hand, preincubation with high glucose plus insulin increased UDP-HexNAc by only ~30%, but the subsequent insulin response was downregulated to the same extent as after incubation with 2.5 mmol/l GlcN plus insulin, which elevated UDP-HexNAc 9-fold. Furthermore, preincubation with 0.5 mmol/l GlcN plus insulin increased UDP-HexNAc maximally without significantly affecting basal or insulin-stimulated glucose transport. Thus, in a glucose-poor milieu, accumulation of the major HSP products is insufficient for the development of insulin resistance. Because GlcN competes poorly with glucose for transport into muscle cells, it is difficult to assess whether the effects of GlcN and high glucose are additive.

Under physiological conditions, glucose flux via HSP represents a relatively small fraction of total glucose flux, and its entry into the pathway is limited by GFAT activity (7,8). In our experiments, GlcN-6-P accumulation was insulin and GlcN dose-dependent. This result is consistent with insulin accelerating GlcN transport into the cell (7) and suggests that when GlcN enters cells bypassing GFAT, its downstream metabolism is limited at 1 of 2 early steps distal to hexokinase, i.e., acetylation of GlcN-6-P to GlcNAc-6-P or conversion of GlcNAc-6-P to UDP-HexNAc. The marked GlcN dose-dependent decline in UTP concentration suggests that the latter step may be limiting. Accumulation of GlcN-6-P in heart (~700-fold) and to a lesser extent in skeletal muscle (~500-fold) has been recently observed in vivo in rats infused with GlcN during a euglycemic insulin clamp (14). Trapping of high-energy phosphate as GlcN-6-P likely contributed to the decline in ATP observed in cells pre-exposed to GlcN plus insulin, consistent with the data of Hresko et al. (27). The degree of ATP depletion in response to similar GlcN doses was smaller in our model than in the study by Hresko et al. This likely reflects the fact that in the study by Hresko et al., cells were exposed to GlcN in glucose-free media or in the presence of much higher insulin concentrations than those used in our studies; both conditions would markedly enhance GlcN entry into the cells. In the study by Hresko et al., GlcN inhibited the acute insulin response of GLUT4 translocation and insulin-stimulated activation of the insulin receptor, IRS-1, and PI-3 kinase as a consequence of ATP depletion. In contrast, our
milder experimental conditions, GlcN inhibited insulin-stimulated glucose transport and GLUT4 translocation but not PI-3 kinase activation, consistent with impaired signal transduction distal to PI-3 kinase. In the presence of insulin, we observed GlcN dose-dependent increases in GDP. Although GTP was not quantitated, it likely declined in parallel with ATP during incubation with GlcN. The small GTP binding protein Rab4 is thought to be critical in insulin-stimulated GCV translocation (47,48). A decline in the GTP/GDP ratio may be inhibitory by limiting Rab4 activation.

Our data agree with the major conclusion of Hresko et al. (27), which is GlcN has metabolic effects different from those of high glucose. Both agents induce insulin resistance of glucose transport in 3T3-L1 adipocytes but via different mechanisms. Our data does not infer that products of HSP may not contribute to glucose-induced insulin resistance. Numerous in vivo studies in different models that do not involve GlcN administration show correlations between insulin resistance and enhanced accumulation of UDP-HexNAc in muscle (15-17,49,50). GFAT activity is increased in muscles of genetic rodent models of insulin resistance (16,17) and in muscles of patients with uncontrolled type 2 diabetes (51). Transgenic mice overexpressing GFAT in muscle and fat develop insulin resistance (52,53). 3T3-L1 adipocytes are differentiated from an immortal fibroblast cell line that is chronically maintained in media containing high glucose concentrations (20–25 mmol/l) before and during differentiation. This may cause selection toward cells that are best adapted to this milieu. Although data in 3T3-L1 adipocytes may not be readily extrapolated to other systems, e.g., in vivo studies of skeletal muscle, we feel that investigations using GlcN to model the role of HSP in glucose-induced insulin resistance need to be interpreted with caution.

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