

# Dynamic Actions of Glucose and Glucosamine on Hexosamine Biosynthesis in Isolated Adipocytes

DIFFERENTIAL EFFECTS ON GLUCOSAMINE 6-PHOSPHATE, UDP-N-ACETYLGLUCOSAMINE, AND ATP LEVELS\*

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Glucose and glucosamine (GlcN) cause insulin resistance over several hours by increasing metabolite flux through the hexosamine biosynthesis pathway (HBP). To elucidate the early events underlying glucose-induced desensitization, we treated isolated adipocytes with either glucose or GlcN and then measured intracellular levels of glucose-6-P (G-6-P), GlcN-6-P, UDP-GlcNAc, and ATP. Glucose treatment rapidly increased G-6-P levels ( $t_{1/2} < 1$  min), which plateaued by 15 min and remained elevated for up to 4 h (glucose  $ED_{50} = 4$  mM). In glucose-treated cells, GlcN-6-P was undetectable; however, GlcN treatment (2 mM) caused a rapid and massive accumulation of GlcN-6-P. Levels increased by 5 min ( $\sim 400$  nmol/g) and continued to rise over 2 h ( $t_{1/2} \sim 20$  min) before reaching a plateau at  $>1,400$  nmol/g ( $ED_{50} = 900$   $\mu$ M). Thus, at high GlcN concentrations, unrestricted flux into the HBP greatly exceeds the biosynthetic capacity of the pathway leading to a rapid buildup of GlcN-6-P. The GlcN-induced rise in GlcN-6-P levels was correlated with ATP depletion, suggesting that ATP loss is caused by phosphate sequestration (with the formation of GlcN-6-P) or the energy demands of phosphorylation. As expected, GlcN and glucose increased UDP-GlcNAc levels ( $t_{1/2} \sim 14$ –18 min), but greater levels were obtained with GlcN (4–5-fold for GlcN, 2-fold for glucose). Importantly, we found that low doses of GlcN ( $<250$   $\mu$ M,  $ED_{50} = 80$   $\mu$ M) could markedly elevate UDP-GlcNAc levels without increasing GlcN-6-P levels or depleting ATP levels. These studies on the dynamic actions of glucose and GlcN on hexosamine levels should be useful in exploring the functional role of the HBP and in avoiding the potential pitfalls in the pharmacological use of GlcN.

The distinguishing hallmark of type 2 diabetes mellitus is an inability of insulin to elicit a normal biological response in peripheral, insulin-responsive target tissues (a condition known as insulin resistance). To investigate the detrimental role of hyperglycemia under defined *in vitro* conditions, we

developed a method to maintain isolated adipocytes in primary culture (1, 2), and we used this system to examine the induction of insulin resistance (3–5). In 1991, we reported (6) the discovery of a unique metabolic pathway mediating glucose-induced desensitization of the insulin-responsive glucose transport system (GTS).<sup>1</sup> Because a major function of this pathway is the formation of hexosamine metabolites, we postulated that enhanced flux of incoming glucose through the hexosamine biosynthesis pathway (HBP) culminates in the induction of insulin resistance (6). Numerous studies have now confirmed the hexosamine hypothesis and extended the regulatory actions of the HBP to muscle tissue and other cell types (7–11).

Enhanced flux into the HBP begins with glucose uptake and the rapid phosphorylation of glucose to glucose 6-phosphate (G-6-P). G-6-P can then be routed to the glycogen biosynthesis pathway, shunted to the pentose phosphate pathway, or converted to fructose 6-phosphate and subsequently catabolized through the glycolytic pathway. Although these pathways represent the major routes traversed by the vast majority of glucose, a small percentage of incoming glucose can be enzymatically converted to glucosamine 6-phosphate (GlcN-6-P) by glutamine:fructose-6-phosphate amidotransferase (GFAT), the first and rate-limiting enzyme of the HBP (10). Once formed, GlcN-6-P undergoes a series of rapid conversions to UDP-N-acetylglucosamine (UDP-GlcNAc). Several studies now indicate that the generation of UDP-GlcNAc plays an important role in the induction of insulin resistance by serving as the precursor product for O-linked glycosylation of various cytosolic and nuclear proteins (7, 10–13).

In adipocytes, GlcN is 40 times more potent than glucose in mediating desensitization (6). As such, GlcN can be a useful tool for studying hexosamine-mediated desensitization because it directly enters the HBP at the level of GlcN-6-P. However, such a potent pharmacological compound can have cellular actions that are distinct from glucose (11). Therefore, in the current study, we have examined the temporal and dose-dependent actions of glucose and GlcN on the formation of hexosamine products. These studies were designed with two goals in mind: to elucidate the early events in glucose-induced desensitization (formation of hexosamine products) and to better understand the pharmacological actions of GlcN.

## EXPERIMENTAL PROCEDURES

**Materials**—Sources of materials were as follows: Porcine insulin, Sigma; collagenase, Worthington; bovine serum albumin, Armour; and penicillin-streptomycin, Dulbecco's modified Eagle's medium, and Dul-

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<sup>1</sup> The abbreviations used are: GTS, glucose transport system; HBP, hexosamine biosynthesis pathway; GFAT, glutamine:fructose-6-phosphate amidotransferase; HBSS, HEPES-buffered balanced saline solution; HPLC, high pressure liquid chromatography.

becco's modified Eagle's medium formulated without D-glucose, Life Technologies, Inc., Grand Island, NY. All other reagents were from Sigma or Fisher unless otherwise specified.

**Preparation and Treatment of Isolated Adipocytes**—Isolated adipocytes were obtained from the epididymal fat pads of male Sprague-Dawley rats (180–225 g) by collagenase digestion as described previously (2). After digestion, cells were washed three times in Hepes-buffered balanced saline solution (HBSS) consisting of 25 mM Hepes, 120 mM NaCl, 0.8 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 5.4 mM KCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% bovine serum albumin, pH 7.6. Cells were then diluted to a final concentration of 5 × 10<sup>5</sup> cells/ml (12 ml of HBSS per 1 g of initial fat weight). From a common pool of cells, 400 μl of adipocytes were aliquoted into 17 × 100 mm of sterile polystyrene tubes containing 1.6 ml of HBSS. Unless otherwise indicated, insulin was added (25 ng/ml final concentration), and the cells were incubated at 37 °C to stimulate the GTS and facilitate the subsequent uptake of glucose or GlcN. After 20 min, glucose, GlcN (or both) was added, and adipocytes were further incubated at 37 °C for either 4 h (dose-response experiments) or for various times (time course experiments).

**Measurement of UDP-N-Acetylglucosamine Levels**—After treatment, adipocytes were transferred to 1.5-ml microfuge tubes, and the cells were washed three times with ice-cold, bovine serum albumin-free HBSS. During the final wash, the cell volume was reduced to 150 μl, and 0.4 ml of cold perchloric acid (0.3 mol/liter) was added to adipocytes for 10 min. The mixture was then centrifuged at 20,800 × g (10 min at 4 °C), and the deproteinized metabolite extract (420 μl) was transferred to a new microfuge tube. After the extract was neutralized by adding a small amount of K<sub>2</sub>CO<sub>3</sub> (5 M), extracts were frozen and stored at -20 °C. The concentration of UDP-GlcNAc was measured by HPLC as described previously (14) by first passing the cell extract through a strong anion-exchange Supelclean LC-SAX column (Supelco, Bellefonte, PA). Analysis was performed using an HPLC system (Waters Associates, Milford, MA) and two reverse phase LC18T columns in series (Supelco). Metabolite concentration was quantitated by UV absorption (260 nm), and levels were determined from a standard calibration curve. Recovery of standards added to adipocyte extract was >95%.

**Measurement of ATP and G-6-P Levels**—ATP concentrations were determined using a standard two-step, coupled reaction developed by Passonneau and Lowry (15, 16). In this method, glucose and hexokinase are added to the adipocyte extract to generate ADP and G-6-P. In the second step, G-6-P is converted to NADPH and 6-phosphogluconolactone (by G-6-P dehydrogenase). The assay was performed in 96-well plates to which we added 150 μl of buffer containing 200 mM triethanolamine-HCl, 10 μM NADP, 5 mM MgCl, plus 150 μl of sample. The relative fluorescence unit was then measured before and 5 min after the addition 10 μl of 44 units/ml G-6-P dehydrogenase. Measurements were obtained using a Beckman spectrophotometer with an excitation wavelength of 355 nm and a 460-nm emission wavelength. Changes in relative fluorescence units were converted to a concentration by use of a calibration curve generated by assaying 0–24 μl of 25 μM G-6-P. Duplicate measurements were obtained for each sample and averaged. Levels of G-6-P were similarly measured fluorometrically (17) using G-6-P standard calibration curves.

**Measurement of GlcN-6-P Levels**—We have developed a fluorometric assay to measure GlcN-6-P levels based on the same enzymatic principles used to measure G-6-P levels. At high concentrations, we found that G-6-P dehydrogenase can act on GlcN-6-P and NADP, resulting in equimolar production of NADPH, whereas at low concentrations (used to measure G-6-P) NADPH was not generated. Thus, the low affinity of G-6-P dehydrogenase for GlcN-6-P allows for the resolution of G-6-P from GlcN-6-P in the same sample by first carrying out the assay for G-6-P with a low amount of G-6-P dehydrogenase (1.2 units/ml) followed by the addition of a much greater concentration of G-6-P dehydrogenase (43 units/ml). Optimization of assay methods revealed that GlcN-6-P measurements are not affected by glucose, GlcN, G-6-P, or GlcNAc-6-P and that standard calibration curves (using GlcN-6-P) are linear (data not shown). To further validate assay results, we incubated adipocytes for 4 h with various concentrations of GlcN, prepared metabolite extracts as described above, and then measured GlcN-6-P levels using two methods. The first method was the fluorometric assay. The second assay method entailed derivatization of GlcN-6-P with o-phthalaldehyde so that it can be detected fluorometrically (following separation by reverse phase HPLC). Both assay methods yielded similar results, but the fluorometric enzymatic assay was faster, less expensive, and allowed for the measurement of numerous samples (using a 96-well assay format).

**Assessment of Glucosamine-induced Desensitization of the Insulin-responsive GTS**—Isolated adipocytes (400 μl) were added to HBSS (1.6 ml) in 17 × 100-mm sterile polystyrene tubes and incubated at 37 °C for 5 h in the absence (controls) or presence of various concentrations of GlcN. After 5h, cells were washed three times with GlcN-free HBSS containing 25 ng/ml insulin. During the final wash, cells were resuspended in HBSS buffer containing 1% bovine serum albumin, pH 7.4, and concentrated to about 2 × 10<sup>5</sup> cells/ml. Maximal insulin-stimulated rates of glucose transport were determined by incubating adipocytes with 25 ng/ml insulin for 30 min at 37 °C. Initial rates of glucose uptake were measured by adding 20 μl of HBSS containing 0.12 μCi of 2-deoxy-D-[<sup>3</sup>H]glucose and 2-deoxyglucose (final substrate concentration of 50 μM). At the end of 3 min, the reaction was terminated by separating cells (180-μl aliquots) from buffer by centrifugation through silicone oil. To correct the 2-deoxyglucose uptake values for uptake by simple diffusion and for nonspecific trapping of radioactivity in the cell pellet, we assessed glucose uptake in the presence of 0.3 mM phloretin. Glucose uptake was derived from the mean of duplicate determinations sampled in duplicate.

**Competitive Effects of Glucose on <sup>14</sup>C-Labeled GlcN Uptake**—Isolated adipocytes (0.2 ml of cells in 12 × 75-mm polystyrene tubes) were incubated for 30 min at 37 °C in the presence of 25 ng/ml insulin and various concentrations of glucose. Initial rates of GlcN uptake were measured by adding 20 μl of HBSS containing 0.1 μCi of <sup>14</sup>C-labeled GlcN. At the end of 3 min, the reaction was terminated by separating cells (170-μl aliquots) from the buffer by centrifugation through silicone oil. To correct uptake values for simple diffusion and for nonspecific trapping of radioactivity in the cell pellet, we assessed GlcN in the presence of 0.3 mM phloretin, which blocks uptake through the glucose transport system.

## RESULTS

**Effects of Glucose on G-6-P and UDP-GlcNAc Levels**—As shown in the inset of Fig. 1A, the addition of 20 mM glucose to insulin-treated cells rapidly elevated intracellular G-6-P levels (*t*<sub>1/2</sub> < 1 min). By 15 min, equilibrium was established, and levels remained elevated for up to 4 h (Fig. 1A). A dose-response curve obtained after 4 h of treatment yielded a glucose ED<sub>50</sub> of 4 mM (Fig. 1B). Fig. 1C shows that the rapid rise in G-6-P levels was followed by a slower increase in UDP-GlcNAc (*t*<sub>1/2</sub> = 18 min). At 1 h, UDP-GlcNAc reached a plateau about 2-fold higher than basal values and remained at this level for up to 4 h. When UDP-GlcNAc levels were assessed after a 4-h treatment time using various concentrations of glucose, we obtained an ED<sub>50</sub> value of 1.2 mM (Fig. 1D). It is important to mention that GlcN-6-P was undetectable in glucose-treated cells (data not shown). These data indicate that extracellular glucose is transported into adipocytes through the insulin-responsive GTS and rapidly phosphorylated to G-6-P. Once internalized, a portion of incoming glucose is routed to the HBP resulting in the subsequent rise in UDP-GlcNAc levels. Flux through the HBP must be relatively rapid because formation of UDP-GlcNAc could be seen as early as 5 min after the addition of glucose (Fig. 1C). The slower rise in UDP-GlcNAc over the 1st h can be explained by rate-limiting steps in UDP-GlcNAc utilization (*i.e.* protein glycosylation) or a slow conversion of UDP-GlcNAc to other metabolites.

**Effects of Glucosamine on GlcN-6-P and UDP-GlcNAc Levels**—After uptake into cells, GlcN is rapidly phosphorylated and enters the HBP at the level of GlcN-6-P. To assess the rapidity and magnitude of metabolite formation, we examined the ability of GlcN to elevate two key hexosamine metabolites, GlcN-6-P and UDP-GlcNAc. Time course studies in insulin-treated cells revealed that 2 mM GlcN causes a rapid and massive accumulation of GlcN-6-P (Fig. 2A). GlcN-6-P levels increased by 5 min (~400 nmol/g) and continued to rise over 2 h (*t*<sub>1/2</sub> ~ 20 min), reaching a plateau at >1,400 nmol/g. Dose-response studies (Fig. 2B) showed that GlcN-6-P levels were unchanged when cells were incubated for 4 h with GlcN concentrations <250 μM. At 500 μM GlcN, levels were only slightly elevated, whereas GlcN-6-P levels were maximally elevated at

FIG. 1. Rapid effect of glucose on elevating intracellular levels of G-6-P and UDP-GlcNAc. Isolated adipocytes were treated for 20 min with 25 ng/ml insulin and then incubated with 20 mM glucose for the indicated times (A and C) or incubated for 4 h with the indicated concentrations of glucose (B and D). At the end of the treatment time, cells were lysed with perchloric acid, and the neutralized cell extract was deproteinated. Concentrations of G-6-P (A and B) and UDP-GlcNAc (C) were measured in cell extracts as described under "Experimental Procedures." Each point represents the mean of duplicate determinations.

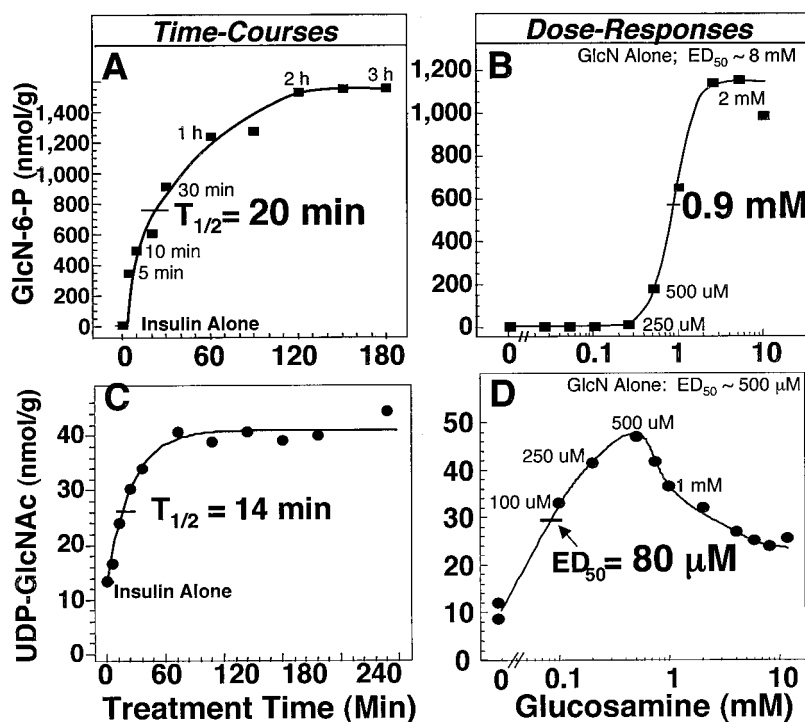
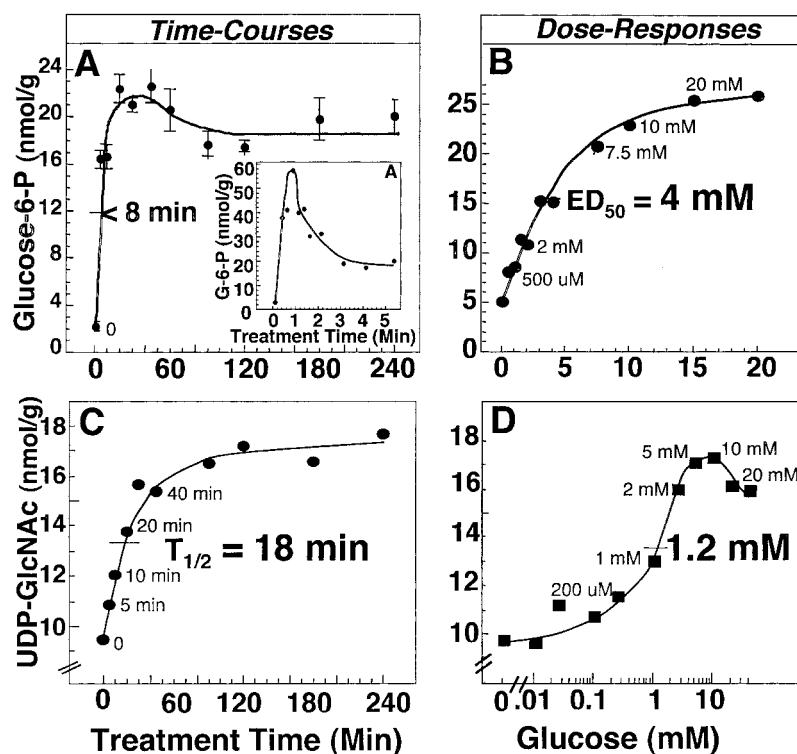


FIG. 2. Ability of glucosamine to elevate intracellular levels of GlcN-6-P and UDP-GlcNAc. Adipocytes were treated for 20 min with 25 ng/ml insulin and then incubated with 2 mM GlcN for the indicated times (A and C) or incubated for 4 h with the indicated concentrations of GlcN (B and D). At the end of the treatment time, cells were lysed with perchloric acid, and the neutralized cell extract was deproteinated. Concentrations of GlcN-6-P and UDP-GlcNAc were measured as described under "Experimental Procedures." Each point represents the mean of duplicate determinations.

2 mM GlcN ( $ED_{50} = 0.9$  mM). In the absence of insulin, similar results were obtained, but a 10-fold higher concentration of GlcN was required ( $ED_{50} = 8$  mM).

Fig. 2, C and D depict the effects of GlcN on UDP-GlcNAc levels as a function of treatment time and dose. When UDP-GlcNAc levels were assessed over time (Fig. 2C), we found that 2 mM GlcN rapidly increased UDP-GlcNAc during the 1st h ( $t_{1/2} \sim 14$  min). Levels remained elevated for up to 4 h. Dose-response studies (at 4 h) revealed an interesting biphasic effect of GlcN treatment (Fig. 2D). Low doses of GlcN (up to 500  $\mu$ M) increased UDP-GlcNAc levels by 4–5-fold ( $ED_{50}$  of 80  $\mu$ M), whereas higher GlcN doses (0.75–10 mM) progressively dimin-

ished maximal levels of UDP-GlcNAc. By comparing the GlcN-induced rise in GlcN-6-P levels (Fig. 2B) with the dose-dependent increase in UDP-GlcNAc levels (Fig. 2D), it is apparent that low concentrations of GlcN can markedly elevate UDP-GlcNAc levels without altering GlcN-6-P levels. From this finding, we conclude that low concentrations of GlcN are rapidly taken up by adipocytes, phosphorylated to GlcN-6-P, and then routed through the HBP resulting in a 4–5-fold increase in UDP-GlcNAc levels. At higher levels of extracellular GlcN (>250  $\mu$ M), we believe that uptake and intracellular phosphorylation of GlcN exceeds the capacity of the HBP, which leads to a progressive accumulation of GlcN-6-P.



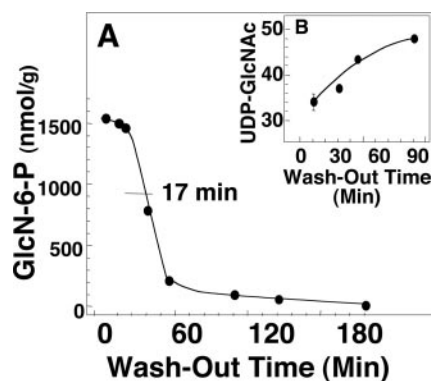


FIG. 3. Temporal changes in GlcN-6-P and UDP-GlcNAc levels after removal of glucosamine. Adipocytes were treated for 20 min with 25 ng/ml insulin and then incubated for 1 h with 2 mM GlcN. Cells were then washed four times and incubated in GlcN-free buffer for the indicated times (Wash-Out Time). Concentrations of GlcN-6-P and UDP-GlcNAc were measured as described under "Experimental Procedures." Each point represents the mean of duplicate determinations.

*Changes in Intracellular Hexosamine Metabolites after Removal of Extracellular Glucosamine*—To gain additional insights into the kinetics of hexosamine flux, we pretreated adipocytes for 1 h with 2 mM GlcN and then washed cells to remove extracellular GlcN. As shown in Fig. 3A, we found that intracellular GlcN-6-P levels were reduced from 1500 nmol/g to about 300 nmol/g after 1 h ( $t_{1/2} = 17$  min). In contrast, UDP-GlcNAc levels actually increased during this time (Fig. 3B). Thus, it appears that the high intracellular levels of GlcN-6-P are reduced by the continued flux of GlcN-6-P through the HBP. Using a similar experimental protocol in which cells were exposed to 20 mM glucose and then extensively washed to remove extracellular glucose, we found that G-6-P levels were also rapidly reduced ( $t_{1/2} = 15$  min) upon the removal of extracellular glucose uptake (data not shown).

*Depletion of Cellular ATP by High Concentrations of Glucosamine*—GlcN has been reported to reduce ATP levels in 3T3-L1 cells (18). To examine the effect of GlcN on ATP levels in adipocytes, we performed the dose-response experiment depicted in Fig. 4A. As can be seen, little or no depletion of ATP was observed at low GlcN concentrations (up to 250  $\mu$ M). However, ATP levels were markedly reduced at higher concentrations (1–4 mM). Because GlcN concentrations that result in ATP loss (Fig. 4A) closely correlated with GlcN concentrations that lead to accumulation of GlcN-6-P (Fig. 2B), we postulate that phosphate depletion (resulting from excessive formation of GlcN-6-P) or the energy demands associated with massive phosphorylation lower cellular ATP levels. This idea is supported by the time course data depicted in Fig. 4B. The rapid decrease in ATP levels upon exposure to 2 mM GlcN ( $t_{1/2} = 8$  min) temporally corresponds to the rapid accumulation of GlcN-6-P that is observable by 5 min (Fig. 2A). It is interesting to note that ATP depletion also correlates with the decrease in UDP-GlcNAc levels seen at higher GlcN concentrations (Fig. 2D). One possible explanation is that ATP depletion (caused by excessive phosphorylation of GlcN) adversely affects one or more biosynthetic steps within the HBP.

*Glucosamine-induced Desensitization of the Insulin-responsive GTS and Corresponding Changes in Hexosamine Metabolites and ATP Levels*—As shown in Fig. 4C, treatment of isolated adipocytes for 5 h with various concentrations of GlcN led to progressive desensitization of the GTS. Substantial insulin resistance was observed using 200  $\mu$ M GlcN, and near maximal desensitization was obtained using 500  $\mu$ M GlcN. At these doses, there was little or no reduction in ATP levels (Fig. 4A), and there were minimal changes in GlcN-6-P levels (Fig. 2B). It

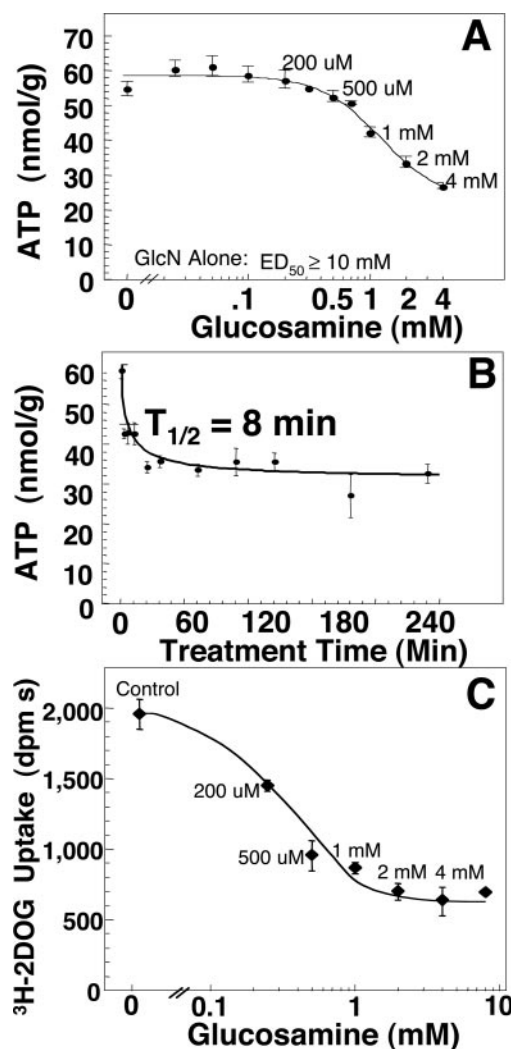


FIG. 4. Effects of glucosamine on ATP levels and induction of insulin resistance. A, adipocytes were treated for 20 min with 25 ng/ml insulin and then incubated for 4 h with the indicated concentrations of GlcN. B, cells were treated for 20 min with 25 ng/ml insulin and then incubated for the indicated times with 2 mM GlcN. Concentrations of ATP were measured as described under "Experimental Procedures." Each point represents the mean of duplicate determinations. C, adipocytes were incubated for 5 h with 25 ng/ml insulin and the indicated concentrations of GlcN. At the end of the incubation period, cells were thoroughly washed and insulin-stimulated rates of glucose uptake were measured using  $^3$ H-labeled 2-deoxyglucose (2-DOG) as described under "Experimental Procedures." Each point represents the mean  $\pm$  S.E. of three replicates.

is important to note that the development of desensitization best correlated with the glucosamine-induced increase in UDP-GlcNAc levels (Fig. 2D). This is consistent with the hypothesis that desensitization through the HBP is mediated by enhanced levels of UDP-GlcNAc and the resulting O-linked glycosylation of key regulatory proteins.

*Ability of Glucosamine to Elevate Hexosamine Metabolites under Euglycemic Conditions (Presence of 5 mM Glucose)*—Fig. 5 depicts the effect of 5 mM glucose on GlcN-mediated increases in the intracellular levels of GlcN-6-P and UDP-GlcNAc. From the two dose-response curves shown in Fig. 5, B and C, we conclude that higher doses of GlcN are required to increase GlcN-6-P in the presence of glucose (as compared with treatment with GlcN alone). In other words, there was a rightward shift in the GlcN dose-response curve from an  $ED_{50}$  of 0.9 mM (Fig. 2B, GlcN alone) to an  $ED_{50}$  of 2.3 mM (Fig. 5B). We also found that co-treatment with glucose reduced the maximal

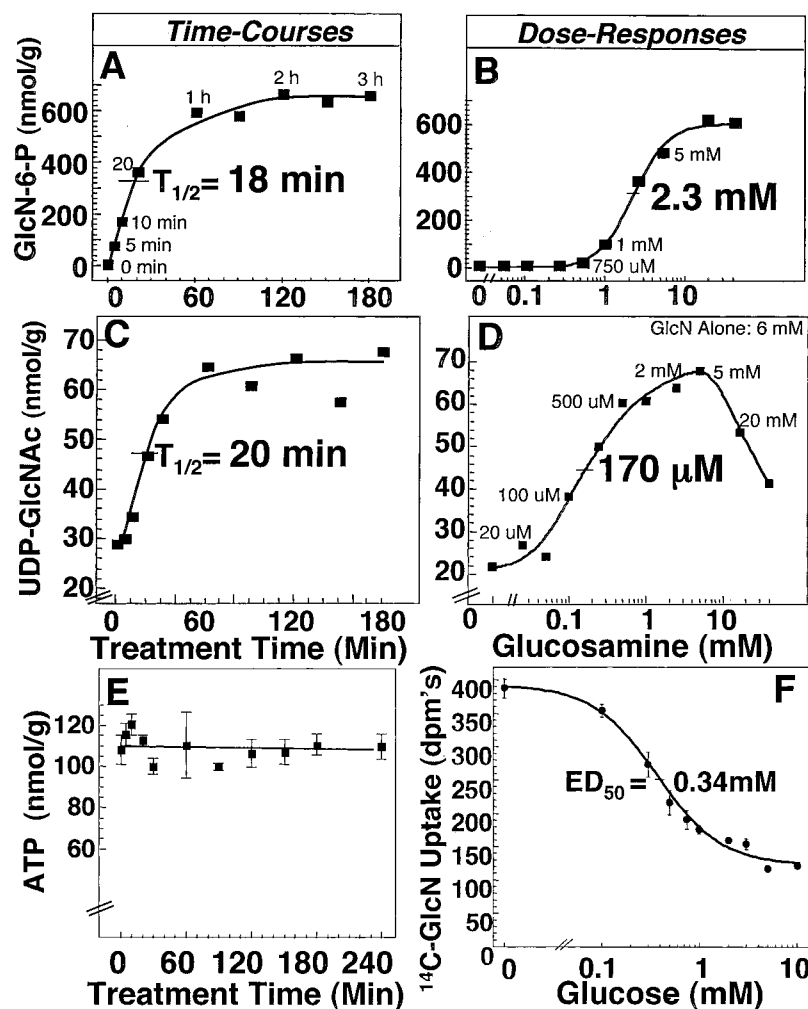


FIG. 5. Combined effect of glucose and glucosamine on GlcN-6-P, UDP-GlcNAc, and ATP levels. *A–E*, adipocytes were treated for 20 min with 25 ng/ml insulin and then incubated with 2 mM GlcN and 5 mM glucose for the indicated times (time courses) or incubated for 4 h with 5 mM glucose plus the indicated concentrations of GlcN (dose responses). Each point represents the mean of duplicate determinations. *F*, isolated adipocytes were pretreated for 20 min at 37 °C with 25 ng/ml insulin and then incubated for 20 min with the indicated concentrations of glucose. Glucosamine uptake was then assessed by adding [<sup>14</sup>C]glucosamine for 3 min and then measuring cell-associated radioactivity. Each point represents the mean ± S.E. of duplicate determinations.

amount of GlcN-6-P that accumulated in the presence high levels of GlcN. With GlcN alone GlcN-6-P levels rose to about 1500 nmol/g (Fig. 2A), whereas levels rose to about 600 nmol/g in the presence of glucose and GlcN (Fig. 5B).

The dose-response study shown in Fig. 5D shows that GlcN (in the presence of 5 mM glucose) increased UDP-GlcNAc levels with an ED<sub>50</sub> of 170 μM. Again, this represents a rightward shift in the GlcN dose-response curve compared with cells similarly treated in the absence of glucose (Fig. 2D). The rightward shift is most likely caused by reduced uptake of GlcN in the presence of glucose because glucose has a higher affinity for cell-surface glucose transporters and can competitively inhibit GlcN uptake. Although glucose was able to reduce the steady-state levels of GlcN-6-P by half (Fig. 5B), no such reduction was observed when measuring UDP-GlcNAc levels (Fig. 5D). In fact, the combination of glucose and GlcN increased UDP-GlcNAc levels by about 30% above those obtained with maximally effective concentrations of GlcN alone (compare Fig. 2D with Fig. 5D). When the dose-response curves in Fig. 5, B and D, are compared, it can be clearly seen that that increases in UDP-GlcNAc levels occur before any measurable changes in GlcN-6-P levels. Specifically, 750 μM of GlcN failed to elevate GlcN-6-P, whereas this dose almost maximally increased UDP-GlcNAc levels. This again illustrates that above a certain threshold concentration of GlcN, the ability of the HBP to process incoming substrate becomes increasingly rate-limited resulting in progressive accumulation of GlcN-6-P.

Time course studies, measuring formation of GlcN-6-P and UDP-GlcNAc during a 3-h incubation period with 5 mM glucose

and 20 mM GlcN (Fig. 5, A and C), indicated that the kinetics of hexosamine formation were unaffected by the inclusion of glucose. This conclusion is based on the finding of similar *t*<sub>1/2</sub> values for GlcN-mediated accumulation of GlcN-6-P in the absence (*t*<sub>1/2</sub> of 20 min, Fig. 2A) and presence of glucose (*t*<sub>1/2</sub> of 18 min, Fig. 5A). For UDP-GlcNAc measurements, a *t*<sub>1/2</sub> of 14 min was obtained with GlcN alone (Fig. 2C) compared with a *t*<sub>1/2</sub> of 20 min with glucose and GlcNAc treatment.

The results depicted in Fig. 5E reveal that 2 mM GlcN (plus 5 mM glucose) failed to diminish ATP levels during a 4-h incubation. This can be attributed in part to the ability of glucose to competitively inhibit the uptake of GlcN (Fig. 5F) and possibly the ability of glucose to serve as an energy source to prevent ATP depletion. Regardless of the explanation, it is important to note that GlcN can effectively increase UDP-GlcNAc levels without altering ATP levels. At very high GlcN concentrations (20 mM), we did find that GlcNAc could substantially reduce intracellular ATP (data not shown).

#### CONCLUSIONS

Treatment of isolated adipocytes with either glucose or GlcN causes progressive insulin resistance over several hours. Because glucose routing through HBP and the subsequent formation of hexosamine products underlies the induction of insulin resistance (10, 11), we have focused on the early events triggering desensitization, namely the formation of hexosamine metabolites. Our experimental approach was to pretreat adipocytes for 20 min with insulin (to stimulate the GTS) and then expose cells to glucose or GlcN for various times from 1 min to

4 h (time course studies). Dose-response studies over 4 h were also performed using various concentrations of glucose or GlcN. After treatment, adipocytes were lysed, and extracts were harvested and used to measure intracellular levels of G-6-P, GlcN-6-P, UDP-GlcNAc, and ATP.

When 20 mM glucose was added to insulin-treated cells, we found that intracellular G-6-P levels were rapidly increased. Maximal levels peaked very early ( $t_{1/2} < 1$  min) reflecting rapid initial uptake through the GTS. During the next 15 min, G-6-P levels gradually decreased to a new steady state reflecting an equilibrium between glucose efflux from cells and the rapid conversion of G-6-P to other metabolites. Levels of G-6-P remained elevated for at least 4 h and were related to extracellular glucose concentrations ( $ED_{50}$  of 4 mM). At 20 mM glucose, an increase in UDP-GlcNAc could be seen as early as 5 min. This indicates that glucose uptake and routing through the HBP is rapid. Levels of UDP-GlcNAc continued to rise over 60 min ( $t_{1/2} \sim 18$  min) until a new steady-state level was attained (from 1 to 4 h) at about twice the basal value. Dose-response studies showed that maximal levels were obtained at about 5 mM ( $ED_{50}$  of 1.2 mM). It is important to mention that the first product of the HBP (GlcN-6-P) was undetectable (data not shown). Thus, once GFAT catalyzes the conversion of F-6-P to GlcN-6-P, this product is readily processed through the HBP.

The addition of GlcN (2 mM) to insulin-treated cells resulted in a rapid rise in UDP-GlcNAc levels ( $t_{1/2} \sim 14$  min) that plateaued at a much higher level than seen in glucose-treated cells (4-fold for GlcN, 2-fold for Glc). One possible explanation for this relates to the known ability of UDP-GlcNAc to allosterically inhibit GFAT (19, 20), the first and rate-limiting enzyme of the HBP. With glucose, initial increases in UDP-GlcNAc levels would limit further flux into the HBP through allosteric inhibition of GFAT. This would result in a lower steady-state level of UDP-GlcNAc. Because GlcN enters the HBP at a step distal to GFAT (formation of GlcN-6-P), allosteric inhibition of GFAT would not diminish GlcN-induced formation of hexosamine products.

An important finding of the current study is that low doses of GlcN (up to 250  $\mu$ M) can markedly elevate UDP-GlcNAc levels ( $ED_{50} = 80$   $\mu$ M) without increasing GlcN-6-P. In this respect, GlcN closely mimics the action of glucose on the formation of hexosamine products. However, at higher levels of GlcN ( $ED_{50} = 900$   $\mu$ M), we observed a rapid and massive accumulation of GlcN-6-P. At 2 mM GlcN, a large increase in GlcN-6-P was seen by 5 min ( $\sim 400$  nmol/g) and continued to rise over 2 h ( $t_{1/2} \sim 20$  min) reaching a plateau at  $>1400$  nmol/g. A likely explanation for this rapid and extensive accumulation pertains to the ability of GlcN to bypass the rate-limiting enzyme of the HBP (GFAT). Thus, unrestricted flux into the HBP can rapidly exceed the biosynthetic capacity of the pathway, leading to a rapid buildup of GlcN-6-P.

One adverse consequence of excessive formation and accumulation of GlcN-6-P appears to be the depletion of cellular ATP. With GlcN concentrations below 250  $\mu$ M, neither ATP nor GlcN-6-P levels were altered, whereas from 250  $\mu$ M to 4 mM there was a sharp rise in GlcN-6-P accompanied by a drop in ATP levels. Similarly, GlcN time course experiments (using 2 mM GlcN) showed a rapid increase in GlcN-6-P by 5 min and a rapid decrease in ATP ( $t_{1/2} \sim 8$  min). Thus, it appears that phosphate sequestration (caused by excessive formation of GlcN-6-P) or the energy demands of phosphorylation can lower cellular ATP levels. Another consequence of elevated GlcN-6-P levels is a reduction in UDP-GlcNAc levels (from their maximal levels). Whether this decrease is related to ATP depletion or allosteric effects of GlcN-6-P on one or more enzymes within the HBP remains to be determined.

Several *in vivo* studies have shown that prolonged GlcN infusion in rats can increase skeletal muscle hexosamine levels and induce whole body insulin resistance (21–26). GlcN-induced insulin resistance is apparent after 90 min of infusion and progressively increases over 4–6 h. Levels of UDP-GlcNAc in muscle tissue are modestly increased (3- to 5-fold), whereas GlcN-6-P accumulates to very high levels (500- to 700-fold increase). This finding indicates that most *in vivo* studies use GlcN infusion rates that greatly surpass the capacity of the HBP in muscle tissue. To reproduce euglycemic-hyperinsulinemic clamp studies under defined *in vitro* conditions, we examined the interaction of GlcN and glucose in isolated adipocytes. Under our conditions, we also observed a modest increase in UDP-GlcNAc (about 4-fold) and a massive accumulation of GlcN-6-P (undetectable to  $>600$  nmol/g). However, we were better able to examine the kinetics of metabolite change because of the inherent advantages of an *in vitro* cellular system. Specifically, we found that steady-state levels were rapidly achieved ( $t_{1/2}$  of 18 min for GlcN-6-P;  $t_{1/2}$  of 20 min for UDP-GlcNAc). Moreover, we also found that low doses of GlcN could increase UDP-GlcNAc levels to near maximal levels without affecting GlcN-6-P levels.

Glucosamine is often used to investigate the basic mechanism(s) underlying glucose-induced insulin resistance because of its demonstrated ability to selectively enter the HBP. However, it is important to recognize that the cellular actions of GlcN may differ from those of glucose (11). For example, in contrast to the more selective flux of GlcN into the HBP, glucose can readily enter several other metabolic pathways (glycolysis, pentose, glycogen, etc.). Thus, glucose utilization through alternate pathways may modify the ultimate response of the HBP. Another important difference between glucose and GlcN pertains to our finding that high GlcN concentrations can overwhelm the biosynthetic capacity of the HBP resulting in massive accumulation of GlcN-6-P. The resulting rise in GlcN-6-P has been shown to alter cellular function through allosteric regulation of various enzymes such as hexokinase and glycogen synthase (24, 27–29). What constitutes an excessive concentration of GlcN depends on a number of variables. For *in vivo* studies, these would include circulating levels of glucose and insulin as well as GlcN infusion rates. For *in vitro* cellular studies, variables would include cell type, presence of competing sugars, type of glucose transport system, and whether insulin is used (for insulin-responsive glucose transport systems). Ultimately, meaningful interpretation of experimental results requires measurements of UDP-GlcNAc levels and assessment of GlcN-6-P levels.

Informed use of GlcN can facilitate our understanding of hexosamine-induced insulin resistance, whereas use of excessive concentrations of GlcN can generate data that lead to erroneous conclusions and generalizations. A case in point is the study of Hresko *et al.* (18), which concluded that GlcN-induced insulin resistance in 3T3-L1 cells is caused by depletion of intracellular ATP. As clearly shown in subsequent reports (30, 31), this conclusion was derived from a toxic side effect of GlcN that could be minimized by the judicious use of this potent agent.

Several studies have concluded that GlcN and glucose induce insulin resistance by different mechanisms in 3T3-L1 adipocytes and in rat skeletal muscle (32–34). Rather than postulating differences in mechanisms of action, we believe that GlcN can mimic hyperglycemic conditions by rapidly increasing flux through the HBP resulting in elevated levels of UDP-GlcNAc and increased O-linked glycosylation of regulatory proteins. Over several hours, these changes culminate in desensitization of the GTS. In this respect, the actions of both GlcN and glucose



are similar. However, unlike glucose high concentrations of GlcN can cause a massive intracellular accumulation of GlcN-6-P that results in allosteric changes in various enzymes and depletes ATP. These secondary changes impair insulin-stimulated glucose transport. Thus, it appears likely that the non-physiological actions of high dose GlcN treatment can obscure (or mask) the more physiological mechanisms operative at lower concentrations. Our current study clearly illustrates this point by showing that a low dose of GlcN (200  $\mu$ M) can enhance hexosamine flux, increase UDP-GlcNAc levels, and induce insulin resistance of the GTS. Importantly, such actions occur with little or no changes in GlcN-6-P levels and with no depletion of cellular ATP. By recognizing the multifaceted and complex actions of GlcN, it should be possible to avoid the potential pitfalls in the use of GlcN and improve the design and interpretation of future GlcN studies.

Acute and chronic hyperglycemia trigger a cascade of cellular events that culminates in impaired insulin action and insulin secretion. Glucose toxicity also underlies the progressive development of diabetic complications, which include retinopathy, nephropathy, neuropathy, and other microvascular and macrovascular disorders. Thus, hyperglycemia is not only a consequence of type 2 diabetes but is a pathophysiological factor that perpetuates and sustains the diabetic state. Numerous studies have implicated the HBP in the induction of insulin resistance. It is also thought that formation of hexosamine products may play a role in the development of secondary complications (35). Therefore, it is important to understand the sequence of events from initial influx of glucose to the resulting abnormalities of cellular function. In the current study, we used isolated adipocytes to obtain new insights into the dynamic and early actions of glucose and GlcN on hexosamine flux and the generation of GlcN-6-P and UDP-GlcNAc. These studies are particularly relevant because adipocytes represent a widely used cellular model system that has proven useful in elucidating the mechanism(s) underlying glucose-induced insulin resistance (5, 6, 10, 11) and in identifying various genes that are regulated through the HBP (36).

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