Glucosamine Regulation of Glucose Metabolism in Cultured Human Skeletal Muscle Cells: Divergent Effects on Glucose Transport/Phosphorylation and Glycogen Synthase in Non-Diabetic and Type 2 Diabetic Subjects*

THEODORE P. CIARALDI, LESLIE CARTER, SVETLANA NIKOULINA, SUNDER MUDALIAR, DONALD A. McCLAIN, AND ROBERT R. HENRY

VA San Diego Healthcare System and Department of Medicine (0673) (T.P.C., L.C., S.N., S.M., R.R.H.), University of California, San Diego, La Jolla, California 92093; and Veterans Affairs Medical Center and Department of Medicine (D.A.M.), University of Mississippi Medical Center, Jackson, Mississippi 39216

ABSTRACT

Chronic exposure (48 h) to glucosamine resulted in a dose-dependent reduction of basal and insulin-stimulated glucose uptake activities in human skeletal muscle cell cultures from nondiabetic and type 2 diabetic subjects. Insulin responsiveness of uptake was also found to be diminished in culture under conditions that retain the characteristics of the in vivo state. Insulin resistance in skeletal muscle of type 2 diabetic subjects may result from both intrinsic or genetic factors as well as acquired components (1). The acquired aspects of insulin resistance may involve metabolic variables such as insulin, glucose and lipids, all of which are elevated in type 2 diabetes. The deleterious effects of hyperglycemia, termed “glucose toxicity” have been shown to have particular importance, especially with regard to the development of diabetic complications (2). The nature of the mechanism by which cells sense and respond to hyperglycemia has been an area of considerable scientific interest. While the major metabolic fate of glucose in principal using tissues such as muscle and liver is primarily into oxidation and storage as glycogen (3), a small (1–3%) fraction is metabolized through the hexosamine pathway, providing substrate for protein glycosylation. Marshall and colleagues, working in primary cultures of rat adipocytes, showed that the ability of high glucose levels to induce insulin resistance required metabolism to glucosamine (4). Blocking the activity of the rate limiting enzyme for this step, glutamine: fructose-6-P amidotransferase (GFA), prevented this response (5).

Numerous other lines of evidence indicate that flux through the hexosamine pathway can influence glucose metabolism and insulin action (reviewed in Ref. 6). Overexpression of GFA, resulting in accumulation of glucosamine and other intermediates in the pathway, caused impairments in insulin stimulation of glycogen synthase (7). Direct addition of glucosamine, which enters cells via glucose transporters (5) and circumvents the rate limiting action of GFA, prevented this response (5).

Insulin resistance in skeletal muscle of type 2 diabetic subjects may result from both intrinsic or genetic factors as well as acquired components (1). The acquired aspects of insulin resistance may involve metabolic variables such as insulin, glucose and lipids, all of which are elevated in type 2 diabetes. The deleterious effects of hyperglycemia, termed “glucose toxicity” have been shown to have particular importance, especially with regard to the development of diabetic complications (2). The nature of the mechanism by which cells sense and respond to hyperglycemia has been an area of considerable scientific interest. While the major metabolic fate of glucose in principal using tissues such as muscle and liver is primarily into oxidation and storage as glycogen (3), a small (1–3%) fraction is metabolized through the hexosamine pathway, providing substrate for protein glycosylation. Marshall and colleagues, working in primary cultures of rat adipocytes, showed that the ability of high glucose levels to induce insulin resistance required metabolism to glucosamine (4). Blocking the activity of the rate limiting enzyme for this step, glutamine: fructose-6-P amidotransferase (GFA), prevented this response (5).

Numerous other lines of evidence indicate that flux through the hexosamine pathway can influence glucose metabolism and insulin action (reviewed in Ref. 6). Overexpression of GFA, resulting in accumulation of glucosamine and other intermediates in the pathway, caused impairments in insulin stimulation of glycogen synthase (7). Direct addition of glucosamine, which enters cells via glucose transporters (5) and circumvents the rate limiting action of GFA, prevented this response (5).

Numerous other lines of evidence indicate that flux through the hexosamine pathway can influence glucose metabolism and insulin action (reviewed in Ref. 6). Overexpression of GFA, resulting in accumulation of glucosamine and other intermediates in the pathway, caused impairments in insulin stimulation of glycogen synthase (7). Direct addition of glucosamine, which enters cells via glucose transporters (5) and circumvents the rate limiting action of GFA, prevented this response (5).

Received February 18, 1999.

Address all correspondence and requests for reprints to: Robert R. Henry, M.D., VA San Diego Healthcare System (V111G), 3350 La Jolla Village Drive, San Diego, California 92161. E-mail: rhenny@vapop.ucsd.edu.

* This work was supported by funds from the American Diabetes Association, the Whittaker Institute for Diabetes Research, Medical Research Service, Department of Veterans Affairs and Veteran Affairs Medical Center, San Diego, CA, and Grant M01 RR-00827 from the General Clinical Research Branch, Division of Research Resources, NIH.
tions, there was no difference in GFA activity between cells from nondiabetic and type 2 diabetic subjects (19). However, GFA activity was upregulated by either hyperglycemia or hyperinsulinemia and more effectively by the combination (19), suggesting that the differences found in muscle biopsies may reflect the influence of the in vivo metabolic environment. In the current studies we directly tested the effects of altered flux through the hexosamine pathway, distal to GFA, on glucose transport, phosphorylation and glycogen synthesis, comparing the behavior of cultured muscle cells from nondiabetic and type 2 diabetic subjects.

**Materials and Methods**

**Materials**

Human biosynthetic insulin was kindly supplied by Dr. Ron Chance of Eli Lilly & Co., Inc. (Indianapolis, IN). Cell culture materials were purchased from Irvine Scientific (Irvine, CA) except for skeletal muscle basal medium (SkGM), which was obtained from Clonetics Corp. (San Diego, CA). FBS was purchased from Gemini (Calabasas, CA). BSA (Cohn fraction V) was supplied by Roche Molecular Biochemicals (Indianapolis, IN). [1,2-3H]-deoxy-o-glucose, [2,3,4-3H]-glucose, U-[14C]-glucose and UDP-[14C]-glucose were purchased from New England Nuclear (Boston, MA). A polyclonal antibody against human GLUT3 was obtained from Chemicon International (Temecula, CA). Biologicals (Cambridge, MA). A polyclonal antibody against human GLUT3 was obtained from Chemicon International (Temecula, CA). Antirabbit IgG conjugated with horseradish peroxidase and the ECL chemiluminesence kit were obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). Protein assay kits, ion exchange resin and electrophoresis chemicals were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). Pepstatin, leupepin, phenylmethylsulfonyl fluoride (PMSF), 2-deoxyglucose, thymidine, glucosamine, glucosamine-6-P and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

**Human subjects**

Both nondiabetic and type 2 diabetic subjects were recruited for muscle biopsy. Glucose tolerance was determined from a 75 g oral glucose tolerance test (OGTT). Subject characteristics are summarized in Table 1. While both groups were, on average, obese (BMI > 27), the diabetic group was significantly more so. The response to the OGTT was no different in nondiabetic and type 2 diabetic subjects (19). However, glucose transport, phosphorylation and glycogen synthesis, comparing the behavior of cultured muscle cells from nondiabetic and type 2 diabetic subjects.

**TABLE 1.** Subject characteristics

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
<th>5.7 ± 0.5</th>
<th>15.8 ± 1.3*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mM)</td>
<td>274 ± 103</td>
<td>433 ± 147</td>
</tr>
</tbody>
</table>

*P < 0.05 vs. ND.

**Membrane preparation**

Cells for membrane preparation were grown in 100-mm dishes and treated as described for activity assays. Total membranes were prepared by the method developed by Walker et al. (25), as described previously (22). The total membrane pellet was resuspended in homogenization buffer and protein content determined.

**Detection of glucose transporter proteins**

Membrane preparations were diluted 1:1 in 2× Lamelli’s buffer without β-mercaptoethanol (26) and heated for 5 min at 90°C. Proteins were separated on 10% SDS-PAGE gels and then transferred to nitrocellulose (27). GLUT1 was identified using a rabbit polyclonal antisera against the rat brain glucose transporter (RaGLUT1; East Acers Biologicals). Polyclonal antisera specific for GLUT4 (RaIRGT) and GLUT3 were also employed. The second antibody was antirabbit IgG conjugated with horseradish peroxidase. Immune complexes were detected using an enhanced chemiluminescence kit. Exposure was limited to the linear range of density as determined by concentration curves established with human skeletal muscle total membranes, included as an internal control. Quantitation was performed with a scanning laser densitometer (ScanAnalysis, Biosoft).
Glycogen synthase activity

The activity of glycogen synthase was measured as described in detail previously (18) using modifications of the methods of Nuttall et al. (28) and Thomas et al. (29). Glycogen synthase activity was assayed at physiologic concentration of substrate (0.3 mM UDP[14C]glucose) in parallel incubations with 0.1 and 10 mM glucose-6-phosphate. For kinetic studies, total cell homogenates were filtered through a Sephadex G-25 column, to remove small molecular weight modifiers, and GS activity was measured at 0.1, 0.2, 0.6, 1.2, and 5.0 mM UDPG in the absence or presence of 0.1, 0.2, 0.3, 0.5, 2.0, and 10 mM glucose-6-phosphate (G-6-P) or glucosamine-6-phosphate (GlcN-6-P). GS activity is expressed as nmol of UDPG-glucose incorporated into glycogen/min/mg total protein, or as fractional velocity (FV), a percent (%) of the ratio of activity at 0.1 mM G-6-P/10 mM G-6-P.

Hexokinase activity

The same cell extracts were used for assay of glycogen synthase and hexokinase (HK) activities. Spectrophotometric analysis of total hexokinase activity was performed at 0.11 and 11 mM glucose with the method described by Vestergaard et al. (30). Potential effects of GlcN-6-P were tested by adding the metabolite directly to the enzyme assay. A parallel sample was heated at 45°C for 60 min to destroy HKII activity and assayed for heat-stable activity. HKII activity was calculated as the difference between total and heat-stable activity. Activity is presented as nanomoles of glucose converted/min/mg protein.

Glycogen synthesis

Glycogen synthesis was determined in differentiated myotubes as [3H]-glucose incorporation into glycogen during a 1 h incubation at 37°C (31). After incubation, cells were rinsed 4 times with 4°C PBS and solubilized with 1 N NaOH at 55°C for 1 h. An aliquot (100 μl) of the lysate was removed for protein analysis. Lysates were neutralized with 10 x HCl, boiled for 30 min and then cooled on ice. Glycogen was precipitated with 95% ethanol and the pellet washed by resuspension and precipitation. The final glycogen pellets were resuspended in 0.5 ml H2O, mixed with scintillation fluid and radioactivity determined by liquid scintillation counting. Results are expressed as nmol glucose converted to glycogen/mg protein/h.

Glycogen synthase immunoblotting

Western blot analysis was performed by the method of Burnette (32) as detailed previously (18). Glycogen synthase was identified using an affinity purified polyclonal antibody raised in rabbits against an oligopeptide (12-mer) specific for the carboxy terminal sequence of glycogen synthase (a gift from Dr. L. Groop, Malmo, Sweden). The secondary antibody was antimouse IgG conjugated with horseradish peroxidase. Proteins were visualized with the enhanced chemiluminescence Western Blot Detection Kit (Amersham Pharmacia Biotech) and exposed to autoradiograph film (XAR-5, Eastman Kodak Co., Rochester, NY). The intensity of the bands was quantified by scanning laser densitometry.

Statistical analysis

Statistical significance was evaluated using Student’s t test and two-tailed P values calculated. Paired analysis was performed for comparisons of acute and chronic insulin and glucosamine exposures in the same sets of cells. Significance was accepted at the P < 0.05 level.

Results

Glucosamine regulation of glucose uptake and phosphorylation

In initial studies to test the effect of increased flux through the hexosamine pathway on glucose uptake in cultured human muscle cells, cells were treated over the last 48 h of the fusion/differentiation period with 10 mM glucosamine in the presence of the standard 5.5 mM glucose of the media. These conditions resulted in no change in the extent of differentiation into myotubes, as determined by multinucleation (not shown). In cells from nondiabetic subjects, glucosamine treatment resulted in a down-regulation of 2-deoxyglucose uptake (Fig. 1). Basal uptake was reduced to 78 ± 4% of control values (P < 0.0005). Insulin-stimulated uptake was reduced to a similar extent (65 ± 3% of control, P < 0.0001).

![Fig. 1. Influence of glucosamine on deoxyglucose transport in cultured muscle cells from nondiabetic (ND) and type 2 diabetic subjects.](https://academic.oup.com/endo/article-abstract/140/9/3971/2990556/ND)
Glucosamine treatment also led to impaired insulin responsiveness as the maximal insulin stimulated increase in uptake was only $44 \pm 13\%$ of that in control cells ($P < 0.025$).

Deoxyglucose uptake in type 2 diabetic muscle cells was impaired compared with nondiabetic cells ($P < 0.05$ in both the absence and presence of insulin). However, similar effects of glucosamine treatment were observed in muscle cells from diabetic subjects. Both basal ($76 \pm 5\%$ of control, $P < 0.0025$) and insulin-stimulated ($74 \pm 6\%, P < 0.005$) uptake were down regulated after treatment. Insulin responsiveness was also reduced by glucosamine treatment, to $23 \pm 11\%$ of the stimulation observed in control diabetic cells.

Glucosamine effects on glucose uptake were concentration-dependent (Fig. 2). Nondiabetic and diabetic cells were equally sensitive, with half of the maximal down-regulation occurring at $2 \text{ mM}$ for both basal and insulin-stimulated uptake. Higher glucosamine concentrations ($20 \text{ mM}$) were deleterious, leading to a loss of cells (not shown).

Deoxyglucose uptake as measured in these studies represents both transport and phosphorylation of the glucose analog (33). Several approaches were taken to distinguish which, or both, of these processes were influenced by glucosamine. Cells were extracted after glucosamine and insulin treatment and hexokinase activity measured. Such analysis would reflect enzyme activity independent of substrate delivery, subcellular localization or the influence of potential allosteric regulators. Alternatively, cells were treated as for the uptake assay and cell-associated radioactivity separated into nonphosphorylated (free DOG) and phosphorylated (DOG-6-P) forms by ion exchange chromatography. An increase in free DOG following treatment would suggest a reduction in hexokinase activity. The results of these studies are presented in Table 2. Acute insulin exposure did not alter total hexokinase activity. There was no significant difference in hexokinase activity between nondiabetic and diabetic cells. Heat labile activity, representing hexokinase II, accounted for $48 \pm 5\%$ of the total activity; this proportion was similar in diabetic and nondiabetic cells. Hexokinase activity was not significantly influenced by glucosamine treatment in either nondiabetic or type 2 diabetic cells (Table 2).

The balance between free and phosphorylated deoxyglucose inside cells provides a more immediate measure of metabolism, including the influences of substrate entry, hexokinase activity and localization, and levels of intracellular metabolites. DOG phosphorylation in nondiabetic cells in the basal state was significantly reduced (more free DOG) following glucosamine treatment (Table 2). Because results are calculated as a percentage of the total intracellular DOG, this takes into account the reduced uptake of DOG ($20-25\%$ lower after glucosamine treatment in these cells). Thus glucosamine treatment led to absolute and relative decreases in deoxyglucose phosphorylation. Glucosamine treatment also impaired phosphorylation in insulin-stimulated cells. In cells from diabetic subjects glucosamine treatment also increased the fraction of free DOG in both the basal and insulin-stimulated conditions (Table 2). Thus glucosamine was similarly capable of inhibiting glucose phosphorylation in both nondiabetic and diabetic muscle cells, even as isolated hexokinase activity was unaltered. One possible cause for this discrepancy could be the influence of intracellular metabolites,
TABLE 2. Effect of glucosamine on hexokinase activity and deoxyglucose phosphorylation

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>Type 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+GlcN</td>
</tr>
<tr>
<td>Hexokinase (mmol/mg protein/min)</td>
<td>1.33 ± .20</td>
<td>1.27 ± .22</td>
</tr>
<tr>
<td></td>
<td>+Insulin</td>
<td>1.27 ± .21</td>
</tr>
<tr>
<td>DOG phosphorylation (% unphosphorylated)</td>
<td>Basal</td>
<td>23.9 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>+Insulin</td>
<td>16.6 ± 2.5</td>
</tr>
</tbody>
</table>

a P < 0.05 vs. basal.
b P < 0.05 vs. paired control.

TABLE 3. Comparison of glucose-6-P and glucosamine-6-P as allosteric modulators of glycogen synthase

<table>
<thead>
<tr>
<th></th>
<th>0.1 mm</th>
<th>10 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G-6-P</td>
<td>GlcN-6-P</td>
</tr>
<tr>
<td>Nondiabetic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td>0.30 ± 0.08</td>
<td>0.18 ± 0.06ab</td>
</tr>
<tr>
<td>Fractional Velocity</td>
<td>8.6 ± 1.4</td>
<td>5.0 ± 0.96ab</td>
</tr>
<tr>
<td>Type 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td>0.17 ± 0.02</td>
<td>0.07 ± 0.02ab</td>
</tr>
<tr>
<td>Fractional Velocity</td>
<td>5.1 ± 0.5</td>
<td>2.8 ± 0.44ab</td>
</tr>
</tbody>
</table>

Activities measured in the basal state. Results are average ± SEM, n = 4 for each group.
a P < 0.05 vs. G-6-P.
b Units, nmol UDPG-glucose/min/mg protein.
c Units, %.

0.01). To test this question further, GlcN-6-P levels in cells were determined and found to decrease with glucosamine treatment (0.16 ± 0.02 vs. 0.12 ± 0.03 mmol/mg protein, control vs. treated, respectively, P < 0.025), suggesting that added glucosamine is primarily converted to UDP-GlcNAc (9) and that direct inhibition of hexokinase by intracellular glucosamine is primarily converted to UDP-GlcNAc may contribute little to the effects shown in Table 2. These values are for nondiabetic and diabetic cells combined, as there were no consistent differences between groups.

Regulation of glucose transporter expression

One possible mechanism by which glucosamine treatment could lead to down-regulation of glucose uptake would be to reduce glucose transporter protein expression. Total membranes were prepared from nondiabetic cells and GLUT1, GLUT3, and GLUT4 expression measured by Western blotting. Glucose transport was measured in parallel in cells from the same subjects; the treatment conditions caused approximately 30% reductions in both basal and insulin-stimulated transport (not shown). Glucosamine treatment had no consistent effect on either GLUT1 (97 ± 15% of untreated control, n = 6), GLUT3 (102 ± 30%, n = 4) or GLUT4 (107 ± 12%, n = 4) protein expression. It should be emphasized that these measurements were made in total membranes and could not distinguish between cell surface and intracellular pools of transporters.

A major role of the hexosamine pathway is to provide substrates for protein glycosylation (34). Both GLUT1 and GLUT4 are highly glycosylated proteins (35) and it is possible that increased flux through the pathway could lead to altered transporter glycosylation. If this occurred, it was not detectable as a change in transporter mobility with the methods used (not shown).

Influence of glucosamine on glycogen synthase activity

The activity of the rate limiting enzyme for nonoxidative glucose metabolism in muscle, glycogen synthase, was measured after treatment under conditions that caused maximal down-regulation of uptake. As reported previously with other subjects (18), both basal glycogen synthase activity at 0.1 mm G-6-P and fractional velocity were lower (P < 0.05) in diabetic muscle cells compared with nondiabetics (Table 3). In contrast to the effect on transport/phosphorylation, glucosamine (10 mm) treatment resulted in an increase in the fractional velocity (FV) of glycogen synthase (Fig. 3). The extent of stimulation was similar in nondiabetic (129 ± 14% of control, P < 0.05) and diabetic (135 ± 8%, P < 0.05) cells. Insulin was still able to acutely stimulate glycogen synthase. In fact, in nondiabetic cells the insulin effect (to 175 ± 17% of basal) was augmented after glucosamine treatment, compared with control (145 ± 8% stimulation, P < 0.05), suggesting that the insulin and glucosamine effects were additive. Relative insulin stimulation in diabetic cells was not different before (164 ± 13%) or after (161 ± 14%) glucosamine treatment. This effect on glycogen synthase activity was dose dependent, with half maximal effects at 2 mm glucosamine in both nondiabetic and diabetic cells (not shown), similar to that for down-regulation of glucose uptake. The glucosamine-mediated increase in glycogen synthase is most likely due to a change in the activation state of the enzyme as neither total activity (measured at 10 mm G-6-P) nor glycogen synthase protein (as determined by Western blot), were altered by glucosamine treatment (not shown). These
later results were observed in both nondiabetic and type 2 cells.

One possible mechanism by which glucosamine could be increasing glycogen synthase activity would be by influencing intracellular levels of allosteric modulators of the enzyme. The most direct candidate would be glucosamine-6-phosphate (GlcN-6-P), the first fate of added glucosamine. To test this possibility, extracts of nondiabetic control cells were chromatographed over Sephadex G-25 to remove small intracellular modulators and exogenous G-6-P and GlcN-6-P compared for their ability to influence synthase activity. While there was significant enzyme activity in the presence of 0.1 mM GlcN-6-P (Table 3), it was only a fraction (59 ± 6%, P < 0.05) of the activity measured with 0.1 mM G-6-P. Activity at 10 mM GlcN-6-P was similarly reduced compared with 10 mM G-6-P (49 ± 7% of 10 mM G-6-P value). The fractional velocity at 0.1 mM GlcN-6-P was half that at 0.1 mM G-6-P. Thus, GlcN-6-P is only half as effective an allosteric activator of glycogen synthase as G-6-P. Similar results were obtained in diabetic cells (Table 3), where activity at 0.1 mM GlcN-6-P (52 ± 6% of G-6-P, P < 0.05) and 10 mM GlcN-6-P (48 ± 7%) were lower compared with G-6-P.

The results in Table 3 suggest that elevations in GlcN-6-P, arising from increased flux through the hexosamine pathway, could contribute to stimulation of glycogen synthase. However, this mechanism need not account for all of the stimulatory effect, for augmented synthase activity in glucosamine-treated cells was observed even after chromatographic removal of low molecular weight modulators (Fig. 4). In nondiabetic muscle cells, there was a tendency for both basal and insulin-stimulated synthase activities in glucosamine-treated cells to remain higher than controls after chromatography, though these differences did not attain statistical significance (P = 0.078). In diabetic cells the residual effects of glucosamine treatment on synthase were significant (P < 0.05) in both the basal and insulin-stimulated states (Fig. 4). Thus, nonallosteric mechanisms may also be active. These could involve covalent modification of the synthase. The fact that intracellular GlcN-6-P levels actually fell with glucosamine treatment indicate that nonallosteric mechanisms may represent the major means of synthase regulation in this situation.

Several approaches were taken to determine the consequences of the opposing changes in glucose transport/phosphorylation and glycogen synthase activity after glucosamine treatment. The total glycogen content of nondiabetic muscle cells was reduced following glucosamine treatment (from 0.85 ± 0.16 to 0.58 ± 0.16 mg glycogen/mg protein, P < 0.0001), despite the increase in synthase activity (Fig. 3). A similar decrease in cellular glycogen following treatment was observed in diabetic cells (from 0.94 ± 0.09 to 0.67 ± 0.09, P < 0.002).

Net glucose incorporation into glycogen represents the balance between glucose uptake, glycogen synthesis and breakdown during the time of the assay. This activity was measured in control and glucosamine-treated human muscle cells (Fig. 5), under conditions where uptake was reduced by approximately 30%, and synthase activity was elevated by approximately 30%. In nondiabetic cells, basal glucose incorporation was reduced by glucosamine treatment (68 ± 8% of control, P < 0.025), as was insulin-stimulated activity (55 ± 4% of control, P < 0.0001). While insulin stimulation of glycogen synthesis was retained in glucosamine-treated cells (136 ± 12% of basal, P < 0.02), both the absolute and relative increases were less than those seen in control cells (165 ± 13% of basal). Similar
behavior was observed in type 2 diabetic cells: both basal (74 ± 9% of control, \( P < 0.05 \)) and insulin-stimulated (72 ± 6%, \( P < 0.02 \)) synthesis were reduced following glucosamine treatment. Relative insulin stimulation was comparable in control (148 ± 18% of basal) and glucosamine-treated (145 ± 14%) diabetic cells.

**Discussion**

Correction of hyperglycemia improves insulin action in diabetic subjects (36), as well as a number of chemical and genetic animal models of insulin resistance (37, 38). Thus, hyperglycemia represents one of the major acquired factors...
leading to impaired glucose utilization and insulin resistance. Initial insight into the mechanisms by which hyperglycemia leads to this state of glucose toxicity was provided by the work of Marshall and colleagues (5, 39), who showed that the negative effects of hyperglycemia were mediated by flux through the hexosamine pathway (4). This involves conversion of glucose to glucosamine, mediated by glutamine:fructose-6-P amidotransferase (GFA). Studies in a number of model systems, including overexpression of GFA (7, 40) and infusion/treatment with glucosamine (9, 10, 41, 42), have confirmed that increased flux through the hexosamine pathway can lead to impaired glucose metabolism, including insulin resistance. Indeed, GFA may represent a major mechanism by which tissues sense and respond to the glucose level in their environment.

Studies in humans revealed that GFA activity in skeletal muscle biopsies was elevated in type 2 diabetic subjects (16). Such an elevation in GFA activity has the potential to exacerbate the deleterious effects of hyperglycemia by directing more glucose into the hexosamine pathway. Such differences were not seen when cultured skeletal muscle cells from non-diabetic and type 2 diabetic subjects were compared (19). Because GFA activity in cultured muscle cells can be regulated by media glucose and insulin levels (19), it is likely that the differences seen in biopsies reflect the influence of the in vivo metabolic environment. Interestingly, the relationship between insulin-stimulated whole body glucose disposal and GFA activity in cultured muscle cells from the same individuals was different in non-diabetic and type 2 diabetic subjects (19); suggesting that the link between production of GlcN-6-P and the response of glucose uptake to flux through the hexosamine pathway may be altered in diabetes. Further evidence for differences in the sensitivity of nondiabetic and diabetic skeletal muscle to glucose was observed in the effects of chronic hyperglycemia on glucose uptake in cultured muscle cells: hyperglycemia was able to down regulate uptake in diabetic cells while having no effect in nondiabetic cells (22). The current studies were intended to see if such differences extended to processes beyond GFA.

The ability of glucosamine pretreatment to down-regulate glucose uptake in cultured human muscle cells (Fig. 1) is similar to the results observed in primary cultured rat adipocytes (5, 43), fibroblasts (13), intact rats (41), and isolated muscles (11, 13). A report in cultured L6 muscle cells represents the one instance where disruption of the hexosamine pathway (inhibition of GFA) failed to perturb glucose transport (12). The glucosamine-induced reduction of glucose transport in cultured human muscle cells occurred in the absence of any change in total expression of GLUT1, GLUT3, or GLUT4 proteins. A similar lack of effect of glucosamine on transporter levels was seen after in vitro treatment of rat adipocytes (43) or muscle (11, 13), or in skeletal muscle following glucosamine infusion (41). Such observations led to the hypothesis that either insulin signaling or transporter translocation was the site of glucosamine-induced insulin resistance and glucose toxicity. Impaired GLUT4 translocation following glucosamine treatment has been demonstrated in skeletal muscle (41) and 3T3-L1 adipocytes (44). The relative diminution of insulin responsiveness of transport is evidence that increased flux through the hexosamine pathway also leads to a measure of insulin resistance. Glucosamine infusion has recently been shown to impair selected early events in insulin signaling (45, 46), but not others (13, 46); this topic certainly merits further study in human muscle. In addition, the fact that both basal and insulin-stimulated transport are impaired suggests that some basic aspect of transporter function is also influenced by glucosamine. Possibilities include transporter activity (44) and orientation in the membrane.

Both GLUT1 and GLUT4 are extensively glycosylated on a single asparagine residue (47). As a major product of the hexosamine pathway is UDP-N-acetylglucosamine, an important substrate for protein glycosylation (34), it is possible that elevated flux through the hexosamine pathway, with resultant increases in UDP-N-acetylglucosamine, could lead to altered transporter glycosylation. Because transporter glycosylation influences the affinity, activity, stability, and subcellular distribution of glucose transporters (47–49), alterations in transporter glycosylation could contribute to the impairments in transport activity resulting from glucosamine treatment, especially the proposed defect in insulin-stimulated translocation. Changes in transporter glycosylation are often detected by changes in electrophoretic mobility (35). No such differences were observed for either GLUT1 or GLUT4 after glucosamine treatment (not shown). Baron et al. also reported no change in rat skeletal muscle GLUT4 mobility after glucosamine infusion (41). These results suggest that transporter glycosylation is not grossly altered by glucosamine. However, more sensitive and specific analysis is necessary to rule out subtle changes in the extent or identity of transporter glycosylation. In addition, incorporation of labeled glucosamine into GLUT4-containing vesicles from skeletal muscle is elevated following glucosamine infusion (50), suggesting that glycosylation of proteins other than GLUT4 may be influenced and contribute to changes in glucose transport.

Beyond the reduction in glucose uptake, glucosamine treatment was also found to reduce glucose phosphorylation (Table 2). This is in agreement with the report of Furnsinn et al. (11), who found that glucosamine treatment of isolated rat soleus muscles decreased both glucose transport and phosphorylation. It has been demonstrated that hexokinase II activity and expression are reduced in skeletal muscle from type 2 diabetic subjects (51, 52), supporting the possibility that both glucose phosphorylation and transport are impaired in diabetes, just as they are by glucosamine. Evidence suggests that these defects in hexokinase II are reflective of the in vivo metabolic environment of the subject (52). Thus, it is not surprising that we found no difference in either total or hexokinase II activity between cells from nondiabetic and diabetic subjects (Table 2) because cells from all subjects were cultured under matched euglycemic and normoinsulinemic conditions. Hexokinase activity in cells did not respond to acute insulin treatment. In vivo responses of hexokinase II activity to insulin infusion are modest (51, 53), require greater than 3 h of insulin exposure (51), and follow increases in hexokinase II expression (54). It is likely that the time for insulin treatment of cultured muscle cells (1 h) was too brief to observe similar changes, even though this time was sufficient for full activation of glycogen synthase. Recent evi-
dence has indicated that more rapid insulin effects on hexokinase II in skeletal muscle involve a redistribution from the cytosolic to the particulate fraction (55); such a change would not be detectable in our total cell extracts.

It was initially unexpected that the same conditions of glucosamine treatment that resulted in significant impairments in deoxyglucose phosphorylation had no effect on hexokinase activity measured in cell extracts (Table 2). As mentioned previously, measurement of deoxyglucose phosphorylation reflects the combined roles of substrate delivery (transport) and hexokinase activity, as well as the influence of intracellular modulators. One of the most important might be GlcN-6-P because it can cause feedback inhibition of hexokinase (56). In intact cells GlcN-6-P might attain levels sufficient to inhibit hexokinase, yet be diluted to ineffective levels during extraction before the hexokinase assay, an idea supported by the observation that GlcN-6-P can directly inhibit hexokinase from cultured muscle cells. In addition, GlcN-6-P levels attained in rat muscle following glucosamine infusion were capable of decreasing hexokinase activity (57).

These results, together with the fact that hexokinase itself is unaltered following glucosamine treatment, suggests that impaired glucose phosphorylation is due to the intracellular environment, including allosteric factors, and not to changes in hexokinase expression or covalent modification of the enzyme that survive cell lysis.

Unlike the reductions in glucose transport/phosphorylation following glucosamine treatment, glycogen synthase fractional velocity was augmented following treatment (Fig. 3). Similar increases in glycogen synthase activity were also observed in glucosamine treated HIRc cells (13) and rat-1 fibroblasts overexpressing GFA (7). In all cases, total glycogen synthase activity was unaltered; rather it was the activation state of the enzyme that was effected (7). While GlcN-6-P can serve as an allosteric activator of glycogen synthase (7 and Table 3), both the results in human muscle cultures (Fig. 4) and GFA-over expressing fibroblasts suggest that additional mechanisms are also responsible for stimulation of glycogen synthase activity. As glycogen synthase activity is regulated by phosphorylation/dephosphorylation (58), it is possible that flux through the hexosamine pathway may influence a kinase or phosphatase acting on glycogen synthase.

Skeletal muscle from type 2 diabetic subjects displays impairments in glucose transport/phosphorylation (59) and glycogen synthase activity (60). These defects are most often preserved in cultured muscle cells from diabetic subjects (17, 18). The effects of glucosamine treatment on glucose transport, reduction in activity, and insulin responsiveness with no change in GLUT4 expression are similar to those of diabetes, so it is quite possible that flux through the hexosamine pathway could contribute to this aspect of diabetes in muscle. However, the relative effects of diabetes and glucosamine on glycogen synthase are opposite, suggesting that increased flux through the hexosamine pathway alone does not create a complete model for the diabetic state. It may also be that the glucosamine effect on transport/phosphorylation is the predominant influence as both total glycogen stores and net glycogen synthesis are reduced: in light of reduced uptake and augmented synthase, it appears to be uptake that determines nonoxidative glucose utilization. The activation of synthase observed in response to glucosamine could be an attempt to compensate for the other negative effects of hyperglycemia, including those on transport.

One interesting aspect of the effect of glucosamine on glucose uptake is the similar sensitivity of nondiabetic and diabetic cells to the sugar (Fig. 2), unlike the behavior in response to glucose (22). Such results suggest that the increased sensitivity of diabetic muscle to glucose resides before the generation of glucosamine. The most likely candidate for this step would be GFA. In parallel behavior, overexpression of GFA in rat fibroblasts increased sensitivity to the ability of glucose to cause insulin resistance (61). There were no major differences between nondiabetic and type 2 diabetic muscle cells with regard to the various effects of glucosamine treatment observed.

In summary, increased flux through the hexosamine pathway, caused in the current situation by glucosamine treatment, can impair glucose transport/phosphorylation and glycogen synthesis and lead to impaired insulin responsiveness, if not total insulin resistance. While increased flux through the hexosamine pathway might not generate all the features of the diabetic state in cultured muscle cells, it could certainly play a large role. The similar sensitivity of nondiabetic and diabetic muscle to glucosamine suggests that the greater negative impact of hyperglycemia in diabetes may be mediated before the formation of glucosamine, possibly at GFA.

References
14. Robinson KA, Weinstein ML, Linenmayer GE, Buse MG 1995 Effects of
diabetes and hyperglycemia on the hexosamine synthesis pathway in rat muscle and liver. Diabetes 44:1438–1446