Insulin Resistance of Glycogen Synthase Mediated by O-linked N-acetylglucosamine.

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SUMMARY

We have investigated the mechanism by which high concentrations of glucose inhibit insulin stimulation of glycogen synthase. In NIH-3T3-L1 adipocytes cultured in low glucose (2.5 mM, LG) the half-maximal activation concentration (A₀.₅) of glucose-6-phosphate was 162±15 µM. Exposure to either high glucose (20 mM, HG) or glucosamine (10 mM, GlcN) increased the A₀.₅ to 558±61 or 612±34 µM. Insulin treatment with LG reduced the A₀.₅ to 96±10 µM, but cells cultured with HG or GlcN were insulin resistant (A₀.₅ = 287±27 µM or 561±77 µM). Insulin resistance was not explained by increased phosphorylation of synthase. In fact, culture with GlcN decreased phosphorylation to 61% of the levels seen in cells cultured in LG. Hexosamine flux and subsequent enzymatic protein O-glycosylation have been postulated to mediate nutrient sensing and insulin resistance. Glycogen synthase is modified by O-linked N-acetylglucosamine and the level of glycosylation increased in cells treated with HG or GlcN. Treatment of synthase in vitro with protein phosphatase 1 increased basal synthase activity from cells cultured in LG to 54% of total activity, but was less effective with synthase from cells cultured in HG or GlcN, increasing basal activity to only 13% or 16%. After enzymatic removal of O-GlcNAc, however, subsequent digestion with phosphatase increased basal activity to over 73% for LG, HG, and GlcN. We conclude that O-GlcNAc modification of glycogen synthase results in the retention of the enzyme in a glucose-6-phosphate dependent state and contributes to the reduced activation of the enzyme in insulin resistance.
INTRODUCTION

Glycogen synthase, which incorporates activated glucose into glycogen, is a major gatekeeper of carbohydrate metabolism. The enzyme is highly regulated, both allosterically and by several kinases and phosphatases (1,2). Phosphorylation inactivates the enzyme. The process is complex, as there are nine phosphorylation sites, targeted by several kinases, each with different effects on enzyme activity (3-5). The inactivation of glycogen synthase can be overcome by allosteric interaction with glucose-6-phosphate (G6P)\(^1\) (6). Insulin stimulates glycogen synthase primarily through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, resulting in inhibition of glycogen synthase kinase-3 (GSK3) and dephosphorylation of the enzyme by protein phosphatase 1 (1,7,8). Protein phosphatase 1 is itself regulated by stimulation and by specific targeting to glycogen (9-11). With activation by insulin, glycogen synthase becomes more sensitive to G6P and basal, G6P-independent activity is increased.

In type 2 diabetes mellitus there is resistance to the stimulation of glycogen synthase by insulin and a reduction of glycogen synthase activity (12-14). How this insulin resistance is triggered is not known. Work from Marshall’s laboratory originally suggested that insulin resistance could be mediated by an increase in carbohydrate flux through the hexosamine biosynthesis pathway (HBP) (15). Consistent with that hypothesis, acute infusions of glucosamine or transgenic over expression in muscle and fat of the rate-limiting enzyme in the HBP, glutamine: fructose-6-phosphate amidotransferase (GFAT) result in insulin resistance (16,17).

The terminal metabolites of the hexosamine pathway are UDP-hexosamines. UDP-N-acetylglucosamine is a substrate for the cytosolic UDP-N-acetylglucosamine: peptide
glycosyltransferase (or O-glycosyl transferase, OGT) which glycosylates nuclear and cytosolic proteins with a single N-acetylglucosamine moiety on serine and threonine residues (O-GlcNAc) (18,19). This recently described protein modification is in many ways analogous to phosphorylation; it is dynamic and has been shown to occur exclusively on phosphoproteins. Additionally, it has been shown to often have a reciprocal relationship with the degree of phosphorylation of a protein (20,21). Recent studies have suggested possible links between the O-GlcNAc modification on proteins and the pathogenesis of diabetes and insulin resistance. For example, insulin resistance of eNOS stimulation results when the Akt phosphorylation site of eNOS is modified by O-GlcNAc (22). Transgenic over expression of OGT in skeletal muscle and fat results in the development of insulin resistance in mice, mimicking the effects of increased hexosamine flux (23).

We have previously demonstrated that treatment of fibroblasts with high concentrations of glucose or glucosamine results in decreased basal activity of glycogen synthase and decreased stimulation by insulin (24-26). To explore whether these effects might be due to direct modification of glycogen synthase by O-GlcNAc, we have examined glycogen synthase in differentiated NIH3T3-L1 adipocytes, treated with either low glucose, high glucose, or glucosamine. We show that treatment with high glucose or glucosamine results in insulin resistance, a reduction of basal glycogen synthase activity and decreased activation by G6P. These effects are associated with increased levels of O-GlcNAc modification of glycogen synthase, and can be reversed by enzymatic removal of O-GlcNAc.
EXPERIMENTAL PROCEDURES

Antibodies and Reagents: The following antibodies were used in the current study: anti-glycogen synthase (Chemicon International, Inc., Temecula, CA), anti-phospho-glycogen synthase (Oncogene, Boston, MA), anti-O-GlcNAc monoclonal IgM antibody (CTD 110.6, a gift of Dr. Gerald Hart, Johns Hopkins University, Maryland) (27), and horseradish peroxidase conjugated anti-rabbit and anti-mouse IgG (Amersham Pharmacia BioTech, Piscataway, NJ). Succinylated wheat germ agglutinin agarose (sWGA) was obtained from EY laboratories (San Mateo, CA). NIH 3T3-L1 cells were obtained from the ATCC (Rockville, MD). UDP-[6-3H]glucose was obtained from Amersham Pharmacia BioTech, DMEM and fetal calf serum were from Gibco Invitrogen Corporation (Grand Island, NY). The insulin used in this study was recombinant human insulin (NovolinR, NovoNordisk, Bagsvaerd, Denmark). 6-acetaminido-6-deoxy-castanospermine (CaspNAc) was obtained from Industrial Research Limited (Wellington, New Zealand). The protease inhibitors used were the Complete tablets from Roche Molecular Biochemicals (Indianapolis, IN). All chromatography media and columns were obtained from Amersham Pharmacia BioTech, with the exception of UDP-hexanolamine agarose, which was obtained from Sigma (St. Louis, MO). All other enzymes and chemicals were obtained from Sigma.

Differentiation, Treatment and Extraction of NIH 3T3-L1 adipocytes. NIH 3T3-L1 cells were grown in 10 ml of DMEM containing 20 mM glucose and 10% fetal calf serum in 10 cm plates (Corning Incorporation, Corning, NY) and with 10% CO2. Two days after confluence 1 µM dexamethasone, 1µg/ml insulin and 0.5 mM isobutylmethylxanthine were added for 3 days, followed by 3 days with insulin alone. The cells were additionally cultured in DMEM with 20
mM glucose and 10% fetal calf serum for 5 to 10 days, with the medium being changed every 3 days. Cells were passaged every 3 days with care taken to ensure that confluence was not reached before passaging (28,29). Experimental protocols were then begun. The differentiated adipocytes were placed in DMEM containing 1% fetal calf serum and either 2.5 mM glucose (low glucose, LG), 20 mM glucose (high glucose, HG), or 2.5 mM glucose plus 10 mM glucosamine (glucosamine, GlcN) for 24 h and again 30 min prior to harvesting. For insulin-treated dishes, insulin (10 nM) was added 30 minutes prior to harvesting. The cells were harvested by placing on ice, washing twice with ice cold KRBH (25 mM HEPES pH 7.4, 150 mM sodium chloride, 4.4 mM potassium chloride, 1.2 mM sodium phosphate pH 7.4, 1 mM magnesium chloride and 1.9 mM calcium chloride) and then extracted with 0.75 ml of extraction buffer (50 mM HEPES pH 7.4, 100 mM sodium chloride, 5% glycerol (v/v), 1 mM 2-acetamido-1-amino-1,2-dideoxy-glucopyranoside, 40 mM sodium fluoride, and protease inhibitors). If β-D-hexosaminidase or phosphatase digests were to be conducted, then the extraction buffer consisted of 25 mM HEPES pH 7.4, 100 mM sodium chloride, and 5% glycerol with protease inhibitors. The resulting extracts were sonicated with a Sonic Dismembrator F60 for 15 s at setting 4 (Fischer Scientific, Pittsburgh, PA) and centrifuged at 20 000g for 2 minutes at 4°C. The supernatant was aspirated and aliquots immediately frozen in liquid nitrogen.

Glycogen Synthase Assay. The assay for glycogen synthase was based on that published by Thomas et al. (30). Extracts from differentiated adipocytes (7.5 µg protein) were mixed in a volume of 100 µl with 10.0 µmol HEPES pH 7.4, 0.5 µmol EDTA pH 7.4, 0.8 mg glycogen (type-III from rabbit liver), 0.2 µmol UDP-glucose, 10 µl glycerol, 0 – 0.3 µmol G6P and 0.4 µCi UDP-[6-3H]glucose and incubated for 45 min at 37°C. The G6P concentrations used (0 – 3
mM) reflect the physiological range in vivo (31). The incubation was terminated by application to Whatman qualitative filter paper number 3 (Maidstone, England) and immersion in 60% (v/v) ethanol. After 5 washes in 200 ml of 60% ethanol, the paper squares were washed once in acetone, dried and assayed for tritium. All assays were done in duplicate. The incorporation of tritium was found to be optimal at 37°C and linear for 120 minutes. The resulting data were analyzed by the least-squares algorithm, for a single rectangular hyperbola, using SigmaPlot for Windows version 4.0 (SSPS Science, Chicago, IL). G6P-independent glycogen synthase activity (I-form) is defined as basal activity. The half-maximal activation value (A0.5) was defined as the concentration of G6P to achieve 50% activation of the G6P dependent activity (D-form). P values for the A0.5 value and basal activity were determined using the values from each separate experiment and conducting a T-Test on Microsoft Excel 98 (Microsoft Corporation). The total activity (I-form + D-form) was defined as the activity at maximal G6P (3 mM).

**Western Blotting.** Protein concentrations in adipocyte extracts were determined using the Bio-Rad protein reagent (Hercules, CA). Extracts were prepared for gel electrophoresis by dilution with extraction buffer and 5 x Laemmli buffer (32). 10 µg of protein were added to each lane. SDS/PAGE was conducted using the Bio-Rad Mini-PROTEAN 3 electrophoresis cell, and resolved proteins were transferred to immobilon-P SQ transfer membrane (Millipore Corporation, Bedford, MA). Resulting blots were blocked with TBST (20 mM Tris pH 7.4, 150 mM sodium chloride and 0.5% Tween-20) containing 4% (w/v) non-fat dried milk for 1 h at room temperature or overnight at 4°C. 4% (w/v) bovine serum albumin was used in lieu of dried milk for detection with the anti-O-GlcNAc antibody. Blots were incubated with primary antibodies for 1 h at room temp, washed 3 times in TBST, and then incubated with the appropriate
horseradish peroxidase conjugated secondary antibody for 1 h. The blots were washed 5 times in TBST and imaged by treating with Super Signal West Femto reagents (Pierce, Rockford, IL) and exposure to Hyperfilm (Amersham Pharmacia BioTech). Densitometry measurements were obtained using a UMAX Astra 3450 scanner (UMAX technologies, Fremont, CA) and NIH image 1.62 software. All densitometric values were normalized to the signal obtained from the low glucose cultured extract.

*Immobilization of Glycogen Synthase with Wheat Germ Agglutinin.* 400 µl of extracts (2 mg protein /ml) were incubated with 50 µg sWGA and 600 µl of RIPA buffer (11 mM sodium phosphate pH 7.4, 150 mM sodium chloride, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 40 mM sodium flouride, 0.5 mM 2-acetamido-1-amino-1,2 dideoxy-glucopyranose, and protease inhibitors). The preparation was incubated on a rotator for 16 h at 4˚C and washed 3 times with extraction buffer. Bound proteins were eluted by boiling in Laemmli buffer and resolved by SDS/PAGE.

*Purification of Glycogen Synthase from Rabbit Muscle.* Rabbit muscle (500g, Pel-Freez, Rogers, AR) was homogenized in 500 ml 2 x standard buffer to give a final concentration of 100 mM HEPES 7.4, 10 mM EDTA, 4 mM DTT, 20% glycerol, 200 mM GlcNAc (to inhibit hexosaminidases) and protease inhibitors. The homogenate was centrifuged at 13,300 g for 1 h at 4˚C and the resulting supernatant was precipitated with 313 g/l of ammonium sulphate. The pellet was resuspended in standard buffer and applied to a phenyl sepharose column (2.5 x 40 cm) equilibrated in standard buffer containing 1 M sodium chloride and 0.5 M ammonium sulphate. The flow through fraction was dialyzed against 10 volumes of standard buffer using
Spectro/Por dialysis tubing (Spectrum Laboratories Inc, Rancho Dominguez, CA) and applied to a ConA sepharose column (1 x 30 cm) equilibrated in standard buffer. The filtrate from the ConA column was then applied to a HiPrep SephQ XL column (10/10) equilibrated in standard buffer and eluted with a gradient of 50 to 500 mM sodium chloride. The active fractions were pooled and concentrated to 5 ml in Vivaspin-15 centrifugal concentrators (MWCO 10,000, Millipore Corporation, Bedford, MA). The preparation was then applied to an UDP-hexanolamine column HR (5/15) (Sigma) and eluted with a gradient of 0 – 1 M sodium chloride. The active fractions were desalted into standard buffer and concentrated to 250 µl in Vivaspin-0.5 centrifugal concentrators (MWCO 10,000). More than 95% of the protein in the active fractions reacted on western blotting with the anti-glycogen synthase antibody.

**Labeling of Glycogen Synthase with [6-3H]galactose.** Purified glycogen synthase (25 µg) from rabbit muscle was incubated for 16 h on ice with 75 mUnits of autogalactosylated β-1,4galactosyltransferase, 50 nmol of MnCl2, 10 nmol AMP, 0.1 µmol HEPES pH 8.0, 0.1 µmol galactose, and 40 µCi of UDP-[6-3H]galactose in 100 µl (33). The mixture was concentrated to 15 µl using a Vivaspin-0.5 centrifugal concentrator (MWCO 30,000, Millipore Corporation, Bedford, MA), boiled with 5 µl 5 x Laemmli buffer and analyzed by SDS/PAGE and autoradiography.

**Digestion of Adipocyte Extracts with Hexosaminidase and Protein Phosphatase 1.** Phosphatase digests were conducted by treating 120 µg protein at 37°C for 30 min in 100 µl of a buffer consisting of 50 mM HEPES pH 7.4, 10% glycerol, 1.2 mM manganese chloride, protease inhibitors (Roche) and 10 Units of the recombinant active subunit of protein phosphatase 1α.
When extracts were to be treated only with phosphatases, the treatments were done in the presence of 100 µM 2-acetamido-2-deoxy-castanospermine (CaspNAc). Complete digestion had occurred by 15 minutes (data not shown). For digestion with β-D-hexosaminidase the pH of the extract (100 µg protein in 25 µl) was adjusted to 5.5 by the addition of 25 µl hexosaminidase buffer (20 mM sodium citrate pH 4.5, 10% glycerol, 100 mM sodium chloride and protease inhibitors) with or without 2 Units of β-N-acetylglucosaminidase from jack beans (Sigma) in the same buffer. After the addition of 5 µl 1 mg/ml bovine serum albumin (Roche) and 5 µl 20 mg/ml glycogen, the preparation was incubated for 2 h at 30°C. The reaction was stopped by the addition of 20 µl 100 mM HEPES pH 8.8 and 1 µl of 100 mM CaspNAc (35). When CaspNAc was added before hexosaminidase, no change in glycogen synthase was observed (data not shown). After subsequent phosphatase digestion, the glycogen synthase activation was measured by duplicate assays at 0 and 3 mM G6P.

RESULTS

Glycogen Synthase from Cells Cultured with High Glucose or Glucosamine Exhibits Insulin Resistance and Reduced Sensitivity to Glucose-6-Phosphate. Differentiated NIH 3T3-L1 adipocytes were cultured for 24 hours with 2.5 mM glucose (low glucose), 20 mM glucose (high glucose) or 10 mM glucosamine plus 2.5 mM glucose (glucosamine). These treatments had a significant effect on the sensitivity of glycogen synthase to G6P. In cells cultured in low glucose, glycogen synthase was sensitive to G6P (A₀.₅ = 162 ± 15 µM) and became more sensitive when cells were treated with insulin (A₀.₅ = 96 ± 10 µM, p < 0.05, Figure 1a and d). Glycogen synthase from cells cultured in high glucose was less sensitive to G6P (A₀.₅ = 558 ± 61 µM, p < 0.02) and insulin did not increase the sensitivity to G6P to same degree as was observed
with low glucose ($A_{0.5}= 287 \pm 27 \, \mu M$, $p = 0.1$, Figure 1b and d). Culture of cells in glucosamine led to a similar degree of insensitivity to G6P ($A_{0.5}= 612 \pm 35 \, \mu M$, $p < 0.005$) and resulted in complete insulin resistance ($A_{0.5}= 561 \pm 77 \, \mu M$, $p = 0.86$, Figure 1c and d). Similar results were obtained from cells cultured in 5 mM glucosamine ($A_{0.5}= 728 \pm 132 \, \mu M$, data not shown). Total activities ($V_{\text{max}}$) of glycogen synthase did not differ among experimental treatments (see Figure 1 Legend).

Culture with high glucose or glucosamine also affected the G6P-independent, or basal activity of glycogen synthase. When adipocytes cultured with low glucose were treated with insulin the basal activity increased from $8.7 \pm 1.0\%$ to $18 \pm 1\%$ of total activity ($p < 0.002$). Both high glucose and glucosamine treatments resulted in lower basal synthase activities and less insulin stimulation. When cultured with high glucose, insulin treatment increased G6P-independent activity from $1.2 \pm 0.1\%$ to only $4.7 \pm 0.7\%$ ($p < 0.005$ and $p < 0.001$, with and without insulin, compared to low glucose). Cells cultured with glucosamine showed a change of basal activity from $3.0 \pm 0.5\%$ to $7.4 \pm 1.8\%$ with insulin stimulation ($p = 0.075$ and $p < 0.005$, with and without insulin, compared to low glucose).

Changes in Glycogen Synthase Activity Induced by Treatment in High Glucose or Glucosamine Do Not Correlate with the Phosphorylation State of the Enzyme. Normally an increase in the $A_{0.5}$ value for G6P and concomitant decrease in basal activity of glycogen synthase would be associated with increased phosphorylation. We therefore examined the phosphorylation state of the enzyme, using a polyclonal antibody against a phosphopeptide of the GSK-3 recognition region of the enzyme (hGS 642 – 662) (Figure 2). Synthase from cells cultured with high
glucose showed a slight increase in phosphorylation relative to low glucose treatment that was not statistically significant (39 ± 29%; p = 0.17). Paradoxically, cells cultured with glucosamine showed a decrease in phosphorylation to 61 ± 9% of the level seen in low glucose (p < 0.001), despite the high A₀.5 value for G6P and lower basal activity.

The phosphorylation of the enzyme was responsive to insulin (Figure 2). Glycogen synthase from adipocytes cultured with low glucose showed a 70 ± 7% decrease in phosphorylation when treated with 10 nM insulin (p < 0.001). However, adipocytes treated with high glucose or glucosamine exhibited insulin resistance. In these cases, phosphorylation decreased by 44 ± 11% (p < 0.05) and 30 ± 13% (p = 0.10) after insulin treatment, less than that observed for cells in low glucose (p < 0.05).

**High Glucose and Glucosamine Treatments Increase Global Levels of Protein Modification by Terminal O-GlcNAc.** The changes in glycogen synthase seen in cells cultured in high glucose or glucosamine were not paralleled by predicted changes in phosphorylation. For example, the A₀.5 value for G6P was high in glucosamine treated cells despite lower levels of phosphorylation (Figures 1 and 2). Increased flux in the hexosamine pathway, resulting in increased levels of protein modification by O-linked GlcNAc, has been proposed as a general mechanism for regulation of metabolism by carbohydrate levels (21,36,37). The global level of protein O-glycosylation, as determined from immunoblots stained with the CTD 110.6 monoclonal antibody, was increased 190 ± 30% (p<0.05) in adipocytes cultured with high glucose (Figure 3, lanes 3 and 4) relative to low glucose (lanes 1 and 2) (27). Cells treated with glucosamine (lanes
5 and 6) increased levels of O-GlcNAc to 610 ± 70% (p<0.001). Treatment with insulin did not significantly affect the levels of terminal O-GlcNAc (Figure 2, lanes 2, 4 and 6).

**Glycogen Synthase is an O-GlcNAc Protein that is Dynamically Glycosylated.** To determine if glycogen synthase itself is modified by O-GlcNAc, highly purified glycogen synthase from rabbit muscle was resolved on SDS/PAGE. The protein, stained with Coomassie Blue, is shown in Figure 4a lane 1. The stained bands are recognized by anti-glycogen synthase antibody (lane 2) and are consistent with the apparent molecular mass of glycogen synthase (84 kd) and previously described degradation products (38). The purified glycogen synthase also reacted with an O-GlcNAc specific antibody (lane 3) and was radiolabeled after treatment with UDP-[3H]galactose and β1,4 galactosyltransferase, a method that specifically probes for terminal GlcNAc (lane 4) (33). The radiolabeling procedure was also performed using ovalbumin, a protein known to contain terminal GlcNAc, and autogalactosylated galactosyltransferase as positive and negative controls (Figure 4a, lanes 5 and 6).

To demonstrate that glycogen synthase from adipocytes was also modified by O-GlcNAc and that the level of the O-GlcNAc modification was dynamic, extracts from cells treated in low glucose, high glucose and glucosamine were incubated with immobilized sWGA agarose to immobilize hyperglycosylated O-GlcNAc proteins. After SDS/PAGE, the amount of glycogen synthase that bound to sWGA was measured by immunoblotting. The level of glycogen synthase binding to sWGA increased 190 ± 40% in cells cultured in high glucose relative to low glucose (Figure 4b). Glucosamine treatment increased the level of glycogen synthase binding to sWGA by 630 ± 70%. Insulin treatment did not affect the level of binding to sWGA. Although
the blot shows an apparent increase in glycosylation of glycogen synthase with insulin in low glucose, this trend was not significant (42 ± 40 % increase, p = 0.4, n = 4).

*O-GlcNAc Blocks Phosphatase-Mediated Activation of Glycogen Synthase.* In order to gain direct evidence of the role of O-linked GlcNAc in glycogen synthase regulation, cell extracts were treated *in vitro* with β-hexosaminidase and/or the active subunit of human protein phosphatase 1 to remove the O-GlcNAc and/or the phosphate from the protein. Protein phosphatase 1 targets glycogen synthase in vivo, mediating insulin stimulated activation (7,9). When extracts from low glucose cultures were digested with protein phosphatase 1 *in vitro*, basal activity of glycogen synthase increased to 54 ± 7% compared to 12 ± 2% in non-digested extracts (p < 0.001, Figure 5a). When extracts from high glucose cultures were digested with phosphatase, the basal activity increased only to 13 ± 2%. Extracts from glucosamine cultured cells were also resistant to phosphatase treatment, with basal activity increasing to only 16 ± 2%. Consistent with the change in basal activity, the $A_{0.5}$ concentration for G6P decreased with phosphatase digestion of the low glucose cultured extract, from 78 ± 12 µM to 13 ± 5 µM G6P (p < 0.025). The decrease in the $A_{0.5}$ value was less in the high glucose and glucosamine extracts (51 ± 6 µM G6P for high glucose and 37 ± 4 µM G6P for glucosamine treatment). The experiments above were performed in the presence of CaspNAc, an inhibitor of the endogenous O-GlcNAcase activity in the cell extracts.

We next determined if the resistance to activation of glycogen synthase by protein phosphatase 1 was due to modification by O-GlcNAc. Adipocyte extracts without added CaspNAc were predigested with β-D-N-acetylglucosaminidase before phosphatase digestion. With removal of
O-GlcNAc, glycogen synthase could be normally activated (Figure 5b). With combined hexosaminidase and phosphatase digestion, basal activity increased to 74 ± 3%, 77 ± 3% and 80 ± 0% of total activity for low glucose, high glucose and glucosamine cultured extracts respectively. A similar increase also occurred in samples from insulin-treated cells (data not shown). Digestion of the extracts with hexosaminidase alone produced smaller increases in basal activity to 7.3 ± 0.3%, 15 ± 0.0% and 11 ± 1%, respectively. To confirm that the phosphatase digestion was complete, a western blot of each digest condition was stained with the anti-phosphoglycogen synthase antibody (Figure 5c).
DISCUSSION

Acute regulation of net glycogen synthesis by carbohydrate occurs through multiple mechanisms, including substrate availability, hormone signaling and allosteric activation. In addition, chronic hyperglycemia leads to changes in glycogen synthase activity by mechanisms that remain poorly understood. Our data demonstrate that one mechanism affecting synthase activity is mediated by increases in hexosamine flux and subsequent O-glycosylation of the enzyme. A nutrient sensing and signaling role for the hexosamine biosynthetic pathway (HBP) was first demonstrated by Marshall, who showed that the HBP mediated the ability of high glucose to induce insulin resistance in cultured adipocytes (15). Since then, studies in cultured cells and animals have verified that the HBP plays a major role in the regulation of metabolism and growth (16,17,24,36,39-46). The HBP has also been shown to be responsible for glucose regulation of several proteins including TGFα, steroid response element binding protein 1, TGFβ1, plasminogen activator inhibitor-1, leptin, NFκB and endothelial nitric oxide synthase (eNOS) (17,22,47-55). Flux through the HBP is responsive not only to glucose but also to fatty acids and oxidative stress (56-58).

UDP-GlcNAc, the terminal metabolite of the HBP, is a substrate for protein glycosylation, suggesting a possible mechanism for the regulatory effects of the hexosamine pathway. Glycosylation of secreted or plasma membrane proteins is not responsive to glucose flux (59). However, O-linked glycosylation of cytosolic proteins is substrate-limited and proportional to glucose flux, making feasible a nutrient sensing function for that pathway (59-62). OGT, which catalyzes the modification of cytosolic proteins by GlcNAc at serine and threonine residues, has a high $K_m$ value, allowing the production of UDP-GlcNAc to be reflected in levels of protein
glycosylation (19,60,63). Several lines of evidence now support the hypothesis that the effects of the HBP on insulin resistance are mediated by the modification of proteins by O-GlcNAc. Firstly, pharmacological inhibition of O-GlcNAc removal from proteins results in insulin resistance at the level of Akt activation and a reduction of glucose uptake (64). Secondly, a transgenic mouse model that over-expresses OGT in fat and muscle exhibits insulin resistance and hyperleptinemia independently of UDP-GlcNAc levels (23). Direct evidence for a role of O-GlcNAc in modifying protein behavior is the recent demonstration that O-GlcNAc modification of eNOS at the Akt phosphorylation site blocks normal Akt-mediated insulin activation of eNOS and lowers eNOS activity in vascular endothelial cells (22).

The current study confirms the above findings that O-GlcNAc modification of proteins can be responsible for the development of insulin resistance and that O-GlcNAc can directly modify the kinetic properties of an enzyme. Activation of glycogen synthase by insulin has been known to be decreased in cells exposed to high concentrations of glucose or glucosamine (24-26,43). We show here that this insulin resistance of glycogen synthase stimulation can be explained by its modification by O-GlcNAc. Namely, the enzyme becomes intrinsically resistant to activation by protein phosphatase 1, which normally mediates activation of the enzyme upon insulin stimulation (7-9). The current studies do not exclude other HBP-mediated events that might affect signal transduction upstream of glycogen synthase. For example, there is evidence that PI3K and Akt activation are affected by hexosamine flux, and many proteins including IRS1 and GSK3 are known to be glycosylated (64-69). Thus, the HBP may operate on several levels to coordinately alter hormone signaling and metabolism in response to excess nutrient flux.
The simplest model for the effect of O-GlcNAc on glycogen synthase is that O-GlcNAc is inhibitory in a manner analogous to phosphate. Enzyme inhibition in this model would be proportional to flux through the HBP and provide a parallel means of regulation dependent on the nutritional state of the cell. Thus, in situations of excess nutrient flux, synthase activity would be held in a state that could not be activated by insulin because insulin does not stimulate deglycosylation of proteins as it does dephosphorylation. This would be an adaptive response to excess feeding, aiding in the limitation of glycogen accumulation and allowing excess calories to be diverted for storage as fat. This model would predict that the same sites used for inhibitory phosphorylation would also be used for glycosylation, and that loss of phosphorylation sites (e.g. by mutagenesis) would parallel loss of glycosylation sites and loss of inhibition. This model is generally consistent with our observations of a reciprocal relationship between phosphorylation and O-glycosylation (Figure 2a and 4b). An alternative model is that O-GlcNAc indirectly regulates phosphorylation events on the enzyme, perhaps by changing the affinity or specificity of glycogen synthase for one or more of its regulatory phosphatases and kinases. It was noted that in spite of relatively low levels of phosphorylation on glycogen synthase, hexosaminidase treatment alone was insufficient to restore significant basal activity. This implies that a complete understanding of the regulation of synthase by these modifications will require a complete mapping of all such sites. Further investigation of the role of the O-GlcNAc modification on glycogen synthase promises to complement our current understanding of glycogen metabolism and of phosphate-based regulation in general. The model of regulation of glycogen synthase by O-GlcNAc should also further elucidate the mechanisms by which excess nutrients contribute to insulin resistance and type 2 diabetes through physiologic and adaptive pathways that become maladaptive when the organism is faced with a chronic excess of food.
REFERENCES


FOOTNOTES

1The abbreviations used are: G6P, glucose-6-phosphate; PI3K, phosphatidylinositol-3-phosphate; GSK3, glycogen synthase kinase-3; HBP, hexosamine bioynthesis pathway; GFAT, glutamine: fructose-6-phosphate amidotransferase; OGT, O-glycosyl transferase or UDP-N-acetylglucosamine: peptide glycosyltransferase; O-GlcNAc, O-linked N-acetylglucosamine; sWGA, succinyl wheat germ agglutinin; LG, low (2.5 mM) glucose; HG, high (20 mM) glucose; GlcN, glucosamine; CaspNAc, 2-acetamido-2-deoxy-castanospermine.
FIGURE LEGENDS

Figure 1. Sensitivity of glycogen synthase to G6P in 3T3-L1 adipocytes cultured in low glucose, high glucose or glucosamine, with or without insulin. NIH 3T3-L1 differentiated adipocytes were incubated for 24 h in either: (a) 2.5 mM glucose (low glucose, LG, ■, □); (b) 20 mM glucose (high glucose, HG ●, ○); or (c) 2.5 mM glucose plus 10 mM glucosamine (glucosamine, GlcN, ♦, ◆). Adipocytes were also treated in the absence ( □, ○, ◆, open bar) or presence ( ■, ●, ♦, ◆, closed bar) of 10 nM insulin for 30 minutes prior to extraction. Duplicate glycogen synthase assays were conducted in the presence of 0 – 3 mM G6P. The half-maximal activation concentration of G6P (A₀.₅ ± SE µM) was determined using the least-squared algorithm from 4 independent cultures and is shown with each curve. A summary of all the A₀.₅ values (± SE) is presented in panel (d). Significant difference relative to the low glucose culture (*, p  0.05) and to the non-insulin treated cells (+, p  0.05) is also indicated. Absolute values for basal and maximal synthase activity, given as nmol glucose transferred per min per mg protein (± SE), in extracts from the differently treated cultures were as follows: (low glucose, basal = 0.29 ± 0.06, maximal = 3.38 ± 0.76; low glucose with insulin treatment, basal = 0.51 ± 0.13, maximal = 2.87 ± 0.69; high glucose, basal = 0.04 ± 0.01, maximal = 3.70 ± 0.87; high glucose with insulin treatment, basal = 0.17 ± 0.07, maximal = 3.68 ± 1.02; glucosamine, basal = 0.08 ± 0.01, maximal = 2.74 ± 0.67; glucosamine with insulin treatment, basal = 0.24 ± 0.12, maximal = 2.94 ± 0.88).

Figure 2. Phosphorylation of glycogen synthase in different metabolic conditions. (a)
Differentiated NIH 3T3-L1 adipocytes were cultured with low glucose (LG, lanes 1,2), high...
glucose (HG, lanes 3,4) and glucosamine (GlcN, lanes 5,6), in the absence (lanes 1,3,5) or the presence (lanes 2,4,6) of 10 nM insulin, as described in the methods section. Total glycogen synthase and phosphoglycogen synthase were detected by immunoblotting, the latter by using an antibody against a phosphopeptide from the GSK3 recognition domain of the enzyme (hGS 642-662). (b) Densitometric data from 3 independent experiments, each assayed in duplicate blots, were averaged and displayed in Figure 2b (± SE).

**Figure 3. Effect of different metabolic conditions on level of cytosolic O-GlcNAc.** NIH3T3-L1 adipocytes were cultured for 24 h in 2.5 mM glucose (LG, lanes 1,2), 20 mM glucose (HG, lanes 3,4), or in 2.5 mM glucose plus 10 mM glucosamine (GlcN, lane 5,6), in the absence (lanes 1,3,5) or the presence (lanes 2,4,6) of 10 nM insulin. 10 µg of each extract was resolved on SDS-PAGE and stained with an antibody specific for O-GlcNAc. The blot shown is a single representative exposure of 5 separate experiments whose lanes were arranged to maintain consistency of the order of the presentation.

**Figure 4. Glycogen synthase is modified by O-GlcNAc and is dynamically glycosylated.** (a) Glycogen synthase was purified from rabbit muscle as described in the methods, run on SDS/PAGE and stained with Coomassie (lane 1). Immunoblots of the purified protein stained with anti-glycogen synthase (lane 2) and anti-O-GlcNAc (lane 3). Specific radiolabeling of O-GlcNAc with $[^{3}H]$galactose was conducted by incubation of the protein with $\beta$-1,4galactosyltransferase and UDP-$[^{3}H]$galactose (lane 4). Ovalbumin, a known GlcNAc protein and $\beta$-1,4galactosyltransferase alone were also incubated as positive and negative
controls for detection of O-GlcNAc (lanes 5 and 6). (b) NIH3T3-L1 adipocytes were cultured for 24 h in 2.5 mM glucose (lanes 1,2), 20 mM glucose (lanes 3,4), or in 2.5 mM glucose with 10 mM glucosamine (lane 5,6), in the absence (lanes 1,3,5) or the presence (lanes 2,4,6) of 10 nM insulin. Extracts were incubated with sWGA agarose and thoroughly washed. Immobilized proteins were resolved on SDS/PAGE. Glycogen synthase was detected using an anti-glycogen synthase antibody. The presented immunoblot is representative of 2 separate experiments conducted in duplicate.

Figure 5. Digestion of glycogen synthase preparations with protein phosphatase 1 and β-N-acetylglucosaminidase. (a) Extracts from differentiated NIH 3T3-L1 adipocytes cultured in 2.5 mM glucose (LG), 20 mM glucose (HG), or 2.5 mM glucose plus 10 mM glucosamine (GlcN) were incubated in the absence or presence of 10 units recombinant protein phosphatase 1 (PP1) for 30 min and assayed in duplicate with 0 and 3 mM G6P. Basal glycogen synthase activity (± SE) in the absence of G6P is expressed as the % of maximal activity seen in 3 mM G6P. (b) Combined β-N-acetylglucosaminidase (Hex) and phosphatase digestion of the adipocyte extracts occurred by predigestion in the absence or presence of 2 units β-N-acetylglucosaminidase, followed by digestion in the absence or presence of 10 units protein phosphatase 1. Basal glycogen synthase activity was determined by duplicate assays with 0 and 3 mM G6P (± SE). (c) To confirm that the phosphatase digestion was complete, 10 µg of protein from extracts of low glucose cultured adipocytes was digested with β-N-acetylglucosaminidase, with or without subsequent protein phosphatase digestion. The digested extracts were then resolved on SDS-PAGE and the resulting blot stained with the anti-phosphoglycogen synthase antibody. Absolute values for basal and maximal synthase activity, given as nmol glucose
transferred per min per mg protein (± SE), in extracts from the differently treated cultures were as follows: (a) (LG, basal = 4.81 ± 0.02, maximal = 36.31 ± 1.88; LG + PP1, basal = 26.96 ± 0.71, maximal = 48.69 ± 2.80; HG, basal = 1.68 ± 0.30, maximal = 32.56 ± 1.74; HG + PP1, basal = 2.36 ± 0.15, maximal = 17.91 ± 0.38; GlcN, basal = 1.77 ± 0.25, maximal = 39.92 ± 1.33; GlcN + PP1, basal = 7.07 ± 0.42, maximal = 40.24 ± 1.00) and (b) (LG, basal = 0.36 ± 0.14, maximal = 44.12 ± 8.70; LG + Hex, basal = 3.32 ± 0.51, maximal = 52.8 ± 8.25; LG + Hex and PP1, basal = 39.52 ± 6.99, maximal = 55.71 ± 8.84; HG, basal = 0.33 ± 0.15, maximal = 42.99 ± 6.60; HG + Hex, basal = 2.26 ± 0.67, maximal = 52.79 ± 8.29; HG + Hex and PP1, basal = 40.07 ± 9.00, maximal = 54.92 ± 9.10; GlcN, basal = 0.40 ± 0.14, maximal = 35.35 ± 7.75; GlcN + Hex, basal = 2.22 ± 0.30, maximal = 48.84 ± 8.09; GlcN + Hex and PP1, basal = 39.54 ± 8.29, maximal = 55.11 ± 7.98).
a

αGS
αpGS

b

pGSase (AIU)

Insulin

-  +

LG  HG  GlcN
a

1 2 3 4 5

90 kDa
70 kDa

b

Insulin

- + - + - +

GSase

-
a

Basal Activity (% Total Activity)

PP1  
LG  HG  GlcN

b

Basal Activity (% Total Activity)

Hex  PP1  
LG  HG  GlcN

C

pGSase

Hex  PP1

-  +  -  +  -  +  +
Insulin resistance of glycogen synthase mediated by O-linked N-acetylglucosamine
Glendon J. Parker, Kelli C. Lund, Rodrick P. Taylor and Donald A. McClain

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