GLUCOSE DEPRIVATION STIMULATES O-GlcNAc MODIFICATION OF PROTEINS THROUGH UPREGULATION OF O-LINKED N-ACETYLGLUCOSAMINYLTRANSFERASE

Rodrick P. Taylor1,2, Glendon J. Parker2, Mark W. Hazel2, Yudi Soesanto1,2, William Fuller2,
Marla J. Yazzie2, and Donald A. McClain1,2,3

From the Departments of Biochemistry1 and Medicine2, University of Utah School of Medicine, and the Research Service of the Veterans Affairs Medical Center3
Salt Lake City, Utah, 84132 USA

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Address correspondence to: Donald A. McClain, Division of Endocrinology, University of Utah School of Medicine, 30 N. 2030 East, Salt Lake City, Utah 84132, USA. Fax: 801-585-0956; Email donald.mcclain@hsc.utah.edu

O-linked N-acetylglucosamine (O-GlcNAc) is a post-translational modification of proteins that functions as a nutrient sensing mechanism. Here we report on regulation of O-GlcNAcylation over a broad range of glucose concentrations. We have discovered a significant induction of O-GlcNAc modification of a limited number of proteins under conditions of glucose deprivation. Beginning 12 h after treatment, glucose-deprived human hepatocellular carcinoma (HepG2) cells demonstrate a 7.8-fold increase in total O-GlcNAc modification compared to cells cultured in normal glucose (5 mM, p=0.008). Some of the targets of glucose deprivation-induced O-GlcNAcylation are distinct from those modified in response to high glucose (HG, 20 mM) or glucosamine (GlcN, 10mM) treatment, suggesting differential targeting with glucose deprivation and glucose excess. O-GlcNAcylation of glycoprotein synthase is significantly increased with glucose deprivation and this O-GlcNAc increase contributes to a 60% decrease (p=0.004) in glycoprotein synthase activity.

Increased O-GlcNAc modification is not mediated by increased UDP-GlcNAc, the rate limiting substrate for O-GlcNAcylation. Rather, the mRNA for nucleocytoplasmic O-linked N-acetylglucosaminyltransferase (neOGT) increases 3.4-fold within 6 h of glucose deprivation (p=0.006). Within 12 h, OGT protein increases 1.7-fold (p=0.01) compared to normal glucose-treated cells. In addition, 12-h glucose deprivation leads to a 49% decrease in O-GlcNAcase protein levels (p=0.03). We conclude that increased O-GlcNAc modification stimulated by glucose deprivation results from increased OGT and decreased O-GlcNAcase levels and these changes affect cell metabolism, inactivating glycogen synthase.

Dynamic O-linked N-acetylglucosamine (O-GlcNAc) modification is a critical modulator of the fate and function of diverse nuclear and cytoplasmic proteins. O-GlcNAcylation of target proteins is dependent upon substrate synthesis in the hexosamine biosynthetic pathway (HBP) coupled with O-N-acetylglucosaminyltransferase (OGT)-mediated protein modification. In many eukaryotic systems, HBP flux has been shown to parallel substrate (glucose) availability making the HBP a nutrient sensor (1-4). In the cell, the HBP converts imported glucose and glucosamine to uridine 5'-diphospho (UDP)-GlcNAc. Glutamine:fructose-6-phosphate amidotransferase is the rate-limiting enzyme in this pathway. OGT catalyzes GlcNAc transfer to serine and threonine residues of target proteins while O-GlcNAcase catalyzes O-GlcNAc removal.

O-GlcNAc is known to affect multiple metabolic pathways and has specifically been implicated as a contributor to insulin resistance and type 2 diabetes (5-7). Chronically elevated HBP flux, a result of hyperglycemia, is known to exacerbate metabolic dysregulation in part by targeting metabolic enzymes. For example, in diabetic mice glycogen synthase (GS) becomes resistant to insulin stimulation as its level of O-GlcNAc modification increases (8,9). AMP-activated protein kinase (AMPK) is activated in adipocytes with elevated HBP flux resulting in O-GlcNAc-mediated elevation of fatty acid oxidation (10). To date, the majority of reports of O-GlcNAc-
mediated metabolic changes attribute increased O-GlcNAc modification to increased HBP flux. We report a novel and significant induction of O-GlcNAc modification in glucose-deprived HepG2 cells that is independent of increased HBP flux and appears distinct from previously reported stress-induced O-GlcNAc induction. Rather, increased O-GlcNAc with glucose deprivation is mediated by induction of OGT and downregulation of O-GlcNAcase. Increased O-GlcNAcylation of GS in these conditions contributes to decreased GS activity.

**Experimental Procedures**

*Antibodies and Reagents-* The following antibodies were used in the current study: anti-O-GlcNAc monoclonal IgM (CTD 110.6, a gift of Dr. G. Hart, Johns Hopkins University, Baltimore, MD), anti-O-GlcNAcase (a gift of Dr. S.W. Whiteheart, University of Kentucky, Lexington, KY), anti-GAPDH (Santa Cruz), anti-β-actin (Cell Signaling), anti-OGT DM-17 (Sigma-Aldrich, St. Louis, MO), anti-glycogen synthase (Cell Signaling; Cat. #386), anti-phospho-glycogen synthase (Cell Signaling), and horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (GE Biosciences) and anti-mouse IgM (Calbiochem). Succinylated wheat germ agglutinin-agarose (sWGA) (EY Laboratories, San Mateo, CA). HepG2 cell line (ATCC, Manassas, VA). Protein A/G Plus-Agarose (Santa Cruz Biotechnology). All enzymes and chemicals were obtained from Sigma with the exception of the following: UDP-[6-³H] glucose (GE Bioscience), Dulbecco's modified Eagle's medium and fetal calf serum (Invitrogen), O-GlcNAcase inhibitor, O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenyl carbamate (PUGNAc, Toronto Research Chemicals, Ontario, Canada), complete table protease inhibitors (Roche Molecular Biochemicals), and TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). All chromatography media and columns were obtained from GE Biosciences. The Beckman Glucose Analyzer II (Beckman Coulter) was used for media glucose determination.

*Growth, Treatment, and Extraction of HepG2 cells-* HepG2 cells were grown in 10 ml of Dulbecco's modified Eagle's medium (DMEM) containing 20 mM glucose, 10% fetal calf serum, 100 units/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, in 10-cm plates (Corning Glass) at 37°C in 5% CO₂. Medium was replaced one day prior to experimental treatment initiation (media glucose concentrations at treatment initiation averaged 10 mM). Experimental treatments were initiated once cells reached 70% confluence. We found 70% confluence to be optimal for promoting the glucose deprivation effect; under- and over-confluent cells demonstrated a diminished glucose deprivation effect. Experimental treatment of each plate comprised 10 ml of glucose-free DMEM, 1% fetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine, and 0 to 40 mM glucose. Glucosamine treatments included glucose-free DMEM, 1% FCS, 1mM sodium pyruvate, 4 mM L-glutamine, 2.5 mM glucose, and 10 mM D-glucosamine. Because media glucose concentrations depleted significantly over time in pilot experiments, media glucose concentrations were assayed every 3 h (using the Beckman Glucose Analyzer II) and glucose was replenished to achieve consistent glucose concentrations throughout treatment. Experimental treatment lasted 0 to 24 h. No cell death was observed for any of the treatment durations. For protein extracts, plates were placed on ice and washed 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 harvested in 0.75 ml of extraction buffer (50 mM HEPES, pH 7.4, 100 mM sodium chloride, 5% glycerol (v/v), 50 µM PUGNAc, and protease inhibitors). The resulting cell suspension was sonicated with a Sonic Dismembrator F60 for 15 sec at setting 6 (Thermo) and centrifuged at 20,000 x g for 2 min at 4°C. Supernatant aliquots were immediately frozen in liquid nitrogen. Cells whose lysates were subsequently digested with hexosaminidase were harvested as above, but in a modified extraction buffer lacking PUGNAc and EDTA to prevent hexosaminidase inhibition. For cells used for RNA determination, the medium was aspirated/discarded and 1 ml of TRI Reagent was immediately applied to the cells. Cells were scraped, then disrupted by repeated pipeting and immediately frozen in liquid nitrogen.
Western Blotting- Protein concentrations of HepG2 lysates were determined using BioRad protein reagent. Lysates were prepared for gel electrophoresis by dilution with extraction buffer and 5X Laemmli buffer. 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<sup>®</sup> transfer membrane (Millipore Corp., 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) nonfat 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 temperature or overnight at 4°C, washed three times in TBST, and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The blots were washed five times in TBST and imaged by treatment with Super Signal West Dura reagents (Pierce) and exposure to Classic Blue Autoradiography Film BX (Molecular Technologies, St. Louis, MO). Densitometry measurements were obtained using an EPSON Perfection 3200 Photo scanner (EPSON, Long Beach, CA) and NIH Image version 1.62 software (rsb.info.nih.gov/ij). In all experiments, GAPDH protein levels were used to normalize changes in protein/modification. GAPDH protein levels are not affected by the various cell treatments of these studies (Fig. 1A, C, 2C).

Immunoprecipitation of Glycogen Synthase-We followed the glycogen synthase antibody manufacturer's protocol for immunoprecipitation. Briefly, 300 µl (600 µg of protein) of cell lysate combined with glycogen synthase antibody (1:25) was rotated overnight at 4°C (incubation with rabbit IgG served as the negative control). 20 µl of protein A/G Plus-Agarose beads (50% bead slurry) was added and mixtures were rotated for 3 hours at 4°C. Beads were pelleted by centrifugation (20,000 x g for 30 seconds at 4°C) and supernatant was discarded (some supernatant was retained and run as a positive control). Beads were washed five times with 500 µl of 1X lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphosphate, 1mM β-glycerophosphate, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml Leupeptin). Pellet was resuspended in 20 µl 3X SDS sample buffer. Samples were boiled at 95°C for 5 minutes then loaded on SDS-PAGE gel (4-15%). Western blotted membranes were probed with α-O-GlcNAc (α-CTD110.6) and α-GS.

Immobilization of O-GlcNAc-modified proteins with Wheat Germ Agglutinin- 400 µl of lysate (1 mg of protein/ml) was incubated with 25 µl of sWGA agarose and 400 µl of radioimmuneprecipitation buffer (11 mM sodium phosphate, pH 7.4, 150 mM sodium chloride, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS, 40 mM sodium fluoride, 0.5 mM 2-acetamido-1-amino-1,2-dideoxyglucopyranose, and protease inhibitors). The preparation was rotated for 16 h at 4°C and washed two times with extraction buffer/radioimmuneprecipitation buffer (1:1). Immobilized proteins were eluted by boiling in Laemmli buffer, resolved by SDS-PAGE, and transferred to Immobilon-P<sup>®</sup> membrane. Levels of O-GlcNAc modification were determined by probing for sWGA-bound/O-GlcNAc-modified target proteins with appropriate antibodies.

Quantitation of mRNA by RT-PCR- RNA was prepared from -70°C frozen TRI Reagent/cell suspensions according to the manufacturer's (TRI Reagent, Molecular Research Center, Inc) protocol and dissolved in water. RNA concentrations were measured spectrophotometrically. First-strand cDNA synthesis was carried out using 3 µg of RNA, oligo(dT) primers (Invitrogen, Carlsbad, CA), and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Real-time PCR was performed with a rapid thermal cycler (LightCycler, Roche Diagnostics, Mannheim, Germany). Reactions (10 µl) were performed using approximately 16 ng cDNA as template with 0.5 µM each primer, 200 µM each deoxynucleotide triphosphate, 50 mM Tris (pH 8.3), 500 µg/ml nonacetylated BSA (Sigma), 3.0 mM MgCl<sub>2</sub>, 0.04 U/µl Platinum Taq DNA polymerase (Invitrogen), and 1:30,000 dilution of SYBR Green I fluorescent dye (Molecular Probes, Eugene, OR). Primers based on human sequences were chosen using the Primer3 program. For nucleocytoplasmic OGT, 5′-
TTTAGCAGCTCTGGCAAATGACAG-3' and 5'-TCAATAAACATGCCTTGCTTC-3'. For mitochondrial OGT, 5'-TTACCCTCTTTCCCTCCATC-3' and 5'-CTGCATAAAATGCGGTG CCTCT-3'. For all OGT isoforms, 5'-CTGCCCAGAACCCTATCA-3' and 5'-TTCCAGACTTTGCGACAGTACT-3'. For O-GlcNAcase, 5'-AGCCTTGAGTGGACCGACAT-3' and 5'-TCTGAGGATTTTATTGACCAGC-3'. For NONO (Non-POU domain-containing octamer-binding protein), 5'-CAAGTGGACCGCAACATCA-3' and 5'-CGCCGCATCTTCCTCTCAC-3'. We assayed expression of 6 different potential normalizer genes and found that NONO expression was consistent across all cell treatments. Amplification occurred over 26 to 45 four-step cycles, with a rate of temperature change between steps of 20°C/sec. The steps were 95°C with a 0-sec hold, 60°C with a 0-sec hold, 72°C with an 11-sec hold, and 80°C with a 1-sec hold. Fluorescence was detected during the fourth step at a temperature previously determined to be below the melting temperature of the PCR products. After amplification, a melting curve was generated by slowly heating the double-stranded DNA product. Analysis of the postamplification melting curves confirmed the absence of nonspecific DNA products. For each amplification's fluorescence vs. cycle line, the LightCycler software determined the second derivative maximum (the threshold cycle at which fluorescence clearly increased above background). Standard curves of log cDNA vs. second derivative maximum (fractional cycle number) were constructed for each quantitated transcript, and for the NONO normalization transcript, from cDNA mixes comprising equal amounts of all cell treatment condition cDNAs. Standard curve points of 0, 6, 10, 16, 26, and 32 ng of combined cell cDNA were always included with the same PCR run with the entire set of individual cDNA amplifications of the same transcript. Results for each individual cDNA were normalized by dividing the relative amount of each transcript by the relative amount of NONO transcript from the same experiment. Within each experiment the same cocktail mix was used, containing everything but the specific primers.

**Uridine diphospho (UDP)-N-acetyl-hexosamine assay**- Levels of UDP-N-acetyl-hexosamines (consisting of UDP-GlcNAc and UDP-GalNAc), products of the hexosamine biosynthesis pathway, were measured in cell extracts as previously described (11). Cell extracts were homogenized at 4°C in 4 vol perchloric acid (300 mM). The precipitates were centrifuged (10,000 x g for 15 min at 4°C), and the lipid was extracted from the supernatants with 2 vol tri-n-octylamine: 1,1,2-trichlorofluoroethane (1:4). The aqueous phase was stored at –80°C until analysis by HPLC. The extracts were filtered (0.45 μm), and HPLC was performed on a Partisil 10.5Ax column (25 cm x 4.6 mm, Waters Corp., Taunton, MA), eluted with a concave gradient from 5 mM potassium phosphate (pH 7.2) to 750 mM potassium phosphate (pH 7.2) over 48 min at a flow rate of 1 ml/min. UDP-HexNAc levels were quantified by UV absorption at 254 nm, compared with external standards.

**Glycogen Synthase Assay**- The assay for glycogen synthase was performed as previously described (8). HepG2 lysate (7.5 μg of protein) was incubated in 100 μl final volume with HEPES (100 mM), pH 7.4, EDTA (5 mM), pH 7.4, 0.8 mg glycogen (type III from rabbit liver), UDP-glucose (2 mM), 10 μl glycerol, glucose 6-phosphate (G6P) (0 or 10 mM), and 0.4 μCi of UDP-[6-3H]glucose for 45 min at 37°C. The incubation was terminated by application to Whatman qualitative filter paper number 3 (Maidstone, UK) and immersion in 60% (v/v) ethanol. After five washes in 400 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 min. Total GS activity was defined as the activity at maximal G6P (10 mM).

**Digestion of HepG2 Extracts with Hexosaminidase-Hexosaminidase digestions were conducted by treating cell lysates (50 μg of protein) with 1 Unit of N-acetylglucosaminidase from jack beans (Sigma) and 25 μl of hexosaminidase buffer (20 mM sodium citrate, pH 4.5, 10% glycerol, 100 mM sodium chloride, and protease inhibitors) and incubating the preparation for 1 h at 30°C. Lysates not digested with hexosaminidase were protected from deglycosylation by the addition of PUGNAs (50 μM):**
PUGNAs inhibits endogenous O-GlcNAcase). Immediately following digestion, glycogen synthase activation was measured as described above.

Statistical procedures- Descriptive statistics are represented as average ± standard error of the mean. Each average represents data from at least three independent experiments. The Student t-test (two tail) was used to compare differences between groups.

Results

Glucose deprivation of HepG2 cells stimulates O-GlcNAc protein modification. We assayed the HepG2 human liver cell line for changes in O-GlcNAcylation of proteins in response to changes in ambient glucose concentration. We chose to study O-GlcNAc changes in liver cells because of the liver’s central role in carbohydrate metabolism and its glucose responsiveness. HepG2 cells were cultured for 12 h with 0 - 40 mM glucose. O-GlcNAc levels were determined by Western Blot analysis of HepG2 cell lysates using the O-GlcNAc-specific antibody CTD110.6. Media glucose concentration had a significant effect on O-GlcNAc modification of a number of cellular proteins. Cells cultured in 0 mM glucose showed a total increase in O-GlcNAc of 7.8-fold (p=0.008) and 4.1-fold (p=0.05) compared with cells cultured in normal and high glucose, respectively (Fig. 1). Appreciable increases of O-GlcNAc with glucose deprivation first appear at 12 h and continue to increase through 18 and 24 h (Fig. 1C). Glucosamine treatment has been shown to stimulate O-GlcNAc modification even more potently than high glucose in a variety of systems (12). However, we observe 3-fold greater O-glycosylation with glucose deprivation than with glucosamine treatment (p=0.02, Fig. 1A,B).

While some protein targets demonstrate increased O-GlcNAc modification with both glucose deprivation and high glucose/glucosamine conditions, others are highly glycosylated exclusively with glucose deprivation. For two protein bands (Fig. 1A, bands b & c, ~250 & ~150 kDa, respectively), O-glycosylation is maximal with glucose deprivation and also increases with high glucose/GlcN treatment, with the lowest glycosylation levels at 1 mM glucose. Other proteins (Fig. 1A, bands a, >250 kDa) fail to show glycosylation with high glucose/glucosamine treatment but demonstrate robust glycosylation exclusively with glucose deprivation. Yet other targets (Fig. 1A, band d, ~75 kDa) demonstrate bimodal modification in which glycosylation is lowest at normal (5 mM) glucose and increases both with lower and higher glucose concentrations, suggesting that some targets are regulated by O-GlcNAc modification throughout the entire range of physiologic glucose concentrations.

Increased O-GlcNAcylation of glycogen synthase (GS) in glucose-deprived cells contributes to a decrease in its activity. The O-GlcNAc modification induced by glucose deprivation is functionally significant. We have previously shown in adipocytes that O-GlcNAc modification of GS in conditions of high glucose decreases GS activity (8). This is also the case with glucose deprivation of HepG2 cells. HepG2 cells cultured in 0 mM glucose for 12 h exhibit a 60% decrease in maximal glycogen synthase activity compared to 5 mM glucose treatment (p=0.004, Fig. 2A). We observed no differences in GS activity among cells treated with 5 and 20 mM glucose or glucosamine (Fig. 2A). The decrease in activity observed with glucose deprivation correlates with a 675% increase in O-GlcNAc-modified GS levels (p=0.05, Fig. 2B) and a 330% increase in the amount of GS precipitated by sWGA (p=0.02, Fig. 2C), a lectin that specifically binds terminal GlcNAc. We observed no difference in glycogen synthase protein amount across glucose treatments or phospho-glycogen synthase levels among treatments (Fig. 2B,D). We also detected no change in the K_M of GS from glucose-deprived cells for its substrate UDP-glucose or its allosteric activator glucose-6-phosphate (not shown).

To demonstrate directly that O-GlcNAc modification contributes to the observed decrease in GS activity, we treated cell lysates with hexosaminidase to reduce the levels of O-GlcNAc modification. This treatment resulted in a 40% rescue of total GS activity after glucose deprivation (p=0.003), while digestion of lysates from cells treated with normal glucose demonstrated no change in total activity (Fig. 2E). We observed a significant decrease in O-GlcNAc modification of proteins to background levels after hexosaminidase digestion, confirming deglycosylation (not shown).
Increased O-GlcNAc with glucose deprivation does not result from increased HBP flux. Previous reports have mainly attributed increases of cellular O-GlcNAc to increased HBP flux and synthesis of the end product of the pathway, UDP-GlcNAc (1,13). This is not the case in glucose-deprived HepG2 cells. Cells cultured for 12 h in 0 mM glucose (which demonstrate a 7.8-fold increase in O-GlcNAc, Fig. 1A,B) exhibit a 40% decrease in UDP-GlcNAc levels compared with normal glucose-treated cells (p=0.01). UDP-GlcNAc levels increased 236% (p=0.008) with high glucose and 616% (p=0.01) with glucosamine treatment compared to 0 mM glucose treatment (Fig. 3).

Nucleocytoplasmic OGT mRNA and OGT protein levels are increased in glucose-deprived cells. To explore the mechanisms for increased O-GlcNAc modification in the absence of increased HBP flux, we examined expression of OGT in conditions of glucose deprivation. Multiple OGT variants have been described (14-16), the best characterized in human liver being the nucleocytoplasmic (nc) and mitochondrial (m) isoforms (16,17). Nc and mOGT isoforms are transcribed from a single OGT gene and are generated through alternative splicing and possibly by transcription initiation at a second internal promoter (17).

Increased OGT mRNA was found to correlate with and precede the increase in protein O-GlcNAc modification observed with glucose deprivation. By 6 h, a 3.4-fold increase in ncOGT transcript was observed (Fig. 4A, p=0.006). By 12 h, ncOGT mRNA levels had returned to levels seen in untreated cells. No change was observed in mOGT mRNA preceding O-GlcNAc induction (Fig. 4B). We tested primers directed to the conserved 3′-end of the OGT gene that detect all OGT transcripts. At 6 h, we observed an increase, although blunted, for total OGT: 0 mM glucose treatment promoted a 1.7-fold total OGT mRNA induction compared to 5 mM glucose treatment (Fig. 4C, p=0.001).

OGT protein levels were significantly increased following OGT transcriptional induction in glucose deprivation. At 12 h we observed a 1.7-fold induction of OGT protein in glucose-deprived cells compared with normal glucose-treated cells (Fig. 5, p=0.01). OGT protein levels in high glucose and glucosamine-treated cells were unchanged relative to normal glucose-treated cells. 

O-GlcNAcase protein levels are significantly decreased in glucose-deprived cells. Cellular O-GlcNAc levels are also determined by the activity of the enzyme O-GlcNAcase. We therefore determined if changes in O-GlcNAcase might also contribute to the observed increase in protein O-GlcNAc modification with glucose deprivation. At 12 h, O-GlcNAcase protein levels were decreased 49% in glucose-deprived cells compared to cells cultured in 5 mM glucose (p=0.03). We observed no difference in O-GlcNAcase protein levels in 20 mM glucose and glucosamine-treated cells compared to 5 mM glucose-treated cells (Fig. 6). We observed no difference in O-GlcNAcase transcript levels among all treatments (not shown).

Discussion

To date, much work on O-GlcNAc modification has focused on its contribution to metabolic regulation under hyperglycemic conditions. In this study we have shown that HepG2 cells under conditions of glucose deprivation demonstrate an even greater increase in O-GlcNAcylation of a number of proteins. This increase is not mediated by an increase in the rate-limiting substrate UDP-GlcNAc, as is the case in high glucose, but rather by induction of OGT and downregulation of O-GlcNAcase. The modification of proteins by O-GlcNAc in glucose deprivation may serve a role in metabolic regulation, part of a general response to conserve energy. We show, for example, that glycogen synthase (GS) activity is diminished with glucose deprivation and this decrease is due in part to O-GlcNAcylation of GS. GS, the rate-limiting enzyme for glycogen synthesis is highly expressed in liver and is integral to glucose homeostasis (18,19). GS activity is regulated through hormone-mediated phosphorylation and dephosphorylation (20) (21), but also by modification with GlcNAc. In 3T3-L1 adipocytes and diabetic mice, O-GlcNAc modification of GS inhibits the enzyme in a manner analogous to phosphate and this inhibition contributes to insulin resistance and the diabetic phenotype (8,9). O-GlcNAcylation of GS in glucose deprivation, however, contributes to the prevention of futile cycling of glycogenesis and glycogenolysis.
The mechanism for the decreased activity of O-GlcNAcylated GS is not known. Post-translational modification of GS with phosphate and O-GlcNAc has previously been shown to alter the K_m of the enzyme for UDP-glucose and glucose-6-phosphate. The O-GlcNAc-mediated decrease in GS total activity reported herein, namely a change in total activity (V_MAX) without a change in protein level or K_m for substrate or activators is novel. Why this differs from the regulation of GS in high glucose conditions is not known. Our current data demonstrate differential protein targeting of O-GlcNAc in conditions of high glucose and glucose deprivation, and the same may be true of specific sites of O-GlcNAcylation within proteins. Alternatively, the differences may relate to the degree of glycosylation or to the tissue/cell type in which the glycosylation occurs.

It is well established that low energy states can also mediate downregulation of anabolic reactions such as protein, fat, and complex carbohydrate synthesis through the AMP-activated kinase cascade (22). The proximal signal by which glucose deprivation signals the induction of OGT, however, is not known. Preliminary experiments in which pyruvate failed to rescue the glucose-deprivation O-GlcNAc response suggest that the signal for O-GlcNAc induction is not a general decrease in energy availability, but rather a specific response to decreased glucose availability. These results suggest that protein O-GlcNAcylation may therefore be an additional and distinct mechanism contributing to a general energy conservation response.

In addition to playing a role in metabolic adaptation to glucose deprivation, the observed increase in O-GlcNAcylation with glucose deprivation may also represent a stress response aimed at preserving protein structure and function. Recent publications implicate O-GlcNAc modification as a survival response to a variety of cellular stressors (23,24). Hydrogen peroxide, ultraviolet light, ethanol, NaCl, and thermal stress all lead to increased global levels of O-GlcNAcylation that have been shown to improve survival under stress (25-27). Increased O-GlcNAc modification under these conditions has been attributed in part to stress-induced increased glucose import and a resultant increase in HBP flux (23). It thus represents a response with similar effects on O-GlcNAc, but induction through a different mechanism, particularly as these studies were conducted over a different time frame (23). HSP70, a chaperone protein is known to respond to cellular stress and has been described as an O-GlcNAc specific lectin (24,28,29). The lectin binding activity changes with nutrient deprivation and other stressors (27-29). It is itself a target of O-GlcNAcylation, although O-GlcNAc does not change HSP70’s lectin activity (28,30). Manipulation of O-GlcNAc levels through the use of PUGNAc, diazo-oxo-norleucine (DON) or genetic ablation of OGT, has been shown to correlate with HSP70 protein levels (23). Consistent with the protective effect of O-GlcNAc on proteins, increased O-GlcNAcylation of the 26S proteasome has been found to inhibit proteasome-mediated protein degradation (31). Although no consistent consensus amino acid sequence has been identified for O-GlcNAc modification, many O-GlcNAc sites are ‘PEST’ (Pro-Glu-Ser-Thr) sequences which are known to render proteins more susceptible to proteasomal degradation (32). Modification of PEST sequences with O-GlcNAc is thought to slow protein degradation (33). These and other findings suggest that O-GlcNAcylation plays a role in protecting its targets against degradation, protection that would benefit stressed, injured, or glucose-deprived cells.

We observe hyper-O-GlcNAcylation of some protein bands with both glucose deprivation and HG/GlcN treatment. However, hyper-O-GlcNAcylation of other bands occurs only with glucose deprivation. These findings suggest differential O-GlcNAc targeting. Little is known about OGT targeting, although OGT interaction with other proteins may contribute to the regulation of O-GlcNAcylation. For example, OGT is known to occur in complex with protein phosphatase 1 (PP1) and this interaction has been hypothesized to target OGT to serine and threonine residues that would also be modified by PP1 (34). The binding of OGT to binding partners is mediated largely by its tetratricopeptide repeat (TPR) interactions with target proteins (35). While the nucleocyttoplasmic (nc) and mitochondrial (m) isoforms of OGT share a conserved C-terminal catalytic region, they differ in the number of N-terminal TPRs as well as cellular localization targeting sequences (14,15,36,37). We
have demonstrated ncOGT induction with glucose deprivation without concomitant mOGT induction, suggesting that differential regulation of OGT transcription, splicing, and transcript turnover may also function to regulate target specificity under varying conditions.

The current data demonstrate that metabolic regulation by O-GlcNAc is not simply a phenomenon seen in hyperglycemic states, but operates in glucose concentrations from below to above normal. This modulation of O-GlcNAc levels is achieved through a number of distinct mechanisms, including substrate flux through the HBP, transcriptional regulation of OGT, regulation of OGT enzymatic activity, posttranslational regulation of O-GlcNAcase, and differential substrate targeting. The data on the distinct effects of O-GlcNAc on GS at low and high glucose also underscore the complexities of how protein function is modulated by O-GlcNAc. Combined with previous data demonstrating effects of O-GlcNAc on subcellular targeting, survival and intrinsic activity of a great variety of proteins, the current results are consistent with a central role for O-GlcNAc in the adaptation of cells and tissues both to stress and to changing nutrient availability.

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Footnotes
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Figure Legends

Fig. 1. Glucose deprivation of HepG2 cells stimulates a dramatic increase in total O-GlcNAc protein modification. A. HepG2 cells were cultured for 12 h with 0 - 40 mM glucose (Glc), or 10 mM glucosamine (GlcN). Medium glucose concentration was maintained by glucose re-feeding every 3 h. Resolved proteins from each treatment condition were probed with the O-GlcNAc-specific CTD110.6 antibody. Cells cultured in 0 mM glucose showed a total O-GlcNAc increase of 7.8-fold (p=0.008), 4.1-fold (p=0.05), and 3-fold (p=0.02) compared with normal glucose, high glucose, and glucosamine treatments, respectively (A,B). C. Appreciable O-GlcNAc induction with glucose deprivation first appears at 12 h and O-GlcNAcylation continues to increase through 18 and 24 h. Average O-GlcNAc densities and depicted time course changes are based on at least 3 independent determinations per condition. *, p ≤ 0.05; **, p ≤ 0.001.
Glycogen synthase (GS) O-GlcNAcylation is increased with glucose deprivation and this increase contributes to a decrease in GS activity. A. Glucose-deprived cells exhibit a 60% decrease in maximal glycogen synthase activity compared to 5 mM glucose treatment (p=0.004; N=4 independent determinations). B. Glucose-deprived cells exhibit a 6.75-fold increase in O-GlcNAc-modification of glycogen synthase (p=0.05; N=6 independent determinations). Glycogen synthase was immunoprecipitated from cell lysates and immunoblotted with α-GS to demonstrate equivalent GS pull-down and α-O-GlcNAc (CTD110.6) to demonstrate changes in O-GlcNAc modification of GS. Failure to precipitate GS with rabbit IgG confirmed specific GS precipitation (not shown). C. Glucose-deprived cells exhibited a 3.3-fold increase in sWGA-bound/O-GlcNAc-modified glycogen synthase (p=0.002; N=5 independent determinations) (inset: sWGA precipitated proteins immunoblotted with α-GS). D. Immunoblots of total GS and phospho-GS levels demonstrate no change among treatments, suggesting that changes in glycogen synthase activity are not due to changes in GS protein or phospho-GS levels (representative blots of at least 3 independent determinations per treatment). GAPDH was probed as a loading control (N= at least 3 independent determinations per treatment). E. Glucose-deprived lysates digested with hexosaminidase demonstrate a 40% rescue of glycogen synthase total activity (0,- vs 0,+: p=0.003; N=6 independent determinations). **, p ≤ 0.001.

UDP-GlcNAc and HBP flux are decreased with glucose deprivation. HepG2 cells were cultured for 12 h with 0 - 40 mM glucose, or 10 mM glucosamine (GlcN). UDP-GlcNAc levels were measured, normalized to protein concentration, and calculated as proportions of the level for normal glucose treatments to allow for experiment-to-experiment averaging. Cells cultured in 0 mM glucose exhibited a 40% decrease in UDP-GlcNAc levels (1.8 µM/µg protein/µl) compared with normal glucose (2.9 µM/µg protein/µl; p=0.01). UDP-GlcNAc levels were increased 236% with high glucose (4.1 µM/µg protein/µl; p=0.008) and 616% with glucosamine (12.2 µM/µg protein/µl; p=0.01) compared to 0 mM glucose treatment. Average UDP-GlcNAc levels are based on at least 3 independent determinations per treatment. **, p ≤ 0.001.

Nucleocytoplastic OGT mRNA levels are significantly increased in glucose-deprived cells preceding O-GlcNAc induction. HepG2 cells were cultured for 0 - 24 h in 0 or 5 mM glucose. Transcript levels of nucleocytoplastic (nc), mitochondrial (m) OGT, and all-OGT were measured by RT-PCR and normalized to NONO transcript levels. A. By 6 h, a 3.4-fold induction of ncOGT was observed in glucose-deprived extracts compared with normal glucose extracts (p=0.006). By 12 h, ncOGT levels had returned to those seen under normal glucose conditions. B. No change was observed for mOGT mRNA preceding O-GlcNAc induction. C. At 6 h, OGT mRNA levels for all isoforms combined are 1.7-fold greater with glucose deprivation than with 5 mM glucose treatment (p=0.001). Average mRNA levels are based on at least 4 independent determinations per treatment. *, p ≤ 0.05; **, p ≤ 0.001.

OGT protein is significantly increased in glucose-deprived cells. HepG2 cells were cultured for 12 h in 0, 5, or 20 mM glucose, or 10 mM glucosamine (GlcN). At 12 h, 0 mM glucose promotes an OGT protein increase of 1.7-fold compared with normal glucose conditions (p=0.01). OGT protein levels in high glucose and glucosamine-treated cells were unchanged relative to normal glucose-treated cells. OGT bands from the same gel were re-arranged for presentation purposes. Average OGT protein densities are based on at least 3 independent determinations per treatment. *, p ≤ 0.05.

O-GlcNAcase protein levels are significantly decreased in glucose-deprived cells. HepG2 cells were cultured for 12 h in 0, 5, or 20 mM glucose, or 10 mM glucosamine (GlcN). O-GlcNAcase protein levels were decreased (49%) in glucose-deprived samples compared to samples treated in 5 mM glucose (p=0.03). We observed no difference in O-GlcNAcase protein levels among 5 and 20 mM glucose treatments to allow for experiment averaging. Cells cultured in 0 mM glucose deprived samples compared to samples treated in 5 mM glucose (p=0.03). We observed no difference in O-GlcNAcase protein levels among 5 and 20 mM glucose treatments to allow for experiment averaging.
glucose and glucosamine-treated samples. Average O-GlcNAcase protein densities are based on at least 4 independent determinations per treatment. *, p ≤ 0.05.
Fig. 1

A

| [Glc] mM | 0 | 1 | 2.5 | 5 | 10 | 20 | 40 | 2.5 |
|----------|---|---|-----|---|----|----|----|-----|
| [GlcN] mM | - | - | - | - | - | - | 10 | - |

KDa

250 -

150 -

75 -

37 -

25 -

α-O-GlcNAc

α-GAPDH

B

Treatment medium [Glc] (mM) or GlcN

C

| Treatment duration (hrs) | 0 | 1 | 3 | 6 | 12 | 18 | 24 |
|--------------------------|---|---|---|---|----|----|----|
| [Glucose] mM             | ~10 | 0 | 5 | 0 | 5 | 0 | 5 |

KDa

250 -

100 -

75 -

α-O-GlcNAc

α-GAPDH
Fig. 2

A

![Graph showing relative glycogen synthase total activity](image)

Treatment medium [Glc] (mM) or GlcN

B

![Image of immunoprecipitation (IP) and immunoblot (IB) results](image)

IP: GS
IB: O-GlcNAc

IP: GS
IB: GS

C

![Graph showing WGA-bound GS (A.U.)](image)

$\frac{\text{WGA-bound GS (A.U.)}}{\text{GlcN}}$

D

![Image of Western blots for GS, α-pGS, and GAPDH](image)

Treatment medium [Glc] (mM) or GlcN

α-GS

α-pGS

α-GAPDH

E

![Graph showing relative glycogen synthase total activity](image)

Relative Glycogen Synthase Total Activity

[Glucose] mM ± Hexosaminidase

0 0 5 5
Fig. 3

![Graph showing relative UDP-GlcNAc levels across different treatment medium concentrations of Glc or GlcN.](image-url)
Fig. 4

A

![Graph A: Relative ncOCT mRNA vs. Duration of Glucose Treatment (hours)]

B

![Graph B: Relative mOCT mRNA vs. Duration of Glucose Treatment (hours)]

C

![Graph C: Relative OCT (all variants) mRNA vs. Duration of Glucose Treatment (hours)]
Fig. 5

![Bar chart showing OGT/GAPDH levels](image)

**OGT/GAPDH**

**Treatment medium [Glc] (mM) or GlcN**

- 0
- 5
- 20
- GlcN
Glucose deprivation stimulates O-GlcNAc modification of proteins through upregulation of O-linked N-acetylglucosaminyltransferase
Rodrick P. Taylor, Glendon J. Parker, Mark W. Hazel, Yudi Soesanto, William Fuller, Marla J. Yazzie and Donald A. McClain

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