Up-regulation of O-GlcNAc Transferase with Glucose Deprivation in HepG2 Cells Is Mediated by Decreased Hexosamine Pathway Flux*

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O-Linked N-acetylglucosamine (O-GlcNAc) is a post-translational modification of proteins that functions as a nutrient-sensing mechanism. We have previously shown a significant induction of O-GlcNAc modification under conditions of glucose deprivation. Increased O-GlcNAc modification was mediated by increased mRNA for nucleocytoplasmic O-linked N-acetylglucosaminyltransferase (ncOGT). We have investigated the mechanism mediating ncOGT induction with glucose deprivation. The signal does not appear to be general energy depletion because no differences in AMP-dependent kinase protein levels or phosphorylation were observed between glucose-deprived and normal glucose-treated cells. However, treatment of glucose-deprived cells with a small dose (1 mM) of glucosamine blocked the induction of ncOGT mRNA and subsequent increase in O-GlcNAc protein modification, suggesting that decreased hexosamine flux is the signal for ncOGT up-regulation. Consistent with this, treatment of glucose-deprived cells with an inhibitor of O-GlcNACase (O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenyl carbamate) completely prevented the subsequent up-regulation of ncOGT. Glucosamine treatment also resulted in a 40% rescue of the down-regulation of glycogen synthase activity normally seen after glucose deprivation. We conclude that deglycosylation of proteins within the first few hours of glucose deprivation promotes ncOGT induction. These findings suggest a novel negative feedback regulatory loop for OGT and O-GlcNAc regulation.

Dynamic O-linked N-acetylglucosamine (O-GlcNAc)

O-GlcNAcylation of target proteins is dependent upon substrate synthesis in the hexosamine biosynthetic pathway (HBP) coupled with O-linked N-acetylglucosaminyltransferase (OGT)-mediated protein modification. The HBP converts a portion of imported glucose to uridine 5'-diphospho (UDP)-GlcNAc. OGT catalyzes GlcNAc transfer to serine and threonine residues of target proteins, whereas O-GlcNAcase catalyzes O-GlcNAc removal (1). HBP flux is known to parallel substrate (glucose) availability, making the HBP a nutrient sensor (2–5).

O-GlcNAcylation is regulated principally by substrate availability. Previous work has indicated that protein O-GlcNAcylation is proportional to substrate (glucose) availability (8). However, we have shown that human hepatocellular carcinoma (HepG2) cells demonstrate a robust O-GlcNAc increase when deprived of glucose, and this O-GlcNAc induction is mediated not by substrate-driven HBP flux increase but instead by increased OGT expression and O-GlcNAcase down-regulation (6). It has subsequently been shown that glucose deprivation of Neuro-2a neuroblastoma cells also results in OGT and O-GlcNAc induction (7). We have therefore investigated the mechanism for regulation of OGT in HepG2 cells and determined that the signal responsible for the induction of OGT mRNA in glucose deprivation is an early decrease in HBP flux and O-GlcNAc modification of proteins. Thus, the levels of O-GlcNAc in these cells are maintained through a feedback mechanism responsive to the degree of protein O-GlcNAc modification.

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. Gerald Hart, Johns Hopkins University, Baltimore, MD), anti-GAPDH (Santa Cruz), anti-AMP-dependent kinase (Cell Signaling), anti-phospho-AMPK (Cell Signaling), anti-acetyl-CoA carboxylase (Cell Signaling), and horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG (GE Healthcare) and anti-mouse IgM (Calbiochem). HepG2 cell line (ATCC, Manassas, VA). All of the enzymes and chemicals were obtained from Sigma with the exception of the following: UDP-[6-3H]glucose (GE Healthcare), 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, Toronto, Canada), complete tablet protease inhibitors (Roche Applied Science), and TRIZOL reagent (Molecular Research Center, Inc., Cincinnati, OH). The Beck-
man 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 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. The medium was replaced 1 day prior to experimental treatment initiation (measured 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 Dulbecco’s modified Eagle’s medium, 1% fetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine, and 0–20 mM glucose. Glucosamine treatments included glucose-free Dulbecco’s modified Eagle’s medium, 1% fetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine, and 0–10 mM D-glucosamine for 0–12 h. For PUGNAc experiments, treatment medium was augmented with PUGNAc, resulting in a final PUGNAc concentration of 50 μM. Because media glucose concentrations deplete significantly over time, 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. No cell death was observed for any of the treatment durations. For protein extracts, the plates were placed on ice and washed twice with ice-cold Krebs-Ringer-bicarbonate-HEPES buffer (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 6 s twice at setting 6 (Thermo) and centrifuged at 20,000 × g for 2 min at 4 °C. Supernatant aliquots were immediately frozen in liquid nitrogen. For cells used for RNA determination, the medium was aspirated/discarded, and 1 ml of TRI reagent was immediately applied to the cells. The cells were scraped, then disrupted by repeated pipetting, and immediately frozen in liquid nitrogen.

**UDP-N-acetylhexosamine Assay**—The levels of UDP-N-acetylhexosamines (consisting of UDP-GlcNAc and UDP-GalNac), products of the hexosamine biosynthesis pathway, were measured in cell extracts as previously described (11). The cell extracts were homogenized at 4 °C in 4 volumes of perchloric acid (300 mM). The precipitates were centrifuged (10,000 × g for 15 min at 4 °C), and the lipid was extracted from the supernatants with 2 volumes of 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.

**Western Blotting**—Protein concentrations of HepG2 lysates were determined using Bio-Rad protein reagent. The lysates were prepared for gel electrophoresis by dilution with extraction buffer and 5× 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-DCO transfer membrane (Millipore Corp., Bedford, MA). The 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. The 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 from non-saturated developed film using an EPSON Perfection 3200 Photo scanner (EPSON, Long Beach, CA) and National Institutes of Health Image version 1.62 software. In all of the experiments, the 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.

**Quantitation of mRNA by Reverse Transcription-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), and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Real time PCR was performed with a rapid thermal cycler (LightCycler; Roche Applied Science). The reactions (10 μl) were performed using ~16 ng of cDNA as template with 0.5 μM each primer, 200 μM each deoxynucleotide triphosphate, 50 mM Tris, pH 8.3, 500 μg/ml nonacetylated bovine serum albumin (Sigma), 3.0 mM MgCl₂, 0.04 unit/μl of 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’-CCTTAGAATTGTGCCATTAAACAG-3’ and 5’-TCAAATATCATGCTTGGCTTTC-3’. For NONO (Non-POU domain-containing octamer-binding protein), 5’-CAAGTGACCGCAAACATC-3’ and 5’-CGCCGATCATCTTCTTAC-3’. We assayed expression of six different potential normalizer genes and found that NONO expression was consistent across all cell treatments. Amplification occurred over 26–45 four-step cycles, with a rate of temperature change between steps of 20 °C/s. The steps were 95 °C with a 0-s hold, 60 °C with a 0-s hold, 72 °C with an 11-s hold, and 80 °C with a 1-s hold. Fluorescence was detected during the fourth step at a temperature previously determined.
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FIGURE 1. NcOGT mRNA levels are inversely proportional to UDP-GlcNAc levels, suggesting negative feedback regulation of O-GlcNAcylation. A, HepG2 cells were cultured for 6 h with 0–20 mM glucose (Glc) or 10 mM glucosamine (GlcN). Medium glucose concentration was maintained by glucose refueling every 3 h. Transcript levels of nucleocytoplasmic OGT were measured by reverse transcription-PCR and normalized to NONO transcript levels. Cells cultured in 0 mM glucose demonstrated the highest relative ncOGT transcript levels, whereas those cultured in 10 mM glucosamine have 4-fold lower ncOGT (p = 0.002). ncOGT transcript levels steadily decrease with increasing glucose concentration (0 mM Glc versus 2.5 mM Glc, p = 0.01; 0 versus 5, p = 0.007; 0 versus 20, p = 0.005). B, HepG2 cells were cultured for 12 h with 0–20 mM glucose or 10 mM glucosamine. 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. The cells cultured in 0 mM glucose exhibited the lowest UDP-GlcNAc levels (1.8 μM/μg protein/μl), whereas those cultured in 10 mM GlcN demonstrated 6.2-fold greater (p = 0.01) UDP-GlcNAc levels (12.2 μM/μg protein/μl). UDP-GlcNAc levels steadily rise with increasing glucose concentration (2.5 mM Glc [UDP-GlcNAc] = 3.05 μM/μg protein/μl; 5 mM Glc [UDP-GlcNAc] = 2.9 μM/μg protein/μl; 20 mM Glc [UDP-GlcNAc] = 4.1 μM/μg protein/μl). Average ncOGT mRNA and UDP-GlcNAc levels are based on at least three independent determinations/treatment. **, p ≤ 0.01.

FIGURE 2. O-GlcNAc levels steadily decrease over 6 h with glucose deprivation. A, HepG2 cells were cultured for 0–6 h in 0 and 5 mM glucose medium. Cell lysates were immunoblotted with α-O-GlcNAc and GAPDH. B, GAPDH normalized O-GlcNAc levels from lysates of glucose-deprived cells are reported. O-GlcNAcylation declines 80% over 6 h (p = 0.002). C, HepG2 cells were cultured for 0–6 h in 5 mM glucose medium. The cell lysates were immunoblotted with α-O-GlcNAc. GAPDH normalized O-GlcNAc levels were unchanged over time. Average normalized O-GlcNAc levels are based on at least three independent determinations/treatment. Apparent differences in banding patterns among these and subsequent O-GlcNAc blots are due only to differences in exposure time. **, p ≤ 0.01.

Glycogen Synthase Assay—The assay for glycogen synthase was performed as previously described (9). HepG2 lysate (7.5 μg of protein) was incubated in 100 μl of 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 (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 of the assays were done in duplicate. The incorporation of trit-
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ium was found to be optimal at 37 °C and linear for 120 min. Total glycogen synthase activity was defined as the activity at maximal glucose 6-phosphate (10 mM).

Statistical Analysis—The descriptive statistics are represented as the averages ± standard errors of the mean. Each average represents data from at least three independent experiments. The Student’s t test (two tail) was used to compare differences between groups.

RESULTS
An inverse relationship between ncOGT mRNA and UDP-GlcNAc levels suggests negative feedback regulation of O-
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We have previously reported a robust increase in protein O-GlcNAcylation in HepG2 human liver cells deprived of glucose (6). To identify the mechanism for this up-regulation, we assayed changes in ncOGT mRNA levels after 6 h of incubation of HepG2 cells in medium containing 0 or 5 mM glucose concentration. As previously reported, we observed a 3.4-fold increase in ncOGT mRNA levels with glucose deprivation compared with cells in normal (5 mM) glucose (6). In this study we measured ncOGT mRNA levels over a wider range of glucose concentrations and with glucosamine treatment. We measured ncOGT levels at 6 h, the time point at which we previously observed maximal ncOGT induction with glucose deprivation. We report that ncOGT levels are maximal with 0 mM glucose and decrease with increasing glucose concentration. Furthermore, under conditions of maximal HBP flux stimulated by 10 mM glucosamine treatment, ncOGT levels are further suppressed (Fig. 1A).

The levels of ncOGT are inversely related to the levels of the end product of the HBP, UDP-GlcNAc. We previously reported changes in UDP-GlcNAc levels with varying glucose concentrations (6). In cells deprived of glucose (which demonstrate maximal ncOGT expression), UDP-GlcNAc levels are the lowest (Fig. 1B). Assaying UDP-GlcNAc levels at 12 h is informative because this is the time point at which O-GlcNAc induction first appears. UDP-GlcNAc levels increase steadily, with increasing glucose concentration reaching a maximum with glucosamine treatment. Glucosamine is a potent stimulator of the HBP because it enters the HBP directly downstream of the feedback-regulated rate-limiting enzyme in hexosamine synthesis, glutamine fructose-6 phosphate amidotransferase (10).

**O-GlcNAc Levels Steadily Decrease over 6 h after Glucose Deprivation**—The observed inverse relationship between ncOGT mRNA and UDP-GlcNAc levels suggests a negative feedback mechanism. We therefore hypothesized that ncOGT mRNA induction in glucose-deprived cells represents a specific response to a decline in HBP flux in the first 6 h following glucose deprivation. Consistent with this hypothesis, cells deprived of glucose demonstrate a steady decline of O-GlcNAcylation between 1 and 6 h, before the induction of ncOGT (Fig. 2, A and B). Control cells in normal glucose exhibit no change in O-GlcNAcylation over the same time course (Fig. 2C).

Low Dose Glucosamine Treatment Prevents the Initial Decrease in UDP-GlcNAc and O-GlcNAc Seen with Glucose Deprivation and Blocks Subsequent Induction of OGT and O-GlcNAcylation and Inhibition of Glycogen Synthase—If the induction of OGT mRNA with glucose deprivation is caused by decreased HBP flux, we reasoned that if we prevented this decline in HBP flux with glucosamine in glucose deprivation, we might prevent OGT induction. This was the case (Fig. 3). The induction of O-GlcNAc modification by glucose deprivation is evident when comparing the first and last lanes (Fig. 3A). Although treatment with high concentrations of glucosamine (5 and 10 mM) leads to an increase in O-GlcNAc modification through greatly increased HBP flux (see also Fig. 1), lower concentrations of glucosamine (0.5 and 1 mM) paradoxically decrease O-GlcNAc modification in glucose-deprived cells to levels observed in cells treated with normal glucose (Fig. 3A, compare first, fourth, and last lanes). 1 mM glucosamine treatment also prevents the decline in UDP-GlcNAc levels and O-GlcNAcylation at 1, 3, and 6 h (Fig. 3, B and C) previously demonstrated to occur with glucose deprivation (Fig. 1, A and B, respectively). 1 mM glucosamine treatment dampens ncOGT induction by 60% in glucose-deprived cells (Fig. 3D; p = 0.05) and therefore prevents the subsequent increase in O-GlcNAcylation seen after 12 h in glucose-deprived cells (Fig. 3E). We previously demonstrated increased O-GlcNAc modification of glycogen synthase with glucose deprivation, which contributed to a 60% decrease in glycogen synthase activity (6). 1 mM glucosamine treatment results in a 44% rescue of glycogen synthase activity (p = 0.0002) in glucose-deprived cells (Fig. 3F). These data demonstrate that prevention of the decline in HBP flux in glucose-deprived cells by 1 mM glucosamine treatment reverses the effects of glucose deprivation on ncOGT expression, O-GlcNAcylation, and glycogen synthase activity.

**PUGNAc Treatment Completely Prevents ncOGT Induction with Glucose Deprivation**—Our data suggest that glucose depre-

![FIGURE 3. Low dose glucosamine treatment of glucose-deprived cells prevents initial UDP-GlcNAc and O-GlcNAc decreases, dampens ncOGT and O-GlcNAcylation induction, and partially rescues glycogen synthase inhibition. A, HepG2 cells were cultured for 12 h in medium lacking glucose but supplemented with 0–10 mM glucosamine (GlcN). Cell lysates were immunoblotted with α-O-GlcNAc. O-GlcNAcylation was minimized with 1 mM glucosamine supplementation (lane 4). In fact, O-GlcNAcylation levels returned to levels seen in normal glucose-treated cells (compare lanes 4 and 7). B, HepG2 cells were cultured for 1, 3, or 6 h in medium containing 0 mM Glc, 5 mM Glc, or 0 mM Glc + 1 mM GlcN. UDP-GlcNAc concentrations were determined and normalized to protein concentration and UDP-GlcNAc concentration with 5 mM Glc treatment. 1 mM GlcN prevents the fall-off in UDP-GlcNAc levels seen with glucose deprivation (0 Glc, 1 h versus 0 Glc, 3 and 6 h; p = 0.04 and p = 0.003, respectively; 0 Glc, 1 h versus 0 Glc, 1 GlcN, 1 h, p = 0.003; 0 Glc, 3 h versus 0 Glc, 1 GlcN, 3 h, p = 0.0001; 0 Glc, 6 h versus 0 Glc, 1 GlcN, 6 h, p = 0.0001). C, HepG2 cells deprived of glucose were cultured for 0–6 h in medium containing 1 mM glucosamine. Cell lysates were immunoblotted with α-O-GlcNAc. 1 mM glucosamine supplementation prevents the decrease in O-GlcNAc observed with glucose deprivation over the first 6 h of treatment. O-GlcNAc levels appear to increase with treatment, although this apparent increase is not significant (0 h versus 3 h, p = 0.15; 0 h versus 6 h, p = 0.33; 1 h versus 3 h, p = 0.06; 1 h versus 6 h, p = 0.19). D, 1 mM glucosamine supplementation dampens ncOGT induction by 60% in glucose-deprived cells (p = 0.05). E, 1 mM glucosamine supplementation prevents the O-GlcNAc induction seen after 12 h of glucose deprivation (0 mM Glc, 0 mM GlcN (lane 2) versus 0 mM Glc, 1 mM GlcN (lane 2), p = 0.004; 5 mM Glc, 0 GlcN (lane 1) versus 0 mM Glc, 1 mM GlcN (lane 1), p = 0.07). F, 1 mM glucosamine supplementation results in a 44% rescue of glycogen synthase activity (p = 0.0002). Average O-GlcNAc, ncOGT mRNA, and glucogen synthase activities are based on at least three independent determinations per treatment. Apparent differences in banding patterns among these and other O-GlcNAc blots appearing in this publication are due only to differences in exposure time, *p ≤ 0.05; **, p ≤ 0.01.
PUGNac on cellular O-GlcNAC levels and glycogen synthase activity (data not shown).

The AMP-dependent Kinase Pathway Does Not Mediate Glucose Deprivation Induction of OGT and O-GlcNAC—The AMPK pathway is a central energy-sensing pathway that is activated by increases in the AMP to ATP ratio. One might expect AMPK pathway activation in cells deprived of glucose and predict that this pathway could contribute to the observed increases in ncOGT mRNA and protein O-GlcNAcylation. However, we observed no differences in AMPK phosphorylation (Fig. 5) or total AMPK protein levels (Fig. 5) at 1, 3, 6, or 12 h, or phosphorylation of AMPK target acetyl-CoA carboxylase at 12 h (Fig. 5B) in glucose-deprived compared with normal glucose-treated cells. Thus, the AMPK pathway does not mediate ncOGT and O-GlcNAC induction in HepG2 cells deprived of glucose (Fig. 5).

**FIGURE 4. PUGNac treatment prevents O-GlcNAC decline over the initial 6 h and completely prevents ncOGT induction with glucose deprivation.** HepG2 cells were cultured for 0–6 h with medium containing 0 mM glucose and 50 μM PUGNac. Lysates were immunoblotted for changes in O-GlcNAC levels. Transcript levels of ncOGT were measured by reverse transcription-PCR and normalized to NONO transcript levels. A, PUGNac treatment prevents the O-GlcNAC decline seen with glucose deprivation. In fact, O-GlcNAC levels increase 2.1-fold by 6 h (*p < 0.03). B, PUGNac treatment results in complete reversal of ncOGT induction seen with glucose deprivation (compare second and third bars, *p < 0.002). In fact, PUGNac treatment results in a 52% decrease in ncOGT mRNA levels from those seen with normal glucose treatment (compare first and third bars, *p = 0.0003). O-GlcNAC levels were normalized against GAPDH protein levels. Average O-GlcNac and ncOGT mRNA levels are based on at least three independent determinations/treatment. Apparent differences in banding patterns among these and other O-GlcNac blots appearing in this publication are due only to differences in exposure time.* *p < 0.05; ** *p < 0.01.

**FIGURE 5.** The AMP-dependent Kinase pathway does not mediate glucose deprivation induction of ncOGT and O-GlcNAC. A, HepG2 cells were cultured for 1, 3, or 6 h in 0 or 5 mM glucose. Cell lysates were immunoblotted for total AMPK and pAMPK levels. The densities were normalized against GAPDH protein levels (not shown). We observed no differences in AMPK or pAMPK between glucose-deprived and normal glucose-treated cells. B, HepG2 cells were cultured for 12 h in 0 or 5 mM glucose. Cell lysates were immunoblotted for total AMPK, pAMPK, and phosphoacetyl-CoA carboxylase (pACC) levels. The densities were normalized against GAPDH protein levels (not shown). We observed no differences in AMPK, pAMPK, or phosphoacetyl-CoA carboxylase between glucose-deprived and normal glucose-treated cells. Average AMPK, pAMPK, phosphoacetyl-CoA carboxylase, and GAPDH levels are based on at least three independent determinations per treatment.
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DISCUSSION

Apart from regulation by substrate availability, little is known about the regulation of O-GlcNAc modification of proteins. OGT structure, modification, and regulation suggest complex regulation of O-GlcNAcylation. For example, regulation of OGT activity and substrate affinity are differentially modulated across a range of UDP-GlcNAc concentrations (11). Furthermore, OGT is tyrosine-phosphorylated, and presumably this modification is functionally important, although its effect on OGT is unknown (12). For many targets, phosphorylation and O-GlcNAcylation are reciprocal, and it is thought that this co-regulation of O-GlcNAc and O-phosphate is at least partially explained by the reported interaction of OGT and protein phosphatase 1 (13). Lastly, multiple OGT isoforms have been characterized that differ in the number of N-terminal tetra-ricopeptide repeats, and these differences are thought to influence OGT target specificity (12, 14, 15).

Cellular homeostasis is often achieved through feedback regulation, and our current results demonstrate that regulation of O-GlcNAcylation is no exception. The inverse relationship between UDP-GlcNAc and ncOGT mRNA levels (Fig. 1) and the prevention of ncOGT induction by low concentrations of glucosamine or PUGNAc support this conclusion. As we and others have demonstrated, O-GlcNAcylation increases in response to glucose deprivation are mediated by changes in OGT expression (6, 7). One established mechanism for the effects of O-GlcNAc on protein function is transcriptional regulation. OGT is known to associate with histone deacetylases (16), and O-GlcNAcase contains a histone acetyltransferase domain (17), indirectly linking O-GlcNAcylation with transcription inhibition and activation, respectively. Chromatin is highly O-GlcNAc-modified (18) as are many eukaryotic transcriptional machinery components including RNA polymerase II (19). Given that more than one-quarter of identified O-GlcNAc targets are transcription factors, it is probable that transcription factors modulating OGT expression are themselves O-GlcNAc-modified and that this may be the mechanism linking changes in UDP-GlcNAc with ncOGT expression. We previously reported that ncOGT induction results in a robust increase in O-GlcNAc modification of a number of proteins, but ncOGT mRNA levels return to normal by 12 h (6). In the model outlined above, the return of ncOGT levels to normal after induction by glucose deprivation would be consistent with deglycosylation of OGT targeting transcription factors (among many other proteins) that were deglycosylated early in treatment, resulting in renormalization of ncOGT.

O-GlcNAcylation has been shown to modulate transcription factor turnover, protein binding, DNA binding, and localization (20, 21). For example, Sp1, the first O-GlcNAc-modified transcription factor identified, exhibits a decreased turnover rate (22) and increasing nuclear localization (23) when modified by O-GlcNAc, and other transcription factors, when modified by O-GlcNAc, demonstrate altered interactions with other proteins (24, 25) or increased DNA binding affinity (26). Additionally or alternatively, regulation of OGT expression may occur at the level of transcript splicing. O-GlcNAcylation of RNA polymerase II is known to affect splicing (27, 28). Given the ubiquitous nature of O-GlcNAcylation, it is also possible that O-GlcNAcylation of splicing factors regulates splicing, thus explaining the differential up-regulation of ncOGT but not mOGT with glucose deprivation. Finally, OGT is itself O-GlcNAc-modified, suggesting potential negative feedback regulation of its activity as well as its transcription (12).

It was recently reported that increased O-GlcNAc modification of proteins mediated by increased OGT expression is also seen in glucose-deprived Neuro-2a cells (7). In Neuro-2a cells, the OGT induction is mediated by activation of the AMPK pathway. We, however, observe no increase in AMPK pathway activation with glucose deprivation. Furthermore, in glucose-deprived HepG2 cells, pyruvate addition or deprivation has no effect on O-GlcNAc induction (data not shown). In HepG2 cells, therefore, O-GlcNAcylation increases not in response to a cellular decrease in energy status but rather to a decrease in UDP-GlcNAc and O-GlcNAcylation. Thus, it appears that mechanisms of O-GlcNAc induction in response to glucose deprivation vary according to tissue type, suggesting an additional level of complexity to the regulation of O-GlcNAc.

It has previously been established that increased O-GlcNAcylation in pathologic hyperglycemic states is driven by increased substrate availability. Herein, we report that O-GlcNAcylation is also regulated by feedback inhibition across the physiologic and lower range of glucose concentrations. The results suggest that there are important consequences for cell function in regulating protein O-GlcNAc levels at low, high, and normal glucose levels.

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