Regulation of Akt signaling by O-GlcNAc in euglycemia.

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**Abstract**

The hexosamine biosynthesis pathway (HBP) regulates the post-translational modification of nuclear and cytoplasmic protein by O-linked N-acetylglucosamine (O-GlcNAc). Numerous studies have demonstrated that in hyperglycemic conditions, excessive glucose flux through this pathway contributes to the development of insulin resistance. The role of the HBP in euglycemia, however, remains largely unknown. Here we investigated the effect of O-GlcNAc on hepatic Akt signaling at physiologic concentrations of glucose. In HepG2 cells cultured in 5 mM glucose, removal of O-GlcNAc by adenoviral-mediated overexpression of O-GlcNAcase increases Akt activity and phosphorylation. We also observed that Akt was recognized by succinylated wheat germ agglutinin (sWGA), which specifically binds O-GlcNAc. Overexpression of O-GlcNAcase in HepG2 cells reduced the levels of Akt in sWGA precipitates. The increased Akt activity was accompanied by increased phosphorylation of Akt substrates and reduced mRNA for glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK). The increased Akt activity was not a result of activation of its upstream activator phosphoinositide 3-kinase (PI3-kinase). Further demonstrating Akt regulation by O-GlcNAc, we found that overexpression of O-GlcNAcase in the livers of euglycemic mice also significantly increased Akt activity resulting in increased phosphorylation of downstream targets and decreased mRNA for glucose-6-phosphatase. Together, these data suggest that O-GlcNAc regulates Akt signaling in hepatic models under euglycemic conditions.

**Keywords:** O-GlcNAcase, hexosamine, hepatic, gluconeogenic enzymes.
**Introduction**

Insulin resistance is a central feature of type 2 diabetes wherein there is a defect in the ability of insulin to stimulate glucose uptake in muscle and fat and to suppress hepatic glucose production. Insulin resistance may result from a combination of hereditary factors, chronic hyperinsulinemia, and so called “glucose toxicity,” which refers to effects of chronically excessive glucose flux on cellular function and regulation (29, 36). One major mediator of the regulatory effects of glucose is the hexosamine biosynthesis pathway (HBP) (4, 18, 28). A relatively small fraction of cellular fructose-6-phosphate enters the HBP and is converted to glucosamine-6-phosphate (GlcN-6-P) by the rate limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFA). GlcN-6-P is converted to uridine 5’-diphospho N-acetylglucosamine (UDP-GlcNAc) which can be used as a substrate by the enzyme O-linked β-N-acetylglucosamine transferase (OGT) to catalyze covalent attachment of O-linked β-N-acetylglucosamine (O-GlcNAc) to serine and threonine residues of nuclear and cytosolic proteins. The O-GlcNAc modification is removed by the enzyme O-linked β-N-acetylglucosaminidase (O-GlcNAcase). Sites of O-GlcNAc modification have been identified for numerous proteins and are often the same as or near phosphorylation sites, suggesting a regulatory function of O-GlcNAc modification. In fact, O-GlcNAc modification is known to modulate transcription, translation, nuclear transport, and other critical cellular processes (12, 38).

The involvement of the HBP in the development of glucose-induced insulin resistance was first suggested by Marshall et al. who demonstrated a requirement for the HBP in desensitizing insulin-stimulated glucose transport in cultured adipocytes (18). Subsequent studies have confirmed the importance of the HBP in mediating insulin resistance. For example,
transgenic mice overexpressing OGT in muscle and fat have elevated insulin levels and diminished glucose disposal rates (22), GFA transgene expression in mouse adipose tissue also results in defective insulin stimulated glucose uptake (21) and adenoviral overexpression of OGT in mouse liver results in higher glucose and insulin levels, with a concomitant increase in hepatic glucose production (37). Conversely, it has recently been demonstrated that adenoviral expression of O-GlcNAcase in the livers of db/db mice improves glucose tolerance and insulin sensitivity (7). All of the data strongly support the importance of O-GlcNAc modification in regulating insulin signaling in pathologic situations of hyperglycemia and nutrient excess. The question remains whether O-GlcNAc modifications also play a role in metabolic regulation in states wherein glucose concentrations are normal. We therefore sought to characterize the significance of O-GlcNAc modifications under euglycemic condition. Particularly, we overexpressed O-GlcNAcase in HepG2 cells cultured in normal glucose and the livers of euglycemic C57BL/6J mice and investigated the effect of removal of protein O-GlcNAc modifications on Akt signaling.
Materials and Methods

Antibodies. Antibodies to Akt, phospho-Akt (Ser-473), phospho-FOXO1 (Ser-256), phospho-GSK-3β (Ser-9) and phospho-MDM2 (Ser-166) were from Cell Signaling Technology (Danvers, MA). Anti O-GlcNAc monoclonal IgM antibody (CTD 110.6) was a gift from Dr. Gerald Hart. GAPDH antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). IRS-1 antibody was purchased from Upstate (Lake Placid, NY).

Virus purification and titer. HEK293 cells were infected with adenovirus for 48 h. Infected cells were harvested and then frozen/thawed for 3 cycles to release the adenovirus. Cell debris was then pelleted by spinning at 3000 rpm for 10 min. Virus was purified and titered using kits from Cell Biolabs, Inc (San Diego, CA). The purified virus was used for study in the HepG2 cells only.

Overexpression of proteins in HepG2 cells. The HepG2 cell line was purchased from American Type Culture Collection (Manassas, VA). HepG2 cells were maintained in 5 mM glucose Dulbecco’s modified eagle medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, at 37°C in a 5% CO2 atmosphere. HepG2 cells were seeded on 6-cm plates and cultured to 70% confluence. Cells were then infected with 5x10^7 plaque forming units (pfu) of adenovirus encoding either GFP or O-GlcNAcase (a generous gift from Dr. Wolfgang Dillmann, University of California, San Diego). On the following day, media was replaced with fresh media containing 1% FBS. Cells were harvested 48 h after infection.

Overexpression of proteins in C57BL/6J mouse livers. Eight to ten-week old mice were injected through the tail vein with a suspension of 200 μl of 1x10^{11} virus particles (VP) of GFP or O-GlcNAcase adenovirus. We infected the mice for 3, 5, 7 or 9 days and determined that O-GlcNAcase mRNA level is highest after 3 days of infection. Mice were sacrificed and harvested
organs were snap-frozen in liquid nitrogen. The GFP and O-GlcNAcase virus were amplified, purified and titered by Vector Biolabs (Philadelphia, PA).

**Western Blot.** HepG2 cells were collected and briefly sonicated in lysis buffer [50 mM Hepes pH 7.4, 100 mM NaCl, 5% glycerol, 1% Triton X-100, 1 mM EDTA, protease inhibitor cocktail (Roche, Indianapolis, IN), phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO)]. Frozen livers from C57BL/6J mice were homogenized in lysis buffer using a dounce homogenizer. 20-60 μg of denatured protein were resolved on a 10% SDS-polyacrylamide gel and transferred onto immobilon nitrocellulose membrane (Millipore, Billerica, MA). Blots were then blocked with TBS-T (20 mM Tris, pH 7.4, 150 mM sodium chloride, and 0.5% Tween 20) containing 5% (w/v) nonfat dried milk for 1 h at room temperature. For detection with the CTD 110.6 antibody, blots were blocked with TBS-T containing 5% (w/v) bovine serum albumin. Blots were incubated with primary antibodies overnight at 4°C and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature.

**Succinyalted wheat germ agglutinin (sWGA).** The assay was carried out as previously described (27). Briefly, 400 μg of protein were incubated with 30 μl of sWGA (Amersham Biosciences, Piscataway, NJ) beads overnight at 4°C. Beads were collected by centrifugation (14000 rpm, 4°C, 1 min) and washed three times with lysis buffer. Beads were collected again with centrifugation and resuspended in 30 μl of Laemmlı sample buffer, boiled and loaded onto a 10% SDS-polyacrylamide gel.

**Akt kinase assay.** 500 μg of protein were incubated overnight at 4°C with immobilized Akt antibody cross linked to agarose beads (Cell Signaling). GSK-3 fusion protein was later added per manufacturer’s protocol. The reaction was terminated by the addition of SDS sample buffer.
Samples were boiled, loaded onto a 10% SDS-polyacrylamide gel and immunoblotted with phospho-GSK-3β antibody.

**PI3-kinase assay.** PI3-kinase was immunoprecipitated by incubating 200 µg of protein with IRS-1 antibody for 3 h followed by incubation with protein A agarose beads (Santa Cruz Biotechnology, Inc.) for 1 h. All incubations were performed at 4°C. Beads were washed twice with each of the following three buffers [Buffer 1: PBS, 1% Igepal, 100 µM Na₃VO₄. Buffer 2: 100 mM Tris (pH 7.5), 500 mM LiCl, 100 µM Na₃VO₄. Buffer 3: 10 mM Tris (pH 7.5), 100 mM NaCl, 100 µM Na₃VO₄], and then resuspended in 10 mM Tris (pH 7.5), 100 mM NaCl and 1 mM EDTA. Prior to adding phosphatidylinositol (PI) (Avanti Polar Lipids, Alabaster, AL) to the resuspended beads, the lipid was dried under a stream of nitrogen gas, resuspended in buffer [10 mM Tris (pH 7.5), 1 mM EGTA] and then sonicated for 2 min. The reaction was started by adding 5 µCi of ^32^P ATP and incubating for 10 min at room temperature. The reaction was stopped with 8 M HCl, and phospholipids were extracted with CHCl₃:MeOH (1:1). The phospholipids were resolved by thin-layer chromatography (TLC) and visualized by autoradiography. Samples were then scraped from the TLC plate and counted for ^32^P incorporation using a liquid scintillation counter.

**RT-PCR.** HepG2 cells were infected with adenovirus as described above. 48 h after infection, RNA was isolated from HepG2 cells using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH), purified using an RNeasy mini kit (Qiagen, Valencia, CA) and reverse transcribed into cDNA. cDNA was purified using a QIAquick PCR purification kit (Qiagen) prior to RT-PCR studies. mRNA levels from HepG2 cells were normalized to non-POU domain containing octamer-binding protein (NONO) (16, 33); 5’-CAAGTGGACCGCAACATCA-3’ (forward) and 5’-CGCCGCATCTCTTTCTTCAC-3’ (reverse). Other primers used for HepG2
studies were as follows: human phosphoenolpyruvate carboxykinase (PEPCK); 5’-GAGCTGACGGATTCACCCTA-3’ (forward) and 5’-CCACTGCCAAGGAGATGAT-3’ (reverse). Human glucose-6-phosphatase; 5’-TACGTCCTCTTCCCCATCTG-3’ (forward) and 5’-CCTGGTCCAGTCTCACAGGT-3’ (reverse). For animal studies, mice were sacrificed 3 days post injection and liver slices were stored in RNAlater. Tissues were weighed and homogenized with a Tissue Tearor in buffer RLT (Qiagen). RNA was purified and reverse transcribed as mentioned above. mRNA levels from C57BL/6J mice were normalized to ribosomal protein L13a (Rpl13a); 5’-GGAGAAACGGAAGAAGG-3’ (forward) and 5’-ACAGGAGCAGTGCCTAAGGA-3’ (reverse). Other primers used for C57BL/6J studies were as follows: mouse and human O-GlcNAcase; 5’-CCCTCGCCTGGATTACTGCT-3’ (forward) and 5’-AGACAGGAGGCAAGCCATCA-3’ (reverse). Mouse PEPCK; 5’-GTCAACACCGACCTCCCTTA-3’ (forward) and 5’-CCCTAGCCTGTTCCTGTGC-3’ (reverse). Mouse glucose-6-phosphatase; 5’-AGGAAGGATGGAGGAAGGAA-3’ (forward) and 5’-TGGAACCAGATGGGAAGAG-3’ (reverse).

Statistical analysis. Results are presented as mean ± S.E.M. The unpaired Student’s t test (two-tail) was used to assess differences between experimental groups and controls. Probability values of <0.05 were considered statistically significant.
Results

O-GlcNAcase overexpression increased Akt activity in HepG2 cells.

Studies involving O-GlcNAc modification have shown that increased protein O-glycosylation can lead to the development of insulin resistance, and one mechanism is through reduction of Akt activity (26, 35). To determine if reduced O-GlcNAc modification would also affect insulin signaling in normal glucose concentrations, we used adenovirus to overexpress O-GlcNAcase in HepG2 cells cultured in medium containing 5 mM glucose. Adenoviral-mediated overexpression of O-GlcNAcase in HepG2 cells increased O-GlcNAcase protein levels, resulting in a decrease in global O-GlcNAc modification of proteins as detected by O-GlcNAc-specific CTD 110.6 antibody (Fig. 1A). We then examined the effect of O-GlcNAcase overexpression on Akt signaling and observed an increase in Akt phosphorylation at Thr-308 and Ser-473 (Fig. 1B) with a concomitant 2.3-fold increase in Akt activity (p<0.05, Fig. 1B), measured in vitro from extracts of HepG2 cells. The increased activity of Akt was reflected in increased phosphorylation of the Akt substrates glycogen synthase kinase-3β (GSK-3β at Ser-9) and murine double minute-2 (MDM2 at Ser-166) (19) (Fig. 1B). We next examined the effect of insulin on Akt phosphorylation and found that insulin further induced Akt phosphorylation in O-GlcNAcase overexpressing cells when compared to control cells (Fig. 1C). Previous reports have demonstrated O-GlcNAc modification of Akt in cells cultured under hyperglycemic conditions (17, 26). To test whether Akt is also O-GlcNAc modified under normal glucose conditions, we used succinylated wheat germ agglutinin (sWGA), a modified lectin that binds O-GlcNAc, to precipitate the O-GlcNAc modified proteins from HepG2 cell lysates. Akt was detected in sWGA precipitates and overexpression of O-GlcNAcase decreased Akt levels in sWGA
precipitates by 32% (p<0.05) (Fig. 1D). These results indicate that removal of O-GlcNAc modifications increases Akt activity in HepG2 cells grown in normal (5 mM) glucose.

**O-GlcNAcase-mediated Akt activation in HepG2 cells was not a result of upstream activation of PI3-kinase.**

After insulin stimulation, insulin receptor substrates (IRS) interact with the p85 subunit of PI3-kinase leading to activation of the p110 subunit of PI3-kinase. This in turn phosphorylates PIP₂ to PIP₃. PIP₃ then stimulates the translocation of Akt to the plasma membrane where Akt can be phosphorylated by PDK-1 and the mTOR-Rictor complex (1, 30, 32). To determine if increased Akt activity with O-GlcNAcase overexpression was due to increased PI3-kinase activity, we measured PI3-kinase activity in HepG2 cells and found that there was no significant difference in PI3-kinase activity between O-GlcNAcase overexpressing cells and control cells. Insulin treatment increased PI3-kinase activity equally (~6-fold) in control and O-GlcNAcase overexpressing cells (Fig. 2).

**O-GlcNAcase overexpression reduced glucose-6-phosphatase and PEPCK mRNA transcript levels in HepG2 cells.**

After insulin stimulation, phosphorylated Akt enters the nucleus where it can phosphorylate forkhead box O1 (FOXO1). Phosphorylation by Akt disrupts the ability of FOXO1 to bind DNA (40) and thereby prevents upregulation of transcription of the gluconeogenic genes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK) (11, 23, 31), resulting in decreased hepatic glucose production (25). To confirm that activation of Akt by O-GlcNAcase in HepG2 cells also resulted in transcriptional regulation of glucose-6-phosphatase and PEPCK, we examined their mRNA transcript levels by RT-PCR. While we were unable to conclusively demonstrate changes in FOXO1 phosphorylation by Akt in HepG2 cells overexpressing O-
GlcNAcase (data not shown), we did observe 57% (p<0.05) and 48% (p<0.05) decrease in glucose-6-phosphatase and PEPCK transcript levels respectively (Fig. 3).

**O-GlcNAcase overexpression increased Akt activity in livers of C57BL/6J mice.**

To determine whether results observed in the HepG2 cells were physiologically relevant in the intact animal, livers of C57BL/6J mice were infected by tail vein injection with adenovirus expressing either GFP or O-GlcNAcase. These mice exhibited normal fasting blood glucose levels (GFP, 133 mg/dl vs. O-GlcNAcase, 140 mg/dl, n=3-4, p=0.32) (Supplemental fig. 1A). O-GlcNAcase mRNA levels in the livers of O-GlcNAcase infected mice increased 9.7-fold (p<0.05) compared to GFP controls (Fig. 4A). No changes in O-GlcNAcase mRNA level were observed in either muscle or fat (data not shown). O-GlcNAcase overexpression resulted in decreased global protein O-GlcNAc modification in liver (Fig. 4A). Consistent with results from HepG2 cells, O-GlcNAcase overexpression in liver led to increased hepatic Akt phosphorylation on Thr-308 and Ser-473 (Fig. 4B), which was accompanied by increased phosphorylation of Akt substrates; GSK-3β (Ser-9), MDM2 (Ser-166) and FOXO1 (Ser-256) (Fig. 4B). Furthermore, we observed a 36% (p<0.05) reduction in glucose-6-phosphatase mRNA compared to control mice. PEPCK mRNA decreased by 24%; however, the result was not statistically significant (Fig. 5). Together, these results indicate that removal of the O-GlcNAc modification also increases Akt signaling in livers of euglycemic mice.
**Discussion**

Sustained hyperglycemia has been shown to induce insulin resistance, and this has been suggested to be an adaptive mechanism in protecting cells from oxidative stress that results from excess nutrients (4). The hexosamine biosynthesis pathway (HBP) has been shown to be one of the cellular nutrient sensors that plays an important role in the development of insulin resistance (3, 20, 28).

The protein kinase Akt is an important mediator of insulin signaling in the regulation of cell survival and metabolism (5, 9, 39). Akt has previously been shown to be O-GlcNAc modified by OGT (10, 26), and several studies have demonstrated linkage between the HBP, the development of insulin resistance and altered Akt signaling. For example, treatment of 3T3-L1 adipocytes with PUGNAc (an inhibitor of O-GlcNAcase) reduces Akt phosphorylation resulting in a reduction of insulin stimulated 2-deoxyglucose uptake (35). In primary adipocytes isolated from rat epididymal fat pads, PUGNAc treatment results in increased Akt O-GlcNAc modification with a concomitant decrease in Akt phosphorylation, resulting in impaired insulin-stimulated glucose transporter 4 (GLUT4) translocation to the plasma membrane (26). O-GlcNAcase overexpression in db/db mice increases Akt phosphorylation and improves glucose tolerance and insulin sensitivity (7). In a recent report, Yang et al. demonstrates that upon insulin stimulation OGT can be translocated to the plasma membrane through interaction with PI(3,4,5)P3, demonstrating a potential mechanism by which Akt can be O-GlcNAcylated (37). Most studies, including those above, have linked hyperglycemia and/or chronically high glucose flux through the hexosamine pathway with the development of insulin resistance (6, 13, 21, 24, 26, 27, 35). The role of the HBP in euglycemia, however, remains largely unknown. In this study, we have examined the role of O-GlcNAc regulation in HepG2 cells that were cultured in 5
mM glucose and livers of euglycemic C57BL/6J mice fed with normal chow. We showed that overexpression of O-GlcNAcase in the two models reduced O-GlcNAc modification of proteins. We observed a decrease in O-GlcNAc modification of Akt in HepG2 cells and an increase in Akt phosphorylation in both tissue culture and in vivo experiments. These data are consistent with the reciprocal relationship that has been established between phosphorylation and O-GlcNAc modification in many other proteins (8, 27). The results are congruent with previous findings using the opposite approach, where PUGNAc treatment of primary rat adipocytes increases O-GlcNAc modification of Akt while reducing its phosphorylation level (26).

The increased Akt phosphorylation resulted in increased phosphorylation of its downstream targets including FOXO1. Previous studies have shown that increased phosphorylation of FOXO1 protein by Akt causes FOXO1 to be exported to the cytoplasm, through interaction with the 14-3-3 protein, where it is targeted for proteosomal degradation (34). Removal of FOXO1 from the nucleus prevents it from upregulating the transcription of gluconeogenic enzymes, including glucose-6-phosphatase and PEPCK (11, 23, 31). Consistent with these observations, we found that overexpression of O-GlcNAcase increased Akt-FOXO1 signaling that significantly reduced mRNA expression of glucose-6-phosphatase and PEPCK in HepG2 cells, although only glucose-6-phosphatase mRNA was significantly reduced in the animal study. Of note, increased FOXO1 phosphorylation was observed in the animal model only. This may be due to technical limitations; however, O-GlcNAc modification of FOXO1 may also be an independent signal in stimulating transcription of gluconeogenic enzymes in HepG2 cells (15). A separate mechanism by which O-GlcNAc modification could affect gluconeogenesis is through cyclic adenosine monophosphate response element-binding protein 2 (CRTC2 or TORC2). CRTC2 is a critical switch that regulates hepatic gluconeogenesis in part
by upregulating glucose-6-phosphatase and PEPCK gene expression under condition of starvation (14). It has been shown that CRTC2 is O-GlcNAc modified and this modification prevents phosphorylation of CRTC2, thereby allowing CRTC2 to be retained in the nucleus where it plays a role in upregulating expression of gluconeogenic enzymes. While the reverse is true, removing O-GlcNAc modification from CRTC2 increases phosphorylation of CRTC2 and results in downregulation of glucose-6-phosphatase and PEPCK mRNA transcript (7). These data show diverse mechanisms in regulating the hepatic glucose production while highlighting the importance of O-GlcNAc modification for its role in modulating proteins that are involved in hepatic gluconeogenesis.

A previous study has shown that HBP flux can impair insulin induced tyrosine phosphorylation of IRS-1, PI3-kinase activity and subsequent Akt activation in RIN β-cells (2), demonstrating that impaired upstream signaling may lead to impaired Akt activation. In this study, we observed an increase in Akt signaling through overexpression of O-GlcNAcase; however, contrary to the above study, we did not observe an increase in PI3-kinase activity associated with decreased O-glycosylation. A similar lack of an effect of O-GlcNAc on signaling upstream of Akt has been observed in adipocytes treated with PUGNAc (35) or in observations made by Yang that suggest O-GlcNAc does not affect PI3-kinase activity (37). These data suggest that HBP-induced changes in Akt activity do not necessarily require changes in insulin signaling upstream from Akt, although upstream effects may be noted in certain cell or tissue types or with hyperglycemia.

We performed physiological assays 3 days post-infection when O-GlcNAcase mRNA levels were maximal and observed increased Akt signaling in livers of C57BL/6J mice, though we were not able to detect any changes in fasting glucose and insulin levels, glucose tolerance or
glycogen content (Supplemental fig. 1-3). Another study of wild type C57BL/6J mice overexpressing O-GlcNAcase also failed to demonstrate a significant difference in glucose tolerance compared to controls (7). The lack of an effect of O-GlcNAcase on overall glucose homeostasis may be due to compensatory effects in the liver or other tissues and the functional significance of increased Akt signaling through decreased O-GlcNAc modification in euglycemia therefore requires further examination. We did note, however, a small but significant decrease in fasting blood glucose after 5 days of infection, consistent with the lower mRNA level of the gluconeogenic enzymes (Supplemental fig. 4).

We also evaluated whether O-GlcNAcase overexpression in liver is sufficient to alter Akt signaling in ob/ob diabetic mice. We found that there was no change in Akt phosphorylation between GFP control and O-GlcNAcase overexpressing mice and in addition we also showed no statistically significant difference in their glucose tolerance (data not shown). This is in contrast to published results obtained using db/db mice and suggests that differences in mouse model or strain, levels of viral expression, kinetics of expression and/or other unknown variables are also affecting the ultimate physiological phenotype that is observed (7).

In summary we have demonstrated that decreasing the O-GlcNAc modification of proteins under euglycemic conditions increases Akt signaling, resulting in reduced mRNA levels of glucose-6-phosphatase and PEPCK in HepG2 cells and reduced glucose-6-phosphatase mRNA in the liver of intact non-diabetic mice. This adds to the growing body of evidence showing the importance of HBP as a nutrient sensing pathway not only in pathologic states of excess nutrient levels but also in normal physiology.
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References


**Figure Legends**

**Figure 1. Effect of O-GlcNAcase overexpression on Akt activity in HepG2 cells.** (A) HepG2 cells were infected with adenovirus encoding either GFP or O-GlcNAcase for 2 days. Cell extracts were immunoblotted with O-GlcNAcase and CTD 110.6 antibodies. (B) Cell extracts were also immunoblotted with phospho-Akt (Thr-308 or Ser-473), total Akt, phospho-GSK-3β (Ser-9) and phospho-MDM2 (Ser-166) antibodies. To correlate Akt phosphorylation with its activity, Akt activity was assessed in cell lysates immunoprecipitated with Akt antibody, incubated with GSK-3 fusion protein substrate, and immunoblotted with phospho-GSK-3 specific antibody. (C) To determine the effect of insulin on Akt signaling, infected HepG2 cells were treated with or without 10 nM insulin for 30 min prior to harvesting. (D) To determine if Akt was O-GlcNAc modified, HepG2 cell lysates were precipitated with sWGA agarose followed by immunoblotting with Akt antibody. The result shown represents the mean ± S.E.M of at least three independent experiments * p<0.05.

**Figure 2. PI3K activity in HepG2 cells.** HepG2 cells were infected with adenovirus encoding either GFP or O-GlcNAcase for 2 days. The cell lysates were immunoprecipitated with IRS-1 antibody, and immunocomplexes were then incubated with phosphatidylinositol (PI) along with $^{32}$P-ATP in assay buffer. Samples were assayed for $^{32}$P incorporation into PI using a liquid scintillation counter. The result shown represents the mean ± S.E.M of three independent experiments * p<0.05.

**Figure 3. Effect of O-GlcNAcase overexpression on gluconeogenic enzymes in HepG2 cells.** HepG2 cells were infected with adenovirus encoding either GFP or O-GlcNAcase for 2 days. Glucose-6-phosphatase and PEPCK mRNA transcript levels were measured as described in Materials and Methods. Both were normalized to mRNA levels of non-POU domain containing
octamer-binding protein (NONO) message. The results shown represent the mean ± S.E.M of seven independent experiments * p<0.05.

**Figure 4. Effect of O-GlcNAcase overexpression on Akt activity in livers of C57BL/6J mice.** O-GlcNAcase protein was overexpressed in the liver of C57BL/6J mice through tail vein injection of adenovirus encoding the protein. Adenovirus encoding GFP was used as control. (A) mRNA and protein level of O-GlcNAcase were determined by RT-PCR and western blotting. The RT-PCR result represents the mean ± S.E.M of five independent experiments * p<0.05. (B) Liver extracts were immunoblotted with specific antibodies as indicated.

**Figure 5. Effect of O-GlcNAcase overexpression on gluconeogenic enzymes in livers of C57BL/6J mice.** O-GlcNAcase protein was overexpressed in the liver of C57BL/6J mice through tail vein injection of adenovirus encoding the protein. Adenovirus encoding GFP was used as control. Glucose-6-phosphatase and PEPCK mRNA level were measured as described in *Materials and Methods*. Both were normalized to ribosomal protein L13a (Rpl13a) message. The results shown represent the mean ± S.E.M of five independent experiments * p<0.05.
Figure 1

Panel A:
- GFP
- O-GlcNAcase
- O-GlcNAcase
- O-GlcNAc

Panel B:
- GFP
- O-GlcNAcase
- p-Akt Thr-308
- p-Akt Ser-473
- Akt
- p-GSK-3β
- p-MDM2

Panel C:
- GFP
- O-GlcNAcase
- p-Akt
- p-Akt Thr-308
- p-Akt Ser-473

Panel D:
- GFP
- O-GlcNAcase
- p-Akt
- p-Akt Thr-308
- p-Akt Ser-473

Relative Akt Activity

0.00 0.50 1.00 1.50

GFP  O-GlcNAcase

Relative O-GlcNAc Akt

0 20 40 60 80

GFP  O-GlcNAcase

* indicates statistical significance.
Figure 2

PI3K activity (cpm)

- GFP
- O-GlcNAcase
- GFP + insulin
- O-GlcNAcase + insulin

* Indicates statistically significant difference.
Figure 3

(A) Relative G-6-Pase mRNA levels.
(B) Relative PEPCK mRNA levels.
Figure 4

A

B

GFP  O-GlcNAcase

- p-Akt Thr-308
- p-Akt Ser-473
- Akt
- p-FOXO1
- p-GSK-3β
- p-MDM2

Relative O-GlcNAcase mRNA

GFP  O-GlcNAcase
Figure 5

(A) Relative G-6-Pase mRNA

(B) Relative PEPCK mRNA