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A role for *N*-acetylglucosamine as a nutrient sensor and mediator of insulin resistance

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Abstract. The ability to regulate energy balance at both the cellular and whole body level is an essential process of life. As western society has shifted to a higher caloric diet and more sedentary lifestyle, the incidence of type 2 diabetes (non-insulin-dependent diabetes mellitus) has increased to epidemic proportions. Thus, type 2 diabetes has been described as a disease of 'chronic overnutrition'. There are abundant data to support the relationship between nutrient availability and insulin action. However, there have been multiple hypotheses and debates as to the mechanism by which nutrient availability modulates insulin signaling and how excess nutrients lead to insulin resistance. One well-established pathway for nutrient sensing is the hexosamine biosynthetic pathway (HSP), which produces the acetylated aminosugar nucleotide uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) as its end product. Since UDP-GlcNAc is the donor substrate for modification of nucleocytoplasmic proteins at serine and threonine residues with *N*-acetylglucosamine (*O*-GlcNAc), the possibility of this posttranslational modification serving as the nutrient sensor has been proposed. We have recently directly tested this model in adipocytes by examining the effect of elevated levels of *O*-GlcNAc on insulin-stimulated glucose uptake. In this review, we summarize the existing work that implicates the HSP and *O*-GlcNAc modification as nutrient sensors and regulators of insulin signaling.

Key words. *O*-GlcNAc; glycosylation; posttranslational modification; hexosamine biosynthetic pathway; glucosamine; hyperglycemia; insulin resistance; type 2 diabetes.

Type 2 diabetes and insulin resistance

Chronic hyperglycemia is the hallmark of all types of diabetes (see [1, 2] for recent reviews). In type 2 diabetes, insulin resistance is the primary feature, and it is believed that this insulin resistance coupled with 'glucose toxicity' is responsible for the plethora of complications seen in patients, including microvascular and macrovasular disorders [3]. While the genesis of type 2 diabetes is still unclear and under intense study, it appears that certain genetic traits predispose individuals for development of the disease when exposed to certain environmental factors, namely chronic nutrient excess and low energy expenditure [4]. Insulin resistance occurs in three separate systems: pancreatic β-cells, liver and peripheral insulin-responsive tissues (adipocytes and skeletal muscle). The role of insulin resistance modulating insulin secretion in the β- cell and upregulating gluconeogenesis and glucose output in the liver will only be touched upon briefly in this review and has been reviewed elsewhere [1–4]. In skeletal muscle and adipose tissue insulin resistance inhibits insulin-responsive glucose uptake and glycogen synthesis. It has been widely proposed that insulin resistance in insulin-responsive tissues is an 'adaptation' to nutrient excess [4–6]. If this hypothesis is correct, there must be a sensor(s) capable of detecting changes in nutrient levels and initiating a proper response. In order to elucidate these sensors, many investigators have asked, 'How does hyperglycemia induce peripheral insulin resistance?' The answer to this question should elucidate an 'energy' or glucose sensor in muscle and adipose tissue. Various hypotheses have been put forward and have been reviewed elsewhere [4, 7–11]. One key observation made by Traxinger and Marshall was that induction of insulin resistance in cultured adipocytes requires three key components: glucose, insulin and glutamine [12]. Since glut-

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amine : fructose-6-phosphate-amidotransferase (GFAT), the first and rate-limiting enzyme of the hexosamine biosynthetic pathway (HSP), requires glutamine and the glucose metabolite fructose-6-phosphate, researchers began to investigate the possibility that the HSP was serving as an energy sensor.

The hexosamine biosynthetic pathway and peripheral insulin resistance (see fig. 1)

Once glucose enters a cell, it is rapidly converted to glucose-6-phosphate that can be converted to glucose-1-phosphate for glycogen synthesis or converted to fructose-6-phosphate. Fructose-6-phosphate is preferentially used for glycolysis, but a small percentage is converted to glucosamine-6-phosphate with the concomitant conversion of glutamine to glutamate by the rate-limiting enzyme in the HSP GFAT [13]. Glucosamine-6-phosphate is then rapidly converted to uridine 5′-diphospho *N*-acetylglucosamine (UDP-GlcNAc) [13]. Due to the chemical makeup of UDP-GlcNAc, it is well positioned to serve as a glucose sensor in that it is a high-energy compound that requires and/or responds to glucose, amino acid, fatty acid and nucleotide metabolism for synthesis (fig. 2). UDP-GlcNAc serves as the donor sugar nucleotide for lipid and secretory protein complex glycosylation, glycosyl phosphatidylinositol (GPI) anchor synthesis, and *N*-acetylglucosmine (*O*-GlcNAc) modification of nuclear and cytosolic proteins.

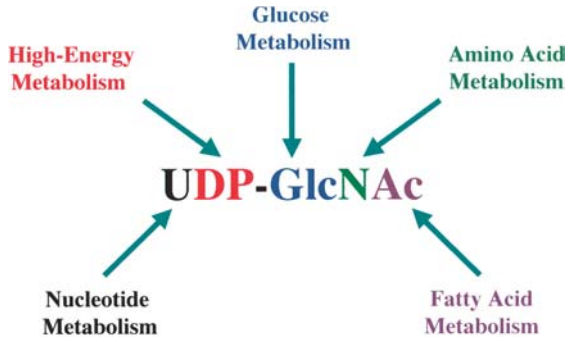

Figure 2. UDP-GlcNAc is well positioned for serving as a glucose sensor in that it is a high-energy compound that is required for synthesis and responds to glucose, amino acid, fatty acid and nucleotide metabolism.

Investigators have studied the HSP in a variety of systems, using both genetic and pharmacological methods (reviewed in [6, 9]). One of the initial observations was that glucosamine that enters the HSP downstream of GFAT and is rapidly converted to UDP-GlcNAc could take the place of hyperglycemia in inducing insulin resistance in cultured adipocytes [14]. Glucosamine has now been used to induce insulin resistance in a variety of cells, tissues and whole organisms, including humans [6, 9]. In 1998, Hresko and colleagues attributed the effects of glu-

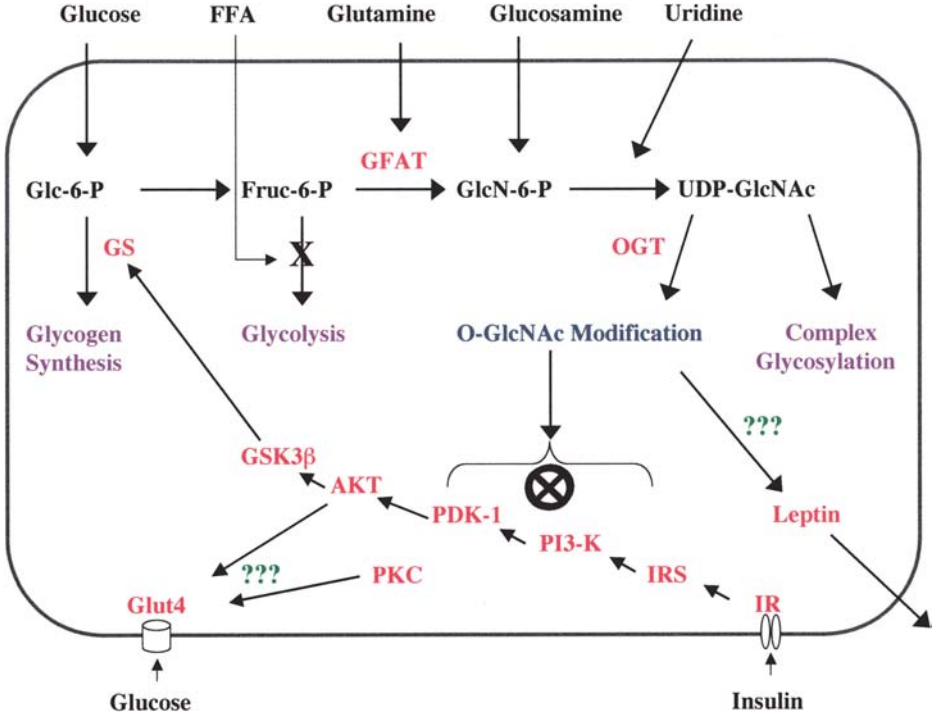

Figure 1. Glucose metabolism and insulin signal transduction in adipocytes. Small molecules are depicted in black, proteins in red and biological processes in purple. Increased flux through the HSP (fruc-6-p to UDP-GlcNAc) results in increased *O*-GlcNAc modification of nucleocytoplasmic proteins and inhibition of insulin-stimulated glucose uptake.

cosamine to ATP depletion [15]. While several other laboratories have disproved this simple explanation [16–18], it is important to note that excessive concentrations of glucosamine can in fact deplete ATP levels, giving rise to secondary toxic effects. While glucosamine leading to insulin resistance was a very exciting finding that implicated the HSP in insulin regulation, the biologically more relevant finding that the effects of hyperglycemia-induced insulin resistance could be blocked by inhibition of activity or suppression of expression of GFAT validated the HSP as a sensor [12, 14]. Further, several groups have now shown in cell culture as well as in animal models and type 2 diabetic patients that hyperglycemia and hyperinsulinemia lead to elevated levels of UDP-GlcNAc [6, 19–21]. Interestingly, increased free fatty acids have also been shown to upregulate the HSP, presumably by inhibiting glycolysis and increasing fructose-6-phosphate levels (see fig. 1) [7, 22, 23]. Both hypo- and hypercaloric intake have also been negatively and positively correlated, respectively, with increased flux through the HSP [23, 24]. Several lines of evidence from genetically engineered rodents also support the role of HSP in modulating insulin resistance and serving as an energy sensor. In mice, targeted overexpression of GFAT to skeletal muscle and adipose tissue leads to peripheral insulin resistance [25]. It is also interesting to note that targeted overexpression of GFAT to β-cells of mice leads to hyperinsulinemia and insulin resistance, implicating the HSP in insulin regulation in β-cells as well [26]. Furthermore, ob/ob mice, which lack leptin and are insulin resistant, have elevated UDP-GlcNAc levels [27]. In conjunction with this, increased levels of hexosamines lead to an increase in leptin release from adipocytes, and glucose-stimulated release of leptin can be reduced by inhibition of GFAT [28, 29]. Leptin, an adipocyte-derived signal, alters nutrient flux such that energy expenditure is favored over energy storage [30]. HSP flux regulating leptin secretion is in agreement with the model of the HSP serving as an energy sensor and a negative feedback system to limit uptake of glucose under hyperglycemic and hyperinsulinemic conditions.

If the HSP hypothesis is correct, the next important question is, How does the energy sensor (increased flux through the HSP resulting in elevated UDP-GlcNAc levels) transduce the signal to cause insulin resistance? Under normal conditions, insulin induces glucose uptake and glycogen synthesis in skeletal muscle and adipocytes (reviewed in [4]). While the mechanism of this signal transduction cascade has not been completely elucidated, several key components and pathways have been identified (fig. 1). Glut4-containing vesicles translocate and fuse with the plasma membrane in response to insulin stimulation, and it is primarily the Glut4 glucose transporter that is responsible for insulin-dependent glucose uptake in adipocytes and skeletal muscle. Several groups have shown that insulin-dependent Glut4 translocation is inhibited in response to increased flux through the HSP [31, 32]; however, alternative mechanisms including activity and total protein levels of Glut 4 have been proposed [33, 34]. The protein munc-18 also translocates to the plasma membrane in response to insulin and is believed to play a role in Glut4 vesicles fusing with the plasma membrane [35]. At the other end of the signaling pathway, binding of insulin activates the intrinsic tyrosine kinase activity of the insulin receptor. Insulin receptor substrate (IRS) proteins bind activated receptor, are tyrosine phosphorylated and recruit active phosphoinositide 3-kinase (PI3)-kinase to the plasma membrane. This leads to activation of PDK-1, which phosphorylates and activates AKT. AKT has a variety of substrates, including GSK3β. While it is unknown exactly how AKT activation leads to Glut4 vesicle translocation, several lines of evidence have clearly established the importance of AKT, including *akt2* knockout mice that are insulin resistant [36]. The molecular defects leading to insulin resistance are an area of intense study, and there are conflicting reports as to where the defect(s) and even who the 'players' are in the signal cascade. For reasons of clarity and brevity, we have elected to summarize what we believe is the most commonly held viewpoint at this time. A number of groups have shown a defect in insulin-dependent glut4 translocation, presumably due to a defect in AKT phosphorylation and activation in response to hyperglycemia or glucosamine treatment, and this defect is also observed for munc-18 translocation in both cases [32, 37–39]. Furthermore, proximal insulin signaling events, such as insulin receptor activation and IRS tyrosine phosphorylation, appear normal in hyperglycemia or glucosamine-treated cells. Thus, it would appear that the nutrient sensor (HSP) is acting at or upstream of AKT and downstream of the insulin receptor. Since active AKT isoforms have also been implicated in preventing apoptosis [40, 41], reduced AKT activation under insulin-resistant conditions may contribute to β-cell death in diabetes. Excessive HSP flux has also been shown to induce apoptosis in retinal neurons but not in L6 muscle cells [42]. These data are consistent with the observed retinopathy often seen in type 2 diabetes [2]. Also, Boehmelt and colleagues showed that cells from Emeg32-deficient mice that are defective in the synthesis of UDP-GlcNAc via the HSP have dramatically decreased UDP-GlcNAc levels, express activated AKT and have an increased capacity to withstand apoptotic stimuli [43]. The reason increased HSP flux may be inducing apoptosis in some cell types (retinal neurons and β-cells) but not others (skeletal muscle and adipocytes) is completely unknown and is a question open for investigation. Insulin resistance also impairs glycogen synthesis [44]. Under normal conditions, insulin stimulation activates AKT, leading to phosphorylation and deactivation of GSK3β (fig. 1). This results in

glycogen synthase being active since it is no longer deactivated by phosphorylation. Because excess HSP flux leads to defective AKT activation upon insulin stimulation, glycogen synthesis is inhibited as well since GSK3β is not efficiently phosphorylated and thus can inhibit glycogen synthase.

Excessive flux through the HSP serves as an energy sensor that appears to be mediating its effect, at least in part, by inhibiting insulin signal transduction at or upstream of AKT. Thus, many investigators have established the HSP pathway as a sensor and implicated specific molecular defects leading to insulin resistance. But what is the mechanism by which increased flux inhibits insulin signaling? Since the vast majority of carbohydrate entering the HSP is rapidly converted to UDP-GlcNAc, several investigators have proposed that glycosylation may be the mediator of insulin resistance [4, 31, 45]. *O*-GlcNAc modification of nucleocytoplasmic proteins is one possible candidate for the mediator.

O-GlcNAc's role in nutrient sensing and insulin resistance

O-GlcNAc modification has several features that distinguish it from classical glycosylation and make it an attractive target for the molecular mechanism by which increase flux via the HSP could inhibit insulin signaling [46]. The covalent modification of serine and threonine hydroxyls on nuclear and cytosolic proteins by β-linked *O*-GlcNAc was described by Torres and Hart in 1984 [47]. Several recent reviews have focused on the properties of the modification and the various proteins modified [48–53]. Briefly, *O*-GlcNAc has several distinguishing characteristics that make it more analogous to phosphorylation than to classical complex glycosylation. Namely (i) *O*-GlcNAc is attached to nucleocytoplasmic proteins or to the cytosolic portions of membrane bound proteins, (ii) the modification involves the attachment of a single sugar from a high-energy donor (UDP-GlcNAc) that is not elongated, (iii) the modification is dynamic, (iv) the enzymes responsible for its attachment (*O*-GlcNAc transferase, OGT) and removal (*O*-GlcNAcase) are nucleocytoplasmic, (v) the modification is inducible and (vi) the modification competes with phosphorylation for the same sites on certain proteins. Underscoring the importance of this modification, embryonic stem cells of mice lacking OGT fail to survive [54]. OGT has been found to be responsive to a wide range of UDP-GlcNAc concentrations [55]. Yki-Jarvinen and colleagues found that inducing insulin resistance in rats by glucosamine and hyperinsulinemia led to elevated levels of *O*-GlcNAc on skeletal muscle proteins [56]. It was also established that IRS-1 became *O*-GlcNAc modified in response to increase flux through the HSP [57]. Hyperglycemia has also been shown to elevate *O*-GlcNAc levels on certain proteins [58, 59]. Finally, the diabetes-inducing reagent streptozotocin raises *O*-GlcNAc levels on proteins in β-cells [58].

As an aside, the attractive hypothesis was put forward that streptozotocin was inducing β-cell death by inhibiting *O*-GlcNAcase [60]. However, we and others, using the more potent *O*-GlcNAcase inhibitor PUGNAc [61], have shown that elevated *O*-GlcNAc levels alone do not induce apoptosis in β-cells [62–64]. Streptozotocin is a potent alkylating reagent and is thought to induce cell death via DNA damage [65]. Streptozotocin, however, is also a weak *O*-GlcNAcase inhibitor that is capable of raising *O*-GlcNAc levels on proteins in cells [66]. Thus, the possibility exists that the combination of *O*-GlcNAcase inhibition and DNA damage is necessary to induce β-cell death.

While there was a strong correlation with elevated *O*-GlcNAc levels and insulin resistance, until recently there was no direct proof for *O*-GlcNAc levels modulating insulin action. As a first step towards addressing whether *O*-GlcNAc modification of proteins was directly modulating insulin signaling, we elevated *O*-GlcNAc levels in 3T3-L1 adipocytes via treatment of the cells with the *O*-GlcNAcase inhibitor PUGNAc [67]. PUGNAc treatment significantly elevated the *O*-GlcNAc modification on many nucleocytoplasmic proteins. More important, elevation of *O*-GlcNAc levels impaired insulin-stimulated glucose uptake in the cells. Thus, using a pharmacological approach, we have established a direct causal relationship between elevated *O*-GlcNAc and insulin resistance in 3T3-L1 adipocytes. We also found that proximal insulin signaling was unaffected, while AKT phosphorylation and activation (as measured by GSK3β phosphorylation) was impaired. Thus, elevation of *O*-GlcNAc levels was not only causing insulin resistance but appeared to be inhibiting insulin signaling at the same point in the pathway as increased flux through the HSP [37, 67]. We were also able to show that IRS-1 and β-catenin were modified by *O*-GlcNAc in a PUGNAc-dependent fashion in the 3T3-L1 adipocytes. These findings allow us to put forth a working model in which *O*-GlcNAc acts as a mediator of insulin resistance as well as a metabolic sensor.

Future directions

One interesting question that remains to be answered is, What is the role of hyperinsulinemia in peripheral insulin resistance? Marshall and colleagues established that glucose, glutamine and insulin were necessary for the induction of insulin resistance in adipocytes [13]. Glucose and glutamine are necessary for elevation of flux through the HSP, leading to elevated UDP-GlcNAc levels, and can be substituted for by glucosamine; however, chronic insulin treatment is still necessary for inducing insulin resis-

tance. Interestingly, we found that PUGNAc treatment alone was sufficient to induce insulin resistance, and glucosamine treatment plus insulin elevated *O*-GlcNAc levels higher than either alone [67]. Buse and colleagues showed that glucosamine treatment alone, which did not cause insulin resistance, was able to substantially elevate UDP-GlcNAc levels in adipocytes and that the addition of insulin induced a two-fold increase in the levels of UDP-GlcNAc [18]. Thus, it would appear that PUGNAc is acting at a point after the convergence of hyperglycemia and hyperinsulinemia signaling. The fact that glucosamine treatment still requires hyperinsulinemia would suggest that insulin is acting downstream of GFAT. Based on the above data and since glucosamine-6-phosphate is rapidly converted to UDP-GlcNAc, we would hypothesize that insulin is acting directly on either OGT or *O*-GlcNAcase. This hypothesis is currently under investigation, and preliminary experiments show that OGT becomes tyrosine phosphorylated in response to insulin stimulation [L. Wells, K. Vosseller, G. W. Hart, unpublished results].

Testing the role of *O*-GlcNAc levels regulating insulin signaling by genetic means as opposed to pharmacological methods would greatly strengthen the argument [67a, published during revision of this manuscript]. In this regard, reduced expression and overexpression of OGT and *O*-GlcNAcase in adipocytes is under development. Testing the role of *O*-GlcNAc on insulin action in other tissue types as well as whole animals is also an important area of future research. Obviously, identification of proteins that become modified by *O*-GlcNAc in response to hyperglycemia and hyperinsulinemia is important, as well as the identification of proteins that are hyper-*O*-GlcNAc modified in rodent models of type 2 diabetes. Once candidate proteins are identified, the elucidation of the molecular mechanism by which O-GlcNAc modification is inhibiting insulin signaling will be of great importance in understanding this posttranslational modification and to future therapies for diabetes.

The role of *O*-GlcNAc in the liver and the β-cell, as well as in microvascular and macrovascular disease, remains to be determined. In this regard, exploring whether *O*-GlcNAc plays a role in apoptosis is especially important. The HSP appears to be involved in β-cell death and retinopathy [42, 58]. Also, we have determined that *O*-GlcNAcase is cleaved in vitro by caspase-3, an executioner caspase in programmed cell death [52]. Activation of AKT is also inhibited by elevated flux through the HSP and elevation of *O*-GlcNAc levels [37, 67]. Active AKT has been demonstrated to be antiapoptotic [40, 41], and cells with reduced UDP-GlcNAc levels have elevated levels of active AKT and are refractory to apoptotic stimuli [43]. Thus, there is compelling evidence for the investigation of a possible role for *O*-GlcNAc in apoptosis. Also, determining whether there is a connection between *O*-GlcNAc modification and leptin expression, secretion and action remains to be elucidated.

O-GlcNAc has begun to emerge as an important posttranslational modification in cellular regulation [46, 50]. Many tools to facilitate study of this modification have only recently become available, including the cloning of OGT in 1997 [68, 69] and *O*-GlcNAcase in 2001 [70], the development of a general *O*-GlcNAc specific antibody in 2001 [71] and *O*-GlcNAc site-specific antibodies in 2002 [72], and mass spectrometry based techniques for site mapping [73–75]. All of these developments should help to accelerate our understanding of the *O*-GlcNAc modification.

In conclusion, we and others are using a working model that *O*-GlcNAc modification of proteins serves as a nutrient sensor to modulate extracellular signal transduction cascades. In this model, the cell is not blindly responding to extracellular signals but instead is taking into account its own energy stores and responding appropriately. Further, *O*-GlcNAc, serving as a metabolic sensor, may be modulating the expression, activity, localization and/or secretion of proteins. There have been several other molecules and mechanisms proposed as nutrient sensors [4, 7–11], and it is likely that cells use multiple sensors and mechanisms to regulate such an important process as energy homeostasis. Deciphering how these sensors are mediating their effects is an important goal if we are to understand the pathophysiology of complex pleiotropic metabolic diseases, such as type 2 diabetes. An understanding of the relationship between *O*-GlcNAc and *O*-phosphate in metabolic sensing, signaling cascades and transcriptional regulation should lead to rational new targets for drug development.

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