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Hexosamine Flux, the O-GlcNAc Modification, and the Development of Insulin Resistance in Adipocytes

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Abstract

Excess flux through the hexosamine biosynthesis pathway in adipocytes is a fundamental cause of “glucose toxicity” and the development of insulin resistance that leads to type II diabetes. Adipose tissue-specific elevation in hexosamine flux in animal models recapitulates whole-body insulin-resistant phenotypes, and increased hexosamine flux in adipocyte cell culture models impairs insulin-stimulated glucose uptake. Many studies have been devoted to unveiling the molecular mechanisms in adipocytes in response to excess hexosamine flux-mediated insulin resistance. As a major downstream event consuming and incorporating the final product of the hexosamine biosynthesis pathway, dynamic and inducible O-GlcNAc modification is emerging as a modulator of insulin sensitivity in adipocytes. Given that O-GlcNAc is implicated in both insulin-mediated signal transduction and transcriptional events essential for adipocytokine secretion, direct functional studies to pinpoint the roles of O-GlcNAc in the development of insulin resistance via excess flux through hexosamine biosynthesis pathway are needed.

Keywords

Hexosamine biosynthesis pathway; O-GlcNAc modification; insulin resistance; adipocyte; adipocytokine

1. Introduction

Clinically, insulin resistance is characterized by a chronic elevation in circulating glucose and insulin levels as the peripheral tissues normally executing glucose clearance, namely adipose tissue and striated muscle, become desensitized despite the elevated hormonal signal. These hyperglycemic and hyperinsulinemic conditions also progressively impair insulin secretion, and, in the later stages of diabetes mellitus, lead to pancreatic beta-cell death. The development of insulin resistance along with the subsequent chronic “glucose toxicity” is widely accepted as a prerequisite condition for the disease progression from metabolic syndrome to type II diabetes and a variety of associated micro- and macrovascular diseases (Matveyenko and Butler, 2008).

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In adipocytes, glucose uptake is initiated through the insulin-responsive glucose transporter-4 (GLUT4). Upon entering cells, glucose is converted by glucokinase into glucose-6-phosphate (Glc-6-P), a portion of which can be shuttled into either glycogen synthesis or the pentose phosphate pathway, depending on the metabolic needs of the cell. Glc-6-P is also a substrate for Glc-6-P isomerase to form fructose-6-phosphate (Fru-6-P), the majority of which enters glycolysis and the tricarboxylic acid (TCA) cycle for the support of basic anabolic processes (Bouche et al., 2004). However, 2 to 5 % of the Fru-6-P enters the hexosamine biosynthesis pathway (HBP, Figure 1) to generate uridine diphospho-N-acetylglucosamine (UDP-GlcNAc). While the detailed metabolic reactions and feedback regulation of HBP were unveiled nearly half a century ago (Kornfeld et al., 1964), the modulatory role of HBP in the development of insulin resistance was not established until almost 30 years later (Marshall et al., 1991a). The end product of the HBP, UDP-GlcNAc, is a nucleotide sugar essential for glycan biosynthesis on a myriad of macromolecules. Of all the glycosylation types, global level of O-linked β -N-acetyl-glucosamine (O-GlcNAc), a ubiquitous intracellular single-sugar glycosylation (Figure 2), has been demonstrated to correlate with HBP flux (Boehmelt et al., 2000, Vosseller et al., 2002, Hazel et al., 2004, Park et al., 2005, Yang et al., 2008). In conjunction to the emerging roles of O-GlcNAc modification in multiple aspects of cellular homeostasis (Hart et al., 2007), the molecular mechanism of the HBP in insulin resistance has just begun to be elucidated (Buse, 2006, Copeland et al., 2008).

With its robust rate of hormonally-stimulated nutrient uptake, adipose tissue is classically viewed as a key energy deposit site. Following the discovery of the adipocyte-derived *OB* gene product, leptin, as a central player in energy homeostasis (Campfield et al., 1995, Halaas et al., 1995, Maffei et al., 1995, Pelleymounter et al., 1995), adipose tissue is now also categorized as a major endocrine organ (Halberg et al., 2008). The cross-talk between adipocyte-secreted factors and insulin resistance has been revealed by several lines of evidence in tissue-specific GLUT4 transgenic mouse models. Briefly, mice with either adipocyte- or muscle-specific GLUT4 gene knockout develop whole-body insulin resistance (Abel et al., 2001, Kim et al., 2001). Furthermore, adipose tissue-specific overexpression of GLUT4 in transgenic mice bearing muscle-specific inactivated GLUT4 gene effectively rescue the insulin-resistant phenotype in these animals (Carvalho et al., 2005). These findings suggest that adipose tissue-secreted factors are key regulators in maintaining glucose homeostasis in whole animals. In an attempt to have a broader understanding of the adipocyte secretome, functional proteomics approaches using media collected from adipocyte cultures have been utilized to identify hundreds of secreted proteins, collectively termed adipocytokines (or adipokines), many of which have been established to act as autocrine, paracrine and endocrine factors (Kratchmarova et al., 2002, Wang et al., 2004, Chen et al., 2005, Alvarez-Llamas et al., 2007, Lim et al., 2008).

In this review, we will first discuss the impact of adipocyte-specific excess HBP flux in animal models followed by the signaling events that are impinged on upon HBP-induced insulin resistance in adipocyte cell culture models. Also, we will include O-GlcNAc-induced insulin resistance in both animal and cell culture models in our discussion in an attempt to explore the potential role of O-GlcNAc as an executor of the HBP with regards to insulin resistance in adipocytes. Finally, we will touch on the influence of the HBP and O-GlcNAc modification on the endocrine function of adipocytes in mediating insulin resistance.

2. Hexosamine Biosynthesis Pathway

The first and rate-limiting enzyme in the HBP is glutamine:fructose-6-phosphate aminotransferase (GFAT) that utilizes glutamine and Fru-6-P for the formation of glucosamine-6-P (GlcN-6-P) and glutamate (Figure 1). Following subsequent enzymatic reactions, the pathway eventually leads to the formation of UDP-GlcNAc, a nucleotide sugar

that can be either converted into other types of nucleotide sugars or directly incorporated into a variety of glycosyl-containing macromolecules. In addition to serving as a precursor for a diverse set of glycoconjugates, UDP-GlcNAc also inhibits GFAT activity through a negative feedback mechanism to reduce the flux through the HBP (Kornfeld et al., 1964).

Evidence linking the correlation between the HBP and insulin resistance in adipocytes was illustrated by Marshall and colleagues two decades ago with a series of elegant experiments (Marshall and Monzon, 1989, Traxinger and Marshall, 1989, Marshall et al., 1991a, Marshall et al., 1991b, Traxinger and Marshall, 1991). By using cultured primary rat adipocytes, the authors observed that: (1) A chronic exposure to both insulin and glucose was required for the adipocytes to become insulin resistant. (2) The impairment in insulin-stimulated glucose uptake during hyperglycemic and hyperinsulinemic conditions was exclusively dependent on the presence of L-glutamine. (3) While simultaneous treatment with high glucose, insulin and glutamine led to the accumulation of UDP-GlcNAc, inhibition of GFAT activity, presumably via a negative feedback mechanism, was also observed. (4) Pharmacological inhibition of GFAT using amidotransferase inhibitors such as O-diazoacetyl-L-serine (azaserine) or 6-diazo-5-oxonorleucine (DON) prevented the glucose-induced insulin resistance. (5) A greater reduction in insulin-mediated glucose uptake was observed when the cells were treated with glucosamine (which enters the HBP downstream of GFAT) compared to high glucose condition, although the metabolic machinery that converts both glucose and glucosamine into the HBP's intermediates is more effective in utilizing glucose. (6) The glucosamine-induced insulin resistance did not require L-glutamine, nor was the effect inhibited by azaserine. While glucose and glutamine metabolism are key inducers of HBP flux, free fatty acid (FFA) and uridine are also potent modulators of the HBP (Wang et al., 1998).

After the findings from Marshall and colleagues, many groups have proceeded to manipulate HBP flux using pharmacological or genetic approaches in order to study the biological mechanisms of insulin resistance in both cell culture and animal models. It is noteworthy that the final outcome of excess HBP flux may be manifested in a tissue-specific manner. For the scope of this review, we focus our discussion on studies involving adipose tissue and cultured adipocytes.

3. Manipulation of HBP Flux in Animal Models

To verify that the HBP is the glucose sensing pathway for the development of insulin resistance in whole animals, a series of experiments, using animals with either glucosamine infusion or GFAT overexpression, have been conducted (Table 1).

3.1 Glucosamine Infusion

In vivo glucosamine infusion to rats, with or without pre-exposure to hyperglycemic condition, revealed that euglycemic rats start to develop insulin resistance, yet chronically hyperglycemic rats are insensitive to the treatment (Rossetti et al., 1995). In addition, Virkamäki and colleagues showed that rats subjected to *in vivo* glucosamine infusion have a lower whole body glucose disposal rate than that in saline-infused control animals (Virkamäki et al., 1997). Epididymal fat pads isolated from the insulin resistant animals mirrored the reduction in insulin-stimulated glucose uptake during the *in vitro* measurement (Virkamäki et al., 1997). These studies provided the preliminary observations of the development of insulin resistance in whole animals resulting from excess hexosamine flux.

3.2 GLUT4-GFAT Transgenic Mice

More direct evidence that excess HBP flux modulates insulin sensitivity in adipocytes, contributed mainly by McClain's laboratory, is derived from transgenic mice overexpressing

GFAT. Their first transgenic mouse model with ectopic expression of GFAT under control by the *GLUT4* promoter (GLUT4-GFAT mice, with GFAT overexpression in adipose and striated muscle tissues) led to animals with a classical insulin-resistant phenotype with hyperinsulinemia and reduction in whole-body glucose disposal rate (Hebert et al., 1996). Elevation in serum leptin level was also observed in these transgenic animals (McClain et al., 2000). Interestingly, muscle explants from GLUT4-GFAT mice showed normal insulin-stimulated glucose uptake (Hazel et al., 2004), strongly suggesting that adipocytes play a regulatory role in the HBP-mediated whole-body insulin resistance. However, it has not been ruled out that the degree of insulin resistance exhibited by explanted muscle strips from GLUT4-GFAT mice eluded the detection threshold.

3.3 aP2-GFAT Transgenic Mice

A second strain of transgenic mice utilizing an *aP2* (adipocyte lipid binding protein) gene promoter driven GFAT construct was created, which allowed an adipocyte site-specific overexpression of GFAT. In this transgenic mouse model, an adipose tissue-restricted elevation in UDP-HexNAc and O-GlcNAc levels was detected, which is associated with the increase of GFAT in the target tissue (Hazel et al., 2004). These transgenic animals also developed whole-body insulin resistance characterized by a reduction in both glucose disposal rate and skeletal muscle glucose uptake. An increase in serum leptin and a decrease in serum adiponectin levels were detected in agreement with their transcript levels in adipose tissue. Furthermore, *ex vivo* skeletal muscle cultures from aP2-GFAT mice displayed normal insulin response (Hazel et al., 2004). Intriguingly, regardless the similarity in their body weight, the both adipocytes and epididymal fat pads derived from aP2-GFAT animals are larger in size compared to that from their wild type littermates (McClain et al., 2005). Higher GLUT4 mRNA and protein levels were also detected in the fat pads derived from the transgenic animals. These characteristics reflect a slight increase in the basal and maximal glucose uptake in conjunction to the overall reduction in the insulin sensitivity from the aP2-GFAT adipocytes. However, whether hepatic gluconeogenesis participates in the reduction of whole body insulin sensitivity in aP2-GFAT mice remains unclear. An increase in total fatty acid synthesis and oxidation rates accompanied by activated AMP-activated protein kinase (AMPK) activity was also observed in the fat pad of aP2-GFAT animals (McClain et al., 2005, Luo et al., 2007). While AMPK is well known for its sensitivity toward intracellular nucleotide levels (*i.e.* AMP/ATP ratio), its major function in adipose tissue is to regulate lipid metabolism by reducing the availability of FFA (Daval et al., 2006). Future investigation into the cause of elevated AMPK activity and reduced FFA levels in aP2-GFAT animals should provide insight into the control (McClain et al., 2005)

4. Impact of HBP Flux on Insulin Action

From a signaling perspective, HBP-mediated glucose desensitization can occur at multiple stages, including insulin-mediated signal transduction, predominantly via the insulin receptor substrate (IRS)/phosphatidylinositol-3 kinase (PI3K) cascade, leading to Akt phosphorylation and/or signaling control of cargo/cytoskeletal protein-mediated GLUT4 translocation (Figure 3). To understand the signaling processes that are affected by HBP-induced insulin resistance, researchers have induced insulin resistance in differentiated 3T3-L1 adipocytes (an immortal murine cell line) by chronic administration of either high glucose (25 mM) in the presence of physiological concentration of insulin (0.6 nM), or glucosamine (2 mM) in low glucose (5 mM) containing medium (Table 1).

4.1 The Metabolic Branch of Insulin Signaling in Cell Culture Models

While cumulative data indicate that both glucose- and glucosamine-induced insulin resistance consistently impede insulin-mediated glucose uptake via a deficiency in GLUT4 translocation

(Ross et al., 2000, Nelson et al., 2002a), a slight discrepancy was observed in the action of insulin-mediated PI3K action. Under high glucose-induced insulin resistance, there is a reduction in insulin-stimulated phospho-Akt levels, especially in the subset of plasma-membrane-associated phospho-Akt (Nelson et al., 2002a). This was not observed when cells were pre-exposed to glucosamine (Nelson et al., 2002a). However, no change in IRS-associated PI3K activity was detected in either model (Nelson et al., 2000). The distinct outcomes in Akt phosphorylation in 3T3-L1 adipocytes are unexpected, because both high glucose and glucosamine treatments are equally effective in causing a defect in the insulin-stimulated Akt phosphorylation in rat retinal neurons where insulin acts as a pro-survival factor (Nakamura et al., 2001).

Having ruled out the impact of HBP flux on the cross-talk between IRS and PI3K, Buse's laboratory sought to pinpoint the molecular effector involved in insulin signaling that is responsible for reducing Akt phosphorylation. In a recent publication, they reported a reduction in phosphatidylinositol 3,4,5-triphosphate (PIP₃, a PI3K product) levels correlating with an increase in PTEN (phosphatase and tensin homolog deleted on chromosome 10) protein levels when the cells were exposed to chronic high glucose and insulin (Robinson and Buse, 2008). Since rapamycin treatment inhibits the alteration of PIP₃ and PTEN levels under insulin-resistant condition, it is believed that mammalian target of rapamycin complex 1 (mTORC1) is involved in negatively regulating the IRS/PI3K/Akt signaling cascade downstream of the insulin receptor. Also, an increase in IRS-1 phosphorylation on Ser 636/639 residues (sites known to be substrates of mTORC1) was detected (Robinson and Buse, 2008), further suggesting the potential role of mTORC1 in modulating the development of insulin resistance in adipocytes. Notably, mTORC1 is a primary amino-acid sensor and a direct downstream effector of AMPK (Figure 3). As AMPK is activated in the fat pads of aP2-GFAT mice, it will be informative to see whether AMPK is involved in modulating HBP-mediated downregulation of insulin signaling via mTORC1 in 3T3-L1 adipocytes.

4.2 Insulin-stimulated GLUT4 Translocation

The regulation of insulin-stimulated GLUT4 translocation is a field of active research. We now know that GLUT4 is stored inside intracellular vesicles and readily distributed to the plasma membrane via fusion between a pair of t-(target membrane) and GLUT4-containing v-(vesicle membrane) SNARE (soluble-N-ethylmaleimide-sensitive factor attachment protein receptor) complexes upon insulin stimulation (Cheatham et al., 1996, Volchuk et al., 1996, Brozinick et al., 2007). This step is mediated by AS160 (Akt substrate of 160 kDa) in a PI3K-dependent manner (Zeigerer et al., 2004, Brozinick et al., 2007). Cumulative evidence also convincingly points to the phosphatidylinositol 4,5-bisphosphate (PIP₂)-assisted remodeling of filamentous actin at the inner leaflet of the plasma membrane (cortical F-actin) as another crucial step for insulin-stimulated GLUT4 translocation (Kanzaki and Pessin, 2001, Kanzaki et al., 2004, Brozinick et al., 2007).

In both glucose- and glucosamine-induced insulin-resistant cell culture models, a reduction in the acute insulin-stimulated GLUT4 translocation was detected accompanied by a significant alteration in membrane redistribution of Munc18-c, a negative regulator of t- and v-SNAREs (Nelson et al., 2002b, Chen et al., 2003). A reduction in insulin-stimulated interaction between two Munc-18c targets, syntaxin 4 and VAMP 2 (key components in t-SNARE and insulin-responsive v-SNARE complexes, respectively) was detected upon glucosamine treatment (Chen et al., 2003). However, it is not clear whether the change in HBP-associated Munc18-c membrane distribution is responsible for the inhibition of syntaxin 4 and VAMP4. Collectively, these data suggest a direct involvement of excess HBP flux in desensitizing the fusion between GLUT4-containing intracellular vesicles and the plasma membrane (Figure 3).

Recently, Elmendorf's group has added another possible explanation for the defect in GLUT4 translocation under HBP-induced insulin resistance (Bhonagiri et al., 2009). By exposing 3T3-L1 adipocytes to excess HBP flux, the authors detected a reduction in plasma membrane PIP₂ content with a concomitant loss in cortical F-actin. Interestingly, experimental replenishment of PIP₂ protects cells from the development of insulin resistance, whereas administration of DON reverses the PIP₂ and F-actin levels. While these preliminary observations give an alternative model for HBP-induced insulin resistance-associated glucose uptake in adipocytes, the mechanistic details bridging GLUT4 translocation and PIP₂ and F-actin levels require further exploration.

4.3 Glycogen Synthesis

In addition to GLUT4 translocation, insulin-mediated PI3K/Akt activation also stimulates glycogen synthesis to balance the intracellular glucose metabolism in response to excess glucose influx. Insulin-dependent glycogen synthesis is triggered by activation of glycogen synthase (GS) through (1) Akt-mediated inhibition of glycogen synthase kinase-3 β (GSK3 β , a negative modulator of GS), and (2) dephosphorylation by protein phosphatase 1 (PP1, more details, see accompanying chapter). Presumably, upon insulin stimulation, elevated glycogen synthesis decreases Glc-6-P and subsequently Fruc-6-P levels, and hence restricts HBP flux due to a decrease in GFAT's substrate.

Given that excess HBP flux blunts the insulin-stimulated GLUT4 action, Parker and colleagues also examined the status of insulin-stimulated glycogen synthesis in 3T3-L1 adipocytes. Exposing cells to either high glucose or glucosamine led to a lower insulin-stimulated GS activity. While involvement of GSK3 β was excluded, it was demonstrated that GS becomes more resistant to PP1 activity under excess HBP flux (Parker et al., 2003). However, further investigation is needed to complete the interaction network between these events.

4.4 Lipid Metabolism

Unlike insulin-mediated signal transduction, relatively little is known of the role of HBP flux in lipid metabolism in 3T3-L1 adipocytes. In agreement with the observation in aP2-GFAT mice-derived fat pads, McClain's group showed that glucosamine-treated 3T3-L1 adipocytes also have higher levels of fatty acid oxidation and AMPK activity (Luo et al., 2007). No significant change in the nucleotide ratio was detected under glucosamine treatment, strongly suggesting that the increase in AMPK activity is a direct action of excess HBP flux. Controversially, Ceddia's group showed that activation of AMPK by using an AMPK-specific activator, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), inhibits insulin-stimulated glucose uptake as well as total fatty acid synthesis and fatty acid oxidation rates in primary rat adipocytes (Gaidhu et al., 2006). In addition, the exact action of AICAR in insulin-induced glucose uptake remains a topic of debate (Salt et al., 2000, Yamaguchi et al., 2005, Gaidhu et al., 2006). Further experiments to examine glucosamine-induced AMPK activity in response to acute insulin stimulation are needed to resolve this discrepancy.

5. O-GlcNAc Modification

The end product of the HBP, UDP-GlcNAc, can be either converted into other types of nucleotide sugars or directly incorporated into a variety of glycosyl-containing biomolecules, including N- and O-linked glycoproteins, glycolipids, GPI-anchored proteins, proteoglycans and glycosaminoglycans. Among them, nucleocytosolic O-GlcNAc modification (Figure 2) is a particularly appealing candidate for the HBP sensor for the following reasons: (1) the enzyme responsible for O-GlcNAc modification consumes UDP-GlcNAc with a relatively high K_M that is close to the physiological level of the nucleotide sugar available (Kreppel and Hart, 1999). (2) Global O-GlcNAc levels have been shown to correlate with HBP flux in many cell

culture and animal models (Vosseller et al., 2002, Hazel et al., 2004, Park et al., 2005, Yang et al., 2008). (3) Using EMeg32 knockout mouse embryonic fibroblasts (MEFs) lacking Glc-6-P acetyltransferase (the second enzyme in the HBP), it was observed that perturbation in HBP flux is directly associated with a significant reduction in global O-GlcNAc levels, yet no significant alteration in other glycosylation products was detected (Boehmelt et al., 2000).

O-GlcNAc is an abundant monosaccharide modification found on the serine and threonine residues of nucleocytoplasmic proteins (Figure 2, (Holt and Hart, 1986, Kears and Hart, 1991b)). Since it was first reported in 1984 by Hart's laboratory (Torres and Hart, 1984), O-GlcNAc modification has been found on more than 500 proteins and shown to regulate a variety of cellular processes (Hart et al., 2007). With a distinct spatial localization compared to complex glycosylations, and its inducible and dynamic nature, O-GlcNAc modification is conceptually more related to phosphorylation than other glycosylations (Holt and Hart, 1986, Kears and Hart, 1991a, Wells et al., 2001, Kneass and Marchase, 2004, Khidekel et al., 2007, Wang et al., 2007). Due to the technological improvements for post-translational modification studies, we now know that the dynamic interplay of O-GlcNAc and O-phosphate modifications is more sophisticated than the original 'Ying-yang hypothesis' (Hart et al., 1995, Wells et al., 2002b, Khidekel et al., 2007, Wang et al., 2007, Wang et al., 2008). One such example is the reciprocal action of O-GlcNAc and O-phosphate modifications on CCAAT enhancer binding protein (C/EBP) β (Li et al., 2009). It is clearly demonstrated that the interplay of phosphorylation and O-GlcNAc modification on (C/EBP) β is crucial for determining *in vitro* differentiation of 3T3-L1 adipocytes (Li et al., 2009).

The cycling of O-GlcNAc is achieved by a pair of cycling enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) that control the addition and removal of a β -GlcNAc moiety, respectively (Figure 2, (Haltiwanger et al., 1990, Haltiwanger et al., 1992, Dong and Hart, 1994, Kreppel et al., 1997, Gao et al., 2001, Wells et al., 2002a)). In mammals, there is only a single gene encoding OGT and likewise a single gene encoding OGA (Comtesse et al., 2001, Nolte and Muller, 2002). In contrast to phosphorylation, where numerous kinases and phosphatases are required to achieve its target diversity, accumulative evidence suggest that the occurrence of O-GlcNAc modification is largely regulated at the levels of O-GlcNAc cycling enzymes via differential transcription, post-translational modification and transient complex conformation (Butkinaree et al., 2009). Importantly, two independent epidemiological studies have revealed that nucleotide polymorphisms in *mgea5* (the gene encoding OGA) are associated with the onset of type II diabetes in different populations (Farook et al., 2002, Lehman et al., 2005). These studies further imply that O-GlcNAc modification of intracellular proteins participates in the development of insulin resistance and disease progression in type II diabetes.

5.1 O-GlcNAc Modification and Insulin Resistance in Animals

Complementary to the transgenic mouse models of GFAT overexpression, a transgenic mouse model with OGT driven under *GLUT4* gene promoter was reported from a collaboration between McClain's and Hanover's groups (Table 1). Not surprisingly, the animals, with elevated OGT expression in adipose and striated muscle tissues, developed a classical insulin-resistant phenotype characterized by a reduction in whole-body glucose disposal rate, as well as elevated plasma insulin and leptin levels (McClain et al., 2002). However, no detailed follow-up characterization of the GLUT4-OGT transgenic mice is available. It would be informative to examine whether the insulin-resistant status of different tissues and muscle explants derived from GLUT4-OGT transgenic mice can recapitulate the observation in transgenic mice overexpressing GFAT. Studies using transgenic mice bearing adipocyte-specific OGT overexpression would also give insight into the role of the O-GlcNAc modification in adipocyte differentiation and the development of insulin resistance.

While no published report on the OGA transgenic animals is currently available, Goto-Kakizaki (GK) rats provide a glimpse of information regarding the role of OGA in the development of insulin resistance (Table 1). GK rats are an inbred strain of Wistar rats with spontaneous development of type II diabetes (Goto et al., 1976, Kimura et al., 1982). Unlike many of the diabetic animal models, GK rats are not obese, yet they exhibit phenotypic resemblance to type II diabetes in humans. The adipocytes isolated from GK rats have a reduction in insulin sensitivity in conjunction with a defect in IRS-1 phosphorylation and GLUT4 translocation (Begum and Ragolia, 1998). Genetic studies have identified the major diabetes-associated locus in GK rats to be *Niddm1* (Galli et al., 1999). Interestingly, the rat OGA gene is assigned to the *Niddm1* locus in proximity to the gene encoding the insulin-degrading enzyme (Van Tine et al., 2003). Also, a short variant of OGA was isolated from GK rats by Kudlow's laboratory, which acts in a dominant-negative manner *in vitro* to elevate O-GlcNAc levels (Toleman et al., 2004, Bowe et al., 2006). While increased global O-GlcNAc levels have been found in the cornea and pancreas isolated from GK rats (Akimoto et al., 2003, Akimoto et al., 2007), it will be intriguing to see the O-GlcNAc status in the adipose tissue of GK rats in order to establish the connection between O-GlcNAc levels and the insulin-resistant phenotype in these animals.

5.2. O-GlcNAc Modification and Insulin Resistance in Culture Adipocytes

Direct evidence showing that O-GlcNAc modification is the interconnecting factor between the HBP and insulin resistance comes from the study Hart's group did using a potent OGA inhibitor (PUGNAc, O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenylcarbamate, (Haltiwanger et al., 1998)) to increase global O-GlcNAc level in 3T3-L1 adipocytes (Vosseller et al., 2002). It was shown that PUGNAc treatment suppresses insulin-mediated glucose uptake in the absence of chronic insulin exposure. A concomitant defect in Akt phosphorylation and activation was also observed while no significant inhibition at signaling events proximal to the insulin receptor was detected (Vosseller et al., 2002). The same conclusions were drawn from another study by ectopically overexpressing OGT in 3T3-L1 adipocytes in parallel to the PUGNAc treatment (Yang et al., 2008). Moreover, an increase in phosphorylation on both Ser307 and Ser636/639 of IRS-1 was observed (Yang et al., 2008) (Table 1). These data partially agree with the phenomena obtained from high glucose-induced insulin resistance. However, since none of the experiments from either of the studies were dedicated to examine the status of GLUT4 translocation, it is premature to pinpoint the exact role of O-GlcNAc on this event.

The effect of O-GlcNAc-mediated insulin resistance was examined also in primary rat adipocytes (Park et al., 2005). In addition to the similar defects in insulin-stimulated glucose uptake and Akt phosphorylation as mentioned earlier, a reduction in IRS-1 tyrosine phosphorylation and GLUT4 translocation were also detected in agreement with the findings in the adipocytes extracted from GK rats (Begum and Ragolia, 1998, Park et al., 2005). While a discrepancy in insulin signaling events in response to external stimuli, such as an exposure to branched-chain amino acids, has previously been observed between differentiated immortal adipocytes and freshly isolated primary adipocytes (Hinault et al., 2006), the distinctive impact of O-GlcNAc in IRS-1 phosphorylation between 3T3-L1 adipocytes and primary rat adipocytes may be explained by the specific physiological conditions of these cell culture models.

While increased global O-GlcNAc levels are implicated in the development of insulin resistance, OGT is also regulated by insulin in 3T3-L1 adipocytes. It was found that OGT is tyrosine-phosphorylated by the insulin receptor upon acute insulin stimulation, which subsequently enhances OGT activity. Moreover, a localization shift from nucleus to cytosol is also observed under these conditions (Whelan et al., 2008). Since these experiments were monitored with acute insulin treatment under insulin-responsive condition, it is not clear

whether insulin receptor-mediated OGT tyrosine phosphorylation is involved in the course of insulin resistance. Furthermore, a PIP₃ binding motif was found on OGT using an *in vitro* binding assay (Yang et al., 2008). Although it remains unknown in the case of 3T3-L1 adipocytes, studies from COS-7 and 3T3-A14 cells showed that OGT is translocated to the plasma membrane in a PI3K-dependent manner in response to acute insulin stimulation (Yang et al., 2008). In contrast to animals ectopically overexpressing wild type OGT, introducing an OGT mutant lacking the PIP₃ binding ability in mouse liver does not impair hepatic insulin action (Yang et al., 2008). Future studies are needed to determine whether the insulin sensitivity of transgenic mice with adipose-specific overexpression of PIP₃-binding-deficient OGT are altered.

5.3 O-GlcNAc Modification on Specific Proteins

While dissecting the signaling steps affected by the HBP or O-GlcNAc-induced insulin resistance, many studies have also investigated whether the proteins participating in such an event are O-GlcNAc modified. Indeed, almost all of the proteins discussed in the earlier sections are known to be *in vivo* substrates of OGT. However, due to a lack of robust site-mapping methods and site-specific O-GlcNAc antibodies, the exact functional targets of O-GlcNAc remain elusive. Below, we summarize a series of observations of insulin resistance-associated signaling events that may be modulated by O-GlcNAc modification (Figure 3).

1. O-GlcNAc modification has been found on proteins involved in insulin-mediated signal transduction, including insulin receptor, IRS-1/2, p85 and p110 of PI3K, PDK1 and Akt. To date, only four sites on IRS-1 (Ser914, Ser1009, Ser1036 and Ser1041) and another on Ser473 of Akt-1 have been confirmed to be O-GlcNAc modified in non-adipocyte cell cultures (Ball et al., 2006, Kang et al., 2008, Klein et al., 2009). It is not known whether these sites impact function and if they are preferential substrates for OGT in adipocytes.
2. Munc18-c was found to be O-GlcNAc modified under glucosamine-induced insulin resistance (Chen et al., 2003). Whether the glycosylated form of Munc18c serves as a direct factor causing a reduction in membrane association with syntaxin 4 and VAMP2 requires further exploration.
3. It was found that chronic HBP flux effectively induces O-GlcNAc modification on AMPK subunit in both immortal and primary murine adipocytes. Importantly, the O-GlcNAc modified AMPK has a higher activity compared to the non-glycosylated form of AMPK (Luo et al., 2007). However, since the site on AMPK has not been mapped, one cannot rule out the possibility of O-GlcNAc modification interfering with the protein-protein interaction between AMPK and its binding partners, and subsequently leading to an increase in its activity.
4. Overall O-GlcNAc levels on glycogen synthase fluctuate in a HBP flux-dependent manner. It is believed that glycosylated glycogen synthase is more resistant to PP1-mediated activation (Parker et al., 2003). A previous study has shown that the catalytic subunits of PP1 form a 'ying-yang' complex with OGT in rat brain extracts (Wells et al., 2004). While the formation of a PP1-OGT complex has not been established in adipocytes, it is possible that glycogen synthase is not the only target of O-GlcNAc in modulating glycogen synthesis under insulin-resistant conditions.

5.4 Paradoxes in O-GlcNAc-Induced Insulin Resistance in Culture Adipocytes

Given that pharmacologically and genetically elevated O-GlcNAc levels in cultured adipocytes and mouse models are associated with insulin-resistant phenotypes, one might expect that reducing O-GlcNAc levels in adipocytes should reverse the HBP-induced insulin resistance. However, a study reported by Robinson and colleagues contradicted this conventional thought

(Robinson et al., 2007). They showed that when 3T3-L1 adipocytes are exposed to high glucose and insulin containing medium, genetically overexpressing OGA or knocking down OGT does not protect cells from developing insulin resistance as the reduction in insulin-stimulated glucose uptake and Akt phosphorylation persist. (Robinson and Buse, 2008) (Table 1). However, in an insulin-resistant *db/db* mouse model (a diabetic mouse model with mutated leptin receptor), the overexpression of OGA via adenovirus significantly improved whole-body glucose tolerance and insulin sensitivity (Dentin et al., 2008), suggesting that lowering of O-GlcNAc levels *in vivo* is beneficial. Knowing that many proteins involved in modulating insulin sensitivity are potential substrates for OGT, and that O-GlcNAc modification can either positively or negatively regulate protein functions, further experiments establishing the functional role of O-GlcNAc on each protein are needed to explain the phenomena observed by Robinson and colleagues.

Another disputable input complicating the model of O-GlcNAc-induced insulin resistance comes from a report from Vocadlo's laboratory using 1,2-dideoxy-2'-propyl- α -D-glucopyranoso-[2,1,D]- Δ 2'-thiazoline (NButGT) to elevate global O-GlcNAc levels (Macauley et al., 2008) (Table 1). NButGT is an OGA inhibitor that was designed based on the structural information attained from a bacterial homolog of human OGA (Macauley et al., 2005, Macauley et al., 2008). Kinetic studies showed that NButGT has a better selectivity toward OGA over lysosomal β -hexosaminidases. When 3T3-L1 adipocytes were treated with NButGT, the cells remained insulin-sensitive and no defect in glucose uptake or Akt phosphorylation was detected (Macauley et al., 2008). These observations are puzzling, as O-GlcNAc-induced insulin resistance has been previously demonstrated by both pharmacological and genetic approaches (Table 1). Future investigation for the potential biological difference between PUGNAc and NButGT treated cells is essential to explain these results.

6. Adipocytokines

Several adipocytokines have been implicated in obesity-mediated insulin-resistant models. However, relatively little information is available on the HBP-induced adipocytokines. Rossetti's group first showed that manipulating HBP flux in rats, via glucose, glucosamine or FFA infusions, leads to an increased plasma leptin level, which correlates with an upregulation of leptin mRNA levels in adipose tissue (Wang et al., 1998). This is further supported by a series of findings: (1) Both GLUT4-GFAT and aP2-GFAT transgenic mice are hyperleptinemic (McClain et al., 2000, Hazel et al., 2004). (2) Exposing isolated human subcutaneous adipocytes to glucosamine released more leptin into the medium, whereas treating the cells with DON to block GFAT successfully reduced leptin reproduction at both the mRNA and protein levels (Considine et al., 2000). (3) Increased HBP flux, by either high glucose or glucosamine, induces leptin production in a dose- and time-dependent manner in primary human adipocytes (Considine et al., 2000, Zhang et al., 2002). (4) Hyperleptinemia is also detected in GLUT4-OGT transgenic mice (McClain et al., 2002).

In addition to leptin, several other diabetes-related adipocytokines have been examined in aP2-GFAT transgenic mice. Whereas insignificant changes in the transcript levels of TNF α and resistin were measured, a slight reduction in the mRNA level of adiponectin correlated with a marked decreased in serum adiponectin was observed (Hazel et al., 2004). Further study on HBP-induced leptin secretion by Considine's group showed that Sp1, a housekeeping transcription factor, participates in such events (Zhang et al., 2002). Notably, Sp1 is a heavily O-GlcNAc modified protein (at least 9 sites, of which only Ser491 has been confirmed (Jackson and Tjian, 1988, Roos et al., 1997)). O-GlcNAc modification on Sp1 has been demonstrated to influence its stability, subcellular localization, transcription activity, accessibility to phosphorylation and ability to engage protein-protein interactions (Han and Kudlow, 1997, Roos et al., 1997, Yang et al., 2001, Majumdar et al., 2003, Solomon et al., 2008). Future

experiments to mutate O-GlcNAc sites on Sp1 to address functional impact of O-GlcNAc modified-Sp1-associated adipocytokine production under insulin-resistant condition are needed.

In order to obtain a more comprehensive picture of adipocytokine secretion that is modulated by HBP- or O-GlcNAc-mediated insulin resistance, our laboratory performed a quantitative functional proteomic study to identify regulated adipocytokines under different conditions (Lim et al., 2008). After inducing insulin resistance with high glucose/insulin or PUGNAc treatments in immortal or primary rodent adipocytes, adipocyte-spent media was harvested and subjected to non-isotope-based quantitative mass-spectrometry analysis. Altogether, more than 200 adipocytokines were identified, with 8 and 20 regulated adipocytokines (including quiescin Q6, angiotensin and slit homologue 3) in 3T3 and primary rat adipocytes, respectively. Follow-up experiments to examine whether these proteins are also regulated at the transcriptional level in a manner similar to leptin are in progress.

7. Conclusions

Increased flux through the HBP under excess glucose availability is a direct cause of insulin resistance in adipocytes, though a variety of mechanisms likely regulate this process (Brownlee, 2001). O-GlcNAc modification, in response to hexosamine flux, appears to be one of the main mechanisms directly downstream of the HBP to modulate metabolic changes by dynamically modifying intracellular proteins, thus affecting protein functions and cellular processes. Indeed, many proteins established in mediating insulin resistance at the molecular level are known to be O-GlcNAc modified. With recent improvements in technologies for detecting and site-mapping O-GlcNAc modified proteins, experiments can now be designed to provide insights into the functional role of O-GlcNAc modification at specific sites on individual proteins in the development of insulin resistance in adipocytes.

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Fig. 1.

The hexosamine biosynthesis pathway (HBP). The HBP is posed to act as a “glucose sensor” since the synthesis of UDP-GlcNAc relies on the incorporation of products from glucose, amino acid (glutamine), fatty acid (acetyl-CoA), and nucleotide (uridine) metabolism. While the majority of the glucose entering the cell is committed to glycogen synthesis, the pentose phosphate pathway and glycolysis, 2 to 5 % of it enters the HBP for the formation of UDP-GlcNAc, a precursor for a variety of glycosylations, including O-GlcNAc modification. As is typical to metabolic pathways, the first and rate-limiting enzyme, GFAT, is negatively regulated by the end product, UDP-GlcNAc. Although the HBP requires several enzymatic steps, the two most well-studied enzymes (depicted in bold) in terms of insulin resistance are GFAT, the rate-limiting enzyme, and Emeg32.

O-linked β -N-Acetylglucosamine (O-GlcNAc)

Fig. 2. O-GlcNAc modification. This dynamic and inducible post-translational modification on nucleocytoplasmic proteins is catalyzed by OGT (in green) and OGA (in red) for the addition to, and removal from the serine and threonine residues, respectively. While no single consensus sequence for O-GlcNAc addition has been identified, the sequence shown here is derived from IRS-1 that contains a known O-GlcNAc residue on Ser1046 (shown in blue).

Fig. 3.

Cross-talk between the hexosamine biosynthesis pathway, O-GlcNAc modification of proteins, signaling events downstream of insulin action, and glucose-induced adipocytokine secretion. Many of the proteins involved in this signaling network are known to be O-GlcNAc modified, but the functional roles of this modification in each case remain to be established.

Table 1

A summary of animal and cultured adipocyte models used in studying insulin resistance, HBP flux and/or global O-GlcNAc levels.

Model System	UDP-GlcNAc Level	O-GlcNAc Level	Insulin Resistance?	References
Animal models				
Glucosamine Infusion	+	+	Yes	Rossetti <i>et al.</i> , 1995; Virkamäki <i>et al.</i> , 1997
GFAT Transgenic Mice	+	+	Yes	Hebert <i>et al.</i> , 1996; Hazel <i>et al.</i> , 2004
OGT Transgenic Mice	Ø	+	Yes	McClain <i>et al.</i> , 2002
GK Rats	Ø	+*	Yes	Goto <i>et al.</i> , 1976; Akimoto <i>et al.</i> , 2003; Akimoto <i>et al.</i> , 2007
Cultured Adipocytes				
High Glucose/Insulin	+	+	Yes	Nelson <i>et al.</i> , 2000; Ross <i>et al.</i> , 2000
Glucosamine	+	+	Yes	Nelson <i>et al.</i> , 2000; Ross <i>et al.</i> , 2000
PUGNAc	⊗	+	Yes	Haltiwanger <i>et al.</i> , 1997; Vosseller <i>et al.</i> , 2002; Park <i>et al.</i> , 2005; Yang <i>et al.</i> , 2008
NButGT	Ø	+	No	Macauley <i>et al.</i> , 2008
OGT Overexpression	Ø	+	Yes	Yang <i>et al.</i> , 2008
OGA Overexpression	Ø	–	Yes	Robinson <i>et al.</i> , 2008
OGT Knockdown	Ø	–	Yes	Robinson <i>et al.</i> , 2008

+: Increased; -: Decreased; ⊗: No significant change; Ø: No information available

* Observed in non-adipose tissues.