Hypoxic regulation of glycosylation via the N-acetylglucosamine cycle

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Glucose is an energy substrate, as well as the primary source of nucleotide sugars, which are utilized as donor substrates in protein glycosylation. Appropriate glycosylation is necessary to maintain the stability of protein, and is also important in the localization and trafficking of proteins. The dysregulation of glycosylation results in the development of a variety of disorders, such as cancer, diabetes mellitus and emphysema. Glycosylation is kinetically regulated by dynamically changing the portfolio of glycosyltransferases, nucleotide sugars, and nucleotide sugar transporters, which together form a part of what is currently referred to as the “Glycan cycle”. An excess or a deficiency in the expression of glycosyltransferases, nucleotide sugars, and nucleotide sugar transporters, which together form a part of what is currently referred to as the “Glycan cycle”. An excess or a deficiency in the expression of glycosyltransferases has been shown to alter the glycosylation pattern, which subsequently leads to the onset, progression and exacerbation of a number of diseases. Furthermore, alterations in intracellular nucleotide sugar levels can also modulate glycosylation patterns. It is observed that pathological hypoxic microenvironments frequently occur in solid cancers and inflammatory foci. Hypoxic conditions dramatically change gene expression profiles, by activating hypoxia-inducible factor-1, which mediates adaptive cellular responses. Hypoxia-induced glycosyltransferases and nucleotide sugar transporters have been shown to modulate glycosylation patterns that are part of the mechanism associated with cancer metastasis. Hypoxia-inducible factor-1 also induces the expression of glucose transporters and various types of glycolytic enzymes, leading to shifts in glucose metabolic patterns. This fact strongly suggests that hypoxic conditions are an important factor in modulating various nucleotide sugar biosynthetic pathways. This review discusses some of the current thinking of how hypoxia alters glucose metabolic fluxes that can modulate cellular glycosylation patterns and consequently modify cellular functions, particularly from the standpoint of the N-acetylglucosamine cycle, a part of the “Glycan cycle”.

Key Words: glycane cycle, hypoxia, sugar metabolism, nucleotide sugar, N-acetylglucosaminyltransferase

Biosynthetic Pathways of Uridine Diphosphate (UDP)-N-acetylglucosamine (GlcNAc)

Glucose is used as an energy source to produce adenosine triphosphate (ATP) through metabolic processes, such as glycolysis and cellular respiration. The free energy, generated by the hydrolytic cleavage of the high-energy phosphate linkages of ATP, is then used to drive various cellular activities. However, glucose is essential not only as an energy source but also as a metabolic precursor of nucleotide sugars, which are utilized as donor substrates in protein glycosylation. Glycosylation, one of the most abundant posttranslational modification reactions, is necessary, in terms of protein stability, as well as the localization and trafficking of the proteins. Because approximately 50% of all known proteins in eukaryotes are glycosylated,(1) the fine tuning of glycosylation is important for maintaining biological systems in a normal context.

Glucose, after its incorporation into the cells via glucose transporters (GLUTs), is initially converted to glucose-6-phosphate by hexokinases, and a portion of the glucose-6-phosphate is then metabolized via the pentose phosphate pathway, and this leads to production of nucleotides, including uridine triphosphate (UTP), guanosine triphosphate (GTP) and cytidine triphosphate (CTP), which are structural components of diverse nucleotide sugars, such as UDP-GlcNAc, UDP-N-acetylglucosamine and UDP-glucuronic acid, guanosine diphosphate (GDP)-mannose and GDP-fucose, and cytidine monophosphate (CMP)-N-acetylneuraminic acid (CMP-NeuAc), respectively, or is used in glycosyn synthesis to store surplus glucose in the form of glycogen in cells (Fig. 1).

In addition, under steady state conditions, approximately 1–3% of the glucose-6-phosphate enters the hexosamine biosynthetic pathway after conversion to fructose-6-phosphate,(2) while the remaining glucose-6-phosphate is metabolized by glycolysis after conversion to fructose-6-phosphate (Fig. 1). In the hexosamine biosynthetic pathway, fructose-6-phosphate is first converted into glucosamine-6-phosphate by glutamine-fructose-6-phosphate amidotransferases (GFAT), the rate-limiting enzyme of the hexosamine biosynthetic pathway; and the glucosamine-6-phosphate is then acetylated by glucosamine-6-phosphate N-acetyltransferases (GNA) to form GlcNAc-6-phosphate, which is isomerized to GlcNAc-1-phosphate and uridinylated by the action of UDP-GlcNAc pyrophosphorylases (UAP) (Fig. 1). The synthesized UDP-GlcNAc is transported to the Golgi apparatus via the UDP-GlcNAc transporters,(3,4) and is then utilized as a donor substrate for the N- and O-linked glycosylation of extracellular and membrane proteins; alternatively, it is utilized in the cytosol for the O-GlcNAc modification (O-GlcNAcylation) of intra-cellular proteins.

Biological Activities of N-Acetylglucosaminyltransferases (GnTs)

In mammalian cells, proteins are modified with a variety of glycans, which can be classified into two major groups, N- and O-glycans.(5) N-glycans are linked to certain asparagine residues of proteins that contain the Asn-X-Ser/Thr motif, whereas O-glycans are attached to a subset of serine and threonine residues.(6) The addition of GlcNAc from UDP-GlcNAc to N- and O-glycans and O-GlcNAcylation is catalyzed by enzymatic activity of GnTs and O-linked N-acetylglucosaminyltransferase (OGT), localized

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in the Golgi apparatus and cytosol, respectively. The formation of branched structures and subsequent elongation and processing of \(\text{N}\)-glycans by various types of \(\text{GnT}\)s alter the biological functions of the glycoproteins\(^{[5]}\) and are associated with a variety of disorders, such as cancer and diabetes mellitus.\(^{[6,7]}\)

As shown in Fig. 2, with respect to the GlcNAc cycle, the Golgi apparatus contains at least two enzymes, \(\text{GnT-V}\) and \(\text{GnT-III}\), which play key roles in the processing of \(\text{N}\)-glycans. \(\text{GnT-V}\) catalyzes the transfer of a GlcNAc unit from UDP-GlcNAc to a \(\alpha 1,6\)-mannose in \(\text{N}\)-glycans to form a \(\beta 1,6\)-branched \(\text{N}\)-glycans, thus elongating the polylactosamine structure (Fig. 2), which controls cell surface receptor residency and is associated with some of the invasive characteristics associated with cancer cells.\(^{[1,8,9]}\) Indeed, the overexpression of \(\text{GnT-V}\) has been reported to promote integrin \(\alpha 5\beta 1\)-mediated cell migration.\(^{[10]}\) It has been also reported that \(\text{GnT-V}\) activity and the levels of \(\beta 1,6\)-branched \(\text{N}\)-glycans were increased in highly metastatic cancer cell lines.\(^{[11,12]}\)

On the contrary, \(\text{GnT-III}\) catalyzes the addition of a GlcNAc unit in a \(\beta 1,4\)-linkage to the mannose residue at the base of the trimannosyl core of an \(\text{N}\)-glycan, to produce a so-called “bisection GlcNAc” (Fig. 2).\(^{[13,14]}\) The introduction of a bisecting GlcNAc prevents the formation of a branched structure in \(\text{N}\)-glycans, since other \(\text{GnTs}\) are not able to utilize an \(\text{N}\)-glycan with a bisecting GlcNAc as a substrate.\(^{[15]}\) The overexpression of \(\text{GnT-III}\) suppresses \(\alpha\beta\)-integrin-mediated cell spreading, migration and the phosphorylation of focal adhesion kinase (FAK).\(^{[16]}\) These results suggest that \(\text{GnT-III}\) has antagonizing effects against \(\text{GnT-V}\).

Additionally, Ohtsubo et al.\(^{[17]}\) demonstrated that a loss of \(\text{GnT-IVa}\), which catalyzes the formation of a \(\beta 4\) GlcNAc linkage on the \(\alpha 3\)-linked mannose of \(\text{N}\)-glycans, attenuates the half-life of GLUT2 on the pancreatic \(\beta\)-cell surface, and the resulting impairment of glucose-stimulated insulin secretion leads to metabolic dysfunctions that are characteristic of type 2 diabetes.
Regulation of Glycosylation via GlcNAc Cycle

Furthermore, alterations in intracellular nucleotide sugar levels can also modulate glycosylation patterns (Fig. 3), because GnTs have different Km values for UDP-GlcNAc. Elevated intracellular UDP-GlcNAc enhances the activities of GnT-V and GnT-IV, which have higher Km values; however, low levels diminished the activities. In fact, it was demonstrated that the formation of β1,6-branched N-glycans in Jurkat T-cells is stimulated by supplementation of metabolic precursors of the hexosamine biosynthetic pathway, including glucose, GlcNAc, glutamine, acetate, ammonia and uridine, and regulates autoimmune reactions of T-cells.(19) Furthermore, hyperglycemia involves an enhancement in the hexosamine biosynthetic pathway activity and O-GlcNAcylation levels, resulting in an enhanced susceptibility to apoptosis of pancreatic β-cells.(20) Thus, glycosylation is affected not only by the levels of expression of a single GnT enzyme but rather by a magnitude factors, including the levels of monooligosaccharides, nucleotide sugars, and nucleotide sugar transporters, the localization of GnTs and nucleotide sugar levels in the Golgi, transcription factors, the pro-form and mature form of glycoproteins, and the structure of cell surface glycoproteins (Fig. 3). (8)

Hypoxia and Glycosylation

Pathological hypoxic microenvironments, which are observed in many cases of solid cancers and inflammatory foci, dramatically change gene expression profiles, and confer various malignant characteristics to cancer cells. Hypoxia-inducible factor-1 (HIF-1) plays key roles in adaptive responses of cells under hypoxic conditions through its transcription activity. The HIF-1 protein is a heterodimer, composed of a constitutively expressed subunit of HIF-1β and an oxygen-dependently regulated subunit of HIF-1α.(21) The expression of HIF-1α is predominantly regulated at posttranslational level. Under oxygen-sufficient conditions, prolyl residues 402 and 564 in the N-terminal domain of HIF-1α protein are hydroxylated by the actions of prolyl hydroxylases (PHD) in an oxygen-dependent manner (Fig. 4). This enables the binding of E3 ubiquitin ligase Von Hippel-Lindau to HIF-1α, and leads to proteasomal degradation of the HIF-1α protein (Fig. 4). On the contrary, under hypoxic conditions, because enzymatic activities of PHDs are reduced, the HIF-1α protein is stabilized and is translocated to the nucleus, where induces gene expression (Fig. 4). Furthermore, in this process, it has been demonstrated that reactive oxygen species (ROS) generated from mitochondria under conditions of hypoxia is required for stabilization of the HIF-1α protein.(22)

It is well known that hypoxic microenvironments dramatically shift the pattern of intracellular glucose metabolism from aerobic cellular respiration to anaerobic glycolysis. Under hypoxic conditions, the expression of GLUT1 and various types of the rate-limiting enzymes of glycolysis, such as phosphofructokinases (PFK), aldolases (ALD), phosphoglycerate kinases (PGK), enolases (ENO) and lactate dehydrogenases (LDH), are markedly trans-activated by HIF-1 (Fig. 4). The resulting increased glucose uptake and activation of glycolysis are highly important compensatory responses against a reduction in intracellular ATP levels due to the hypoxic inhibition of cellular respiration.

In a previous study, Koike et al. (23) reported that hypoxia increases the levels of cell surface sialyl Lewis x and sialyl Lewis a determinants, which are specific ligands for E-selectin, and...
findings suggest that activation of the hexosamine biosynthetic pathway in response to hypoxia is not reflected in the intracellular abundance of UDP-GlcNAc.

In order to clarify hypoxia-induced alterations in the synthetic pathway of UDP-GlcNAc, we analyzed changes in the metabolic flow of glucose in response to hypoxia using unpublished LC-MS methods after labeling the starting glucose at C-6 with $^{13}$C. The ratio of metabolic flow was calculated from the intensity of the isotopic pattern of $^{13}$C-labeled nucleotide sugar in the mass spectrum. It was found that a signal intensity 6 mass units higher than that for ordinary UDP-GlcNAc in hypoxic cells was similar to that in normoxic cells. This result indicates that metabolic flow of the hexosamine biosynthetic pathway is maintained under hypoxic conditions, because the mass shift of 6 reflects UDP-GlcNAc, in which the glucosamine (hexose), was replaced by a $^{13}$C-glucosamine, thus leading to an overall reduction in UDP-GlcNAc.

Hypoxia and UDP-GlcNAc

It is unclear how hypoxic microenvironments affect the biosynthetic machinery of the nucleotide sugars. At present, there is only one report dealing with a regulatory mechanism of the hexosamine biosynthetic pathway in response to hypoxia. Manzari et al. demonstrated that hypoxia increases both mRNA and the expressions of the corresponding protein, GFAT, the rate-limiting enzyme of the hexosamine biosynthetic pathway. This study concluded that the transcriptional activity of the $GFAT$ gene is directly regulated by HIF-1. We have also confirmed, using RAW264.7 cells (a murine macrophage cell line), that hypoxic exposure results in an increase in the mRNA levels of GFAT1 but the mRNA levels of other enzymes of the hexosamine biosynthetic pathway downstream of GFAT1, such as GNA1, UAP1 and GlcNAc kinase (NAGK) remain unchanged (unpublished data). These results imply that the biosynthesis of UDP-GlcNAc should be promoted in cells under hypoxic conditions. Thus, we recently determined changes in intracellular UDP-GlcNAc levels in response to hypoxia, using ion-pair reversed-phase high performance liquid chromatography (HPLC$^{(25)}$) and liquid chromatography-mass spectrometry (LC-MS). Surprisingly, the intracellular level of UDP-GlcNAc was reduced by approximately 50% in cells exposed to hypoxia (unpublished data). These findings suggest that activation of the hexosamine biosynthetic pathway in response to hypoxia is not reflected in the intracellular abundance of UDP-GlcNAc.

![Diagram of HIF-1 regulation](image-url)
by hypoxic exposure may inhibit the addition of UDP to GlcNAc.

On the contrary, it is suspected that a diverging pathway of nucleotide sugar synthesis is activated in response to hypoxia. A portion of the synthesized UDP-GlcNAc has been shown to be epimerized by UDP-GlcNAc 2-epimerase (GNE), and is then supplied to the synthetic pathway of CMP-NeuAc,(29) which leads to cell surface sialylation (Fig. 3). We found that, when RAW264.7 cells were exposed to hypoxic conditions or treated with the hypoxia-mimetic agent cobalt chloride (CoCl₂), the intracellular level of CMP-NeuAc, unlike UDP-GlcNAc, was significantly increased (unpublished data). From these results, analyses of the downstream pathway(s) of UDP-GlcNAc may reveal the mechanism behind the hypoxia-induced reduction of UDP-GlcNAc.

Hypoxia and GnTs

It is very important to clarify the mechanism by which GnTs is controlled, because the levels of such enzymes directly affect the patterns of glycosylation and is partly responsible for the GlcNAc cycle. However, to date, the transcriptional regulatory mechanism of GnT genes has not been fully elucidated. Meanwhile, our group previously demonstrated that the expression of the MgaT5 gene, encoding GnT-V, is regulated by a member of the E-twenty six (Ets) family of transcription factors, Ets-1.(29,30) It was also reported that the expression of the ETS1 gene is regulated by HIF-1.(31) These findings suggest the indirect induction of GnT-V expression by HIF-1 in cells that are exposed to hypoxia. However, the level of MgaT5 mRNA was relatively decreased by hypoxic exposure in the case of RAW264.7 cells (unpublished data). This may be because Ets-1 protein is constitutively expressed in monocytes and macrophages, and, as a result, the expression of Ets-1 is not inducible in response to hypoxia in these cells.(52) These findings indicate that the expression of GnT-V is not induced in hypoxic macrophages. Furthermore, we recently reported that the enzymatic activity of GnT-V was gradually reduced along with hypoxic exposure time in RAW264.7 cells, while no GnT-III activity was detected in these cells (unpublished data). On the other hand, it has been shown that the GnT-V protein, residing in the Golgi lumen, is cleaved by γ-secretase (a protease complex composed of presenilin, nicastrin, anterior pharynx-defective (APH)-1, and presenilin enhancer (PEN)-2), a protease complex composed of presenilin, nicastrin, anterior pharynx-defective (APH)-1, and presenilin enhancer (PEN)-2), a protease complex composed of presenilin, nicastrin, anterior pharynx-defective (APH)-1, and presenilin enhancer (PEN)-2), (a protease complex composed of presenilin, nicastrin, anterior pharynx-defective (APH)-1, and presenilin enhancer (PEN)-2), (a protease complex composed of presenilin, nicastrin, anterior pharynx-defective (APH)-1, and presenilin enhancer (PEN)-2), and the secreted type of GnT-V has angiogenesis effects.

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Wang et al.(35) demonstrated that HIF-1 regulates the expression of APH-1A, a component of γ-secretase, and the γ-secretase-mediated secretion of amyloid β (Fig. 4). Thus, it is necessary to examine, not only transcriptional regulation but also the post-translational modification of GnTs.

Conclusion

Hypoxia reduces the activity of a portion of the GlcNAc cycle, including intracellular UDP-GlcNAc levels and GnT-V activity. Analysis of the metabolic flow of glucose using unpublished LC-MS methods using [13C6]-labeled glucose (Nakajima et al., manuscript in preparation) indicates that the hypoxia-induced reduction in intracellular UDP-GlcNAc levels is caused by the suppression of the addition of an acetyl group to glucosamine and intracellular nucleotide levels.

To clarify physiological significances of these hypoxia-induced reductions in GlcNAc cycle activity, it is essential to analyze whether or not the levels of β1,6-branched N-glycans of the target cell surface proteins are reduced. Furthermore, a better understanding of the mechanisms associated with GlcNAc cycle inactivation in response to hypoxia is important, because recovery experiments of GlcNAc cycle activities are essential in terms of identifying whether the resultant carbohydrate structural alterations contribute the hypoxia-induced changes in cellular function. Thus, it needs glucose metabolomic analysis regarding the UDP-GlcNAc biosynthetic pathways, including the pentose phosphate pathway and subsequent nucleotide synthesis, hexosamine biosynthetic pathway, and glycolysis. Finally, other components of the glycan cycle, including nucleotide sugar transporters, the localization of GnTs and nucleotide sugar levels in the Golgi should also be considered, and an understanding of the glycan cycles will promise further advancements in the future for the field of glycobiology.(36)

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Abbreviations

ALD aldolases
APH anterior pharynx-defective
ATP adenosine triphosphate
CMP cytidine monophosphate
CMP-NeuAc cytidine monophosphate-N-acetylmuramic acid
CTP cytidine triphosphate
ENO enolases
Ets E-twenty six
FAK focal adhesion kinase
FUT7 fucosyltransferase VII
GDP guanosine diphosphate
GFAT glutamine-fructose-6-phosphate amidotransferases
GlcNAc N-acetylgalactosamine
GLUTs glucose transporters
GNA glucosamine-6-phosphate N-acetylttransferases
GNE UDP-GlcNAc 2-epimerase
GnT N-Acetylgalactosaminytranferases
GTP guanosine triphosphate
HIF-1 hypoxia-inducible factor-1
HPLC reversed-phase high performance liquid chromatography
LC-MS liquid chromatography-mass spectrometry
LDH lactate dehydrogenases
NAGK GlcNAc kinase
OGT GlcNAcAcetylation O-GlcNAc modification
OGT O-linked N-acetylgalactosaminyltransferase
PDK pyruvate dehydrogenase kinases
PEN-2 presenilin enhancer-2
PFK phosphofructokinases
PGK phosphoglycerate kinases
PHD prolyl hydroxylases
ROS reactive oxygen species
TCA tricarboxylic acid
UAP UDP-GlcNAc pyrophosphorylases
UDP Uridine Diphosphate
UDP-GlcNAc UDP-N-acetylgalactosamine
UTP uridine triphosphate

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