O-GlcNAc modification, insulin signaling and diabetic complications

T. Issad a,*, b, E. Masson a, b, P. Pagesy a, b

a CNRS (UMR 8104), université Paris Descartes, institut Cochin, 22, rue Méchain, 75014 Paris, France
b Inserm, U1016, Paris, France

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Abstract

O-GlcNAc glycosylation (O-GlcNAcylation) corresponds to the addition of N-acetylglucosamine on serine and threonine residues of cytosolic and nuclear proteins. O-GlcNAcylated proteins are dynamic post-translational modifications, analogous to phosphorylation, that regulate the stability, the activity or the subcellular localization of target proteins. This reversible modification depend on the availability of glucose and therefore constitutes a powerful mechanism by which cellular activities are regulated according to the nutritional environment of the cell. O-GlcNAc has been implicated in important human pathologies including Alzheimer disease and type-2 diabetes. Only two enzymes, OGT and O-GlcNAcase, control the O-GlcNAc level on proteins. Therefore, O-GlcNAcylations cannot organize in signaling cascades as observed for phosphorylations. O-GlcNAcylations should rather be considered as a “rheostat” that controls the intensity of the signals traveling through different pathways according to the nutritional status of the cell. Thus, OGT attenuates insulin signal by O-GlcNAcylating proteins involved in proximal and distal steps in the PI-3 kinase signaling pathway. This negative feedback may be exacerbated when cells are chronically exposed to elevated glucose concentrations and could thereby contribute to alterations in insulin signaling observed in diabetic patients. O-GlcNAcylations also appears to contribute to the deleterious effects of hyperglycaemia on excessive glucose production by the liver and deterioration of β-cell pancreatic function, resulting in worsening of hyperglycaemia (glucotoxicity). Moreover, O-GlcNAcylations directly participate in several diabetic complications. O-GlcNAcylations of eNOS in endothelial cells have been involved in micro- and macrovascular complications. In addition, O-GlcNAcylations activate the expression of profibrotic and antifibrinolytic factors, contributing to vascular and renal dysfunctions.

Keywords: O-GlcNAc; Post-translational modifications; Insulin signaling; Insulin resistance; Glucotoxicity; Diabetic complications; Review

Résumé

La glycosylation par O-GlcNAc (O-GlcNAcylaton) correspond à l’addition de N-acétylglucosamine sur les résidus serines ou thréonines des protéines cytosoliques ou nucléaires. C’est une modification post-traductionnelle dynamique, analogue à la phosphorylation, qui régule l’activité, la localisation subcellulaire ou la stabilité des protéines. Cette modification réversible dépend de la disponibilité en glucose et correspond donc à un puissant mécanisme de contrôle des activités cellulaires en fonction de l’environnement nutritionnel. La O-GlcNAcylaton a été impliquée dans des pathologies humaines majeures, comme la maladie d’Alzheimer et le diabète. Les deux enzymes seulement, l’OGT et l’O-GlcNAcase, contrôlent le niveau de O-GlcNAcylaton des protéines. De ce fait, les O-GlcNAcylations ne peuvent s’organiser en cascades de signalisation, comme celles observées pour les phosphorylations. La O-GlcNAcylaton doit plutôt être considérée comme un “rheostat” qui va moduler l’intensité des signaux passant au sein des différentes voies de signalisation en fonction de l’état nutritionnel de la cellule. Ainsi, la O-GlcNAcylaton de protéines impliquées dans les étapes précoces et tardives de la voie de la PI-3 kinase atténué le signal de l’insuline. Ce rétrocontrôle négatif pourrait être excéré lorsque les cellules sont exposées de façon chronique à des concentrations anormalement élevées de glucose et pourrait contribuer aux défauts de signalisation de l’insuline observés chez les patients diabétiques. Les O-GlcNAcylations pourraient également jouer un rôle dans la production excessive de glucose par le foie et la détérioration de la fonction β-pancréatique observées dans les situations d’hyperglycémie chronique, conduisant ainsi à une aggravation de l’hyperglycémie (glucotoxicité). En outre, les O-GlcNAcylations ont été directement impliquées dans certaines

* Corresponding author. Tel.: +33 1 40 51 64 09; fax: +33 1 40 51 64 30.
E-mail address: tarik.issad@inserm.fr (T. Issad).
complications diabétiques. Ainsi, la O-GlcNAcylation de eNOS dans les cellules endothéliales joue un rôle majeur dans les complications micro- 
et macrovasculaires. De plus, les O-GlcNAcylations activent l’expression de facteurs pro-fibrotiques et anti-fibrinolytiques, contribuant ainsi aux 
alité des patients et aux complications rénales.

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Mots clés : O-GlcNAcylation ; Modifications post-traductionnelles ; Signalisation de l’insuline ; Résistance à l’insuline ; Glucotoxicité ; Complications diabétiques ; Revue générale

The sequencing of the human genome at the beginning of this century unexpectedly revealed that it comprises less than 40,000 genes (20–25,000 genes coding for proteins, according to more recent estimations [1]), whereas living cells are complex systems that display much larger number of functional activities. The high diversity of cellular activities, as compared to the low number of genes coding for proteins, may in part be explained by the existence of numerous post-translational modifications (phosphorylation, glycosylation, sumoylation, acetylation, ubiquitination, nitrosylation, palmitoylation, farnesylation, methylation, ADP-riboseylation, hydroxylation, oxidation, etc.) that can considerably expand the functional potentialities of proteins. More specifically, these modifications control the localisation of proteins in different cell compartments, their activity, as well as their interaction with different partners within multimolecular complexes, allowing transmission of information through sophisticated intracellular signaling networks.

Phosphorylations and glycosylations constitute the most abundant and most widely studied post-translational modifications. However, whereas phosphorylations are generally considered as rapid and reversible modifications allowing cell responses to different stimuli, glycosylations have long been considered as stable modifications, that involve the addition of complex carbohydrate chains that generally remain on the protein throughout its lifespan. These complex glycosylations on serine/threonine (O-glycosylations) or asparagine (N-glycosylations) are restricted to specific cell compartments (endoplasmic reticulum, Golgi, extracellular surface of the plasma membrane) and occur essentially on membrane or secreted proteins. However, in 1984, while studying the distribution of terminal N-Acetylglucosamine residues at the surface of T lymphocytes, G.W. Hart discovered a new type of glycosylation: the O-linked-N-acetylglucosaminyltransferase [2]. This glycosylation consists in the addition of a monosaccharide, N-Acetyl-glucosamine (GlcNAc) on the hydroxyl group of serine or threonine residues (Fig. 1). This O-Glycosylation is now generally referred to as “O-GlcNAcylation”, although “O-GlcNAcation” is also being used and would seem more appropriate, as it avoids the misleading “Acylation” term, which may erroneously suggest that an acylation reaction is involved.

Whereas this modification was initially believed to occur on membrane proteins of the lymphocytes, it rapidly appeared that, in contrast to “classical” glycosylations, this modification was essentially found on cytosolic and nuclear proteins [3,4]. It was then soon observed that O-GlcNAc modification was a dynamic process, which turnover on a protein was much higher than the turnover of the protein itself [5]. It is now well established that O-GlcNAcylation is a reversible modification, analogous to phosphorylation, that controls the stability, the subcellular localisation and the activity of proteins, and also regulates protein–protein interactions within molecular complexes [6]. However, in contrast to phosphorylations/dephosphorylations, which are regulated by hundreds of kinases and phosphatases, only two enzymes, O-linked N-acetylglucosaminyltransferase (OGT) and N-Acetyl-β-D glucosaminidase (O-GlcNAcase), cloned in 1997 [7,8] and 2001 [9] respectively, control the O-GlcNAcylation level of proteins (Fig. 1).

Although O-GlcNAcylation has been known for a quarter of a century, the scientific community only recently started to realize its importance in most biological processes. This modification, which is found in most living organisms (fungus, bacteria, plants, animals) could constitute an ancestral mechanism by which protein activities are regulated according to the nutritional environment of the cell. Indeed, glucose is one of the most largely used energy substrate in living cells. A fraction (2–3%) of the glucose entering the cell is converted into UDP–N-Acetyl Glucosamine (UDP-GlcNAc), through the hexosamine biosynthetic pathway (HBP). The level of UDP-GlcNAc in the cell thus reflects the flux of glucose through this pathway (Fig. 1). UDP-GlcNAc is the substrate of OGT, which can therefore be considered as a metabolic sensor, capable of modifying proteins according to glucose availability (Fig. 1). In addition, UDP-GlcNAc is at the crossroad of several metabolic pathways, (glucose, fatty acids (acyetyl moiety), amino-acid (glutamine) and nucleotide (UDP) metabolism), and could therefore constitute a more general integrator of the nutritional state of the cell (Fig. 2).

1. O-GlcNAcylation regulate the activity of cytosolic and nuclear proteins

Numerous studies have demonstrated that O-GlcNAcylations regulate cell function by controlling protein stability, enzymatic activity, subcellular localisation as well as interaction with cellular partners [6,10,11]. An additional refinement in the regulation of proteins emerges from the fact that O-GlcNAcylation may affect the phosphorylation status of a protein, by regulating the phosphorylation of adjacent residues or by competing for the same serine or threonine residue (the so-called Yin-Yang mechanism, in which modification of a serine or threonine residue by either phosphorylation or O-GlcNAcylation differently affects the protein’s function). Interestingly, OGT has been found in protein complexes associated with the protein phosphatase PP1 (isofoms β and γ), suggesting a coordinated action of the two enzymes for the regulation of the O-GlcNAcylation/phosphorylation status of proteins [12].
**Fig. 1.** The hexosamine biosynthesis pathway. The hexosamine biosynthesis pathway (HBP) leads to the production of UDP-GlcNAc (Uridine 5-diphospho N-acetylglucosamine), the substrate used by OGT for O-GlcNAcylation of cytosolic or nuclear proteins. UDP-GlcNAc is also the GlcNAc donor for other types of glycosylations not shown on this figure ("classical" glycosylations in the endoplasmic reticulum and the Golgi apparatus, glycolipids, etc.). The rate-limiting step of the HBP is catalysed by the GFAT (glutamine fructose-6-phosphate amidotransferase) which uses glutamine to convert fructose-6-phosphate into glucosamine-6-phosphate. UDP-GlcNAc is an allosteric inhibitor of GFAT, allowing a negative feedback of the HBP. Experimentally, the level of O-GlcNAc glycosylation of proteins can be increased by incubating cells with high-glucose concentrations, with glucosamine (which bypasses allosteric inhibition of GFAT), or with PUGNac (O-[2-acetamido-2-deoxy-β-d-glucopyranosylidene] amino-N-phenylcarbamate), which inhibits deglycosylation of protein by O-GlcNAcase. Azaserine and DON (6-Diazo-5-oxo-l-norleucine) are commonly used chemical inhibitors of GFAT.

1. **O-GlcNAcylation: a “rheostat” that regulates cell signaling**

As mentioned previously, the analogy between O-GlcNAcylation and phosphorylation is striking. However, it must be kept in mind that in contrast to phosphorylations, protein O-GlcNAcylations, which can only be performed by a single enzyme (OGT), cannot organize in signaling cascades, in which one O-GlcNAcylated protein will transmit a signal by O-GlcNAcating another protein (as observed in phosphorylation cascades involving numerous protein kinases). It rather seems that O-GlcNAcylation should be considered as a transversal regulatory mechanism, a “rheostat” that would control the intensity of the signal traveling through different pathways according to the nutritional status of the cell. This rheostat may actually regulate different signaling pathways in opposite directions in the same cell, decreasing the signal through a given pathway while increasing it through another. For instance, in endothelial cells, inhibition of the PI-3 kinase pathway by O-GlcNAcylation was concomitant to an increase in the activation of Erk1/2 and P38 MAP kinase pathways [13]. Although the mechanisms remain elusive, increased O-GlcNAc levels were also found associated with increased Erk1/2 activity in other cell types [14–16].

1.2. **OGT: the question of its specificity**

Since only one single enzyme is responsible for O-GlcNAcylation of a large number of proteins (more than 1000 proteins identified to date as OGT targets), it is difficult to figure out how is achieved the control of specificity of OGT towards different substrates in different biological situations. However, several mechanisms have been suggested.

A certain level of specificity could be linked to the existence of three isoforms of OGT, generated by alternative splicing of its mRNA. The long nucleo-cytoplasmic form of OGT (ncOGT, 116 kDa), is constituted of a catalytic domain in its C-terminal part and 12 TPR (tetra-tricopeptide repeats) in its structure, which is involved in the recognition of specific substrates. Another mechanism involves the interaction of OGT with other proteins, such as GRAP (glucose-regulated aminoacyl-tRNA synthetase), which enhances OGT activity and substrate specificity. Additionally, the localization of OGT in different subcellular compartments, such as the nucleus, cytoplasm, or endoplasmic reticulum, may contribute to its specificity. Finally, the regulation of OGT activity by phosphorylation, acetylation, or ubiquitination may also play a role in determining substrate specificity.
N-terminal region. Two other isoforms, which differ in their N-terminal region, have also been described: a mitochondrial form of 103 kDa (mOGT, comprising in its N-terminal region a mitochondrial targeting sequence followed by 9 TPR) and a short isoform of 78 kDa (sOGT, comprising only 3 TPR in its N-terminal region, and displaying a subcellular localisation similar to ncOGT) [17,18]. TPR domains are known to be involved in protein–protein interactions, and could constitute important determinants in the regulation of specificity of action of OGT towards different substrates. Thus, the deletion of the three first TPR of ncOGT had no effect on its activity towards peptide substrates, but totally inhibited its activity towards certain proteins such as casein kinase II and nucleoporine p62 [19]. In addition, addressing mOGT to the mitochondria also permits to specifically target a subset of substrates [17,20].

Changes in cellular localisation of the nucleocytoplasmic form of OGT, induced by different hormonal ligands, permit to target OGT towards specific substrates. Thus, as will be discussed below, the production of PIP3 at the plasma membrane constitutes a mechanism by which OGT is specifically addressed to a subset of substrates upon activation of a given signaling pathway.

The specific association of OGT with different partners also constitutes one of the mechanism by which OGT is addressed towards certain substrates. For example, the association of OGT with Trafficking protein kinesis binding 1 (TRAK1) may permit its targeting to RNA polymerase II, suggesting a potential mechanism for regulation of transcription [21]. In neuroblastic Neuro-2a cells, the association of OGT with p38 MAP kinase permits its targeting to the neurofilament protein NF-H, inducing its O-GlcNAcylation and depolymerisation [22].

Finally, OGT is itself regulated by post-translational modifications that could modify its activity and/or specificity of action. Thus, OGT is capable of auto-O-GlcNAcylation, although the consequences of this modification have not been elucidated [8,19]. OGT is also regulated by phosphorylation. The serine-threonine kinase Calcium/calmodulin-dependant protein kinase IV (CaMKIV) phosphorylates OGT and increases its activity in neuronal NG-10815 cells [23]. OGT is also phosphorylated on tyrosine in response to insulin in 3T3L1 adipocytes, resulting in an increase in its activity [24].

Thus, the combination of different mechanisms of regulation of OGT (alternative splicing, targeting to different compartments, membrane relocalisation induced by PIP3, post-translational modifications of OGT) should contribute to ensure, according to the cell type and the cell environment, the specificity of action of OGT, which complexity is still far from being understood.

2. O-GlcNAcylation and human diseases

The biological importance of O-GlcNAcylation is underlined by the fact that abnormal O-GlcNAcylation of proteins has been associated with a number of human diseases [10].

Thus, O-GlcNAcylation may play a role in neurodegenerative diseases [25]. Whereas the gene coding for OGT is a candidate for Parkinson dystony linked to the X chromosome, the gene coding for O-GlcNAcase is close to a candidate region for Alzheimer’s disease. Moreover, alterations in O-GlcNAcylation seem to contribute to the toxicity of proteins involved in Alzheimer’s disease. A defect in O-GlcNAcylation of Tau protein, probably due to a decreased glucose utilisation by the aging brain, could contribute to its hyperphosphorylation, resulting in dysfunctions that lead to the development of the neuronal alterations. A Yin-Yang reciprocal relationship has indeed been described in which O-GlcNAcylation of Tau protein may have a protective role by reducing Tau phosphorylation on numerous residues [26,27].

O-GlcNAcylation have also been shown to regulate the activity of several oncogenes and tumor suppressors [6,28–30], suggesting a potential role for this modification in tumorigenesis.

Finally, anomalies in O-GlcNAcylations are involved in metabolic diseases. The gene coding for O-GlcNAcase corresponds to a susceptibility locus for Type 2 diabetes in an American-Mexican population [31] and SNP variants of the gene coding for GFAT have been associated with Type 2 diabetes mellitus in Caucasian individuals [32]. Beside, O-GlcNAcylations regulate insulin signaling and seem to play an important part in the development of diabetes and its complications [10,11,33], as discussed in further details below.

3. O-GlcNAcylation and glucotoxicity

3.1. O-GlcNAcylation and insulin resistance

Insulin resistance, defined as a decreased efficiency of insulin action on its target tissues, is a major feature of Type 2 diabetes and obesity. It has long been known that chronic hyperglycaemia per se has deleterious effects on insulin sensitivity [34]. The involvement of the HBP in glucose-induced insulin resistance was first proposed in 1991 by Marshall et al., who observed that a marked decrease in insulin sensitivity in primary adipocytes could be induced by the concomitant addition of glutamine, high-glucose concentrations and insulin in the culture medium. The effect of these agents was inhibited by drugs acting on GFAT, the rate-limiting enzyme of the HBP (Fig. 1). Moreover, glucosamine alone, which enters the pathway downstream of the GFAT (Fig. 1), was much more efficient in inducing insulin resistance than the three agents added together [35]. These results led the authors to propose that the HBP played a major role in insulin resistance, although the molecular mechanisms involved were not known at this time [35].

The involvement of the HBP and of protein O-GlcNAcylation in insulin resistance was then clearly demonstrated by McClain et al. [36,37], who developed transgenic mouse models overexpressing either GFAT or OGT in different tissues. These authors showed that overactivation of the pathway in muscle, adipose tissue, liver or pancreatic β-cells resulted in phenotypes similar to those observed in type 2 diabetes and obesity (Fig. 3). More specifically, overexpression of GFAT in tissues involved in insulin-stimulated glucose uptake (muscle and adipose tissue), by using the Glut4 promoter, resulted in insulin resistance
associated with decreased insulin-induced glucose transporter translocation in skeletal muscle. This insulin resistance could be reversed by the antidiabetic drug troglitazone. A more direct evidence of the involvement of O-GlcNAcylation in insulin resistance was obtained by the observation that transgenic mice overexpressing OGT in muscle and adipose tissue under the control of Glut4 promoter display a phenotype similar to Glut4-GFAT mice [18,36].

These observations logically led to the investigation of O-GlcNAcylation of proteins involved in insulin signaling.

### 3.2. O-GlcNAcylation and regulation of insulin signaling

Binding of insulin to its receptor stimulates its autophosphorylation on tyrosine residues [38]. This stimulates the tyrosine kinase activity of the receptor towards intracellular substrates,
such as IRS1 or Shc, which then activate signaling pathways involved in the metabolic and mitogenic effects of insulin [39]. It soon appeared that numerous actors of insulin signaling, from plasma membrane to the nucleus, were modified by O-GlcNAcylation (Fig. 4). Indeed, the intracellular β subunit of the insulin receptor, IRS1 and IRS2, the p85 and p110 subunit of the PI-3 kinase, PDK1, the protein kinase Akt/PKB, and the transcription factor FoxO1 are all targets of OGT. In most cases, O-GlcNAcylation of these proteins has effects opposite to those induced by insulin [10].

A recent study has shed a new light on the mechanism by which OGT is addressed to the plasma membrane upon hormone stimulation, in the vicinity of the proteins involved in early steps of insulin signaling [40]. In the basal state, OGT has essentially a cytosolic and nuclear localisation. Upon insulin stimulation, phosphatidyl-inositol 3-phosphate (PIP3) production by PI-3 kinase induces the recruitment of OGT at the plasma membrane through a domain denominated PPO (PIP-binding domain of OGT), located in the C-terminal part of the enzyme. The recruitment of OGT at the membrane then favors O-GlcNAcylation of insulin signaling proteins, resulting in attenuation of the signal (Fig. 4). This negative feedback could be exacerbated in situation in which cells are bathed in an environment abnormally rich in glucose (and insulin) due to chronic hyperglycaemia in obese or diabetic patients. This would lead to a decrease in insulin signaling efficiency, resulting in cellular insulin resistance and the development of a vicious circle (glucotoxicity) in which hyperglycaemia will worsen itself [11,33].

3.3. Liver glucotoxicity

Excessive production of glucose by the liver is a major cause of fasting hyperglycaemia in human and in animal models of diabetes. The hydrolysis of glucose-6-phosphate by glucose 6-phosphatase (G6Pase) is the final and obligatory step for the release of glucose into the circulation. Insulin inhibits the expression of this enzyme, both in cultured hepatocytes [41] and in animals [42]. This transcriptional regulation is believed to play a major role in the inhibition of hepatic glucose production by insulin. However, and most surprisingly, high concentrations of glucose increased G6Pase mRNA expression in cultured hepatocytes [43,44]. In partially pancreatectomized diabetic animals, hyperglycaemia per se also increased the G6Pase mRNA expression, and this effect was reversed by phlorizin, which normalizes glycaemia independently of insulin [42]. This indicates that, paradoxically, hyperglycaemia per se has a stimulatory effect on G6Pase expression.

The expression of the G6Pase gene is known to be under the control of the Forkhead Box Other-1 (FoxO1) transcription factor. Insulin, through activation of the P3-Kinase/PKB signaling pathway, induces phosphorylation of FoxO1 on serine and threonine residues [45,46], resulting in FoxO1 association with 14-3-3 protein [47], exclusion from the nucleus [48] and consecutive inhibition of its transcriptional activity [49]. This mechanism is believed to play a major role in insulin-induced inhibition of hepatic glucose production through decreased expression of the genes coding for G6Pase and other proteins involved in the control of gluconeogenesis, such as PGC1 and PEPCK [50].

Recent data demonstrated that FoxO1 is O-GlcNAcylated in liver cells, resulting in an increase in its transcriptional activity, without any modification of its subcellular localisation [51]. This led to the proposal that in diabetic patients, chronic hyperglycaemia may induce abnormal level of FoxO1 O-GlcNAcylation, resulting in increased expression of gluconeogenic genes and excessive glucose production by the liver [33]. This vicious circle would lead to further worsening of hyperglycaemia and eventually to overt diabetes [33]. This hypothesis was reinforced by the observation that CRTC2, a transcriptional coactivator of CREB, is also modified by O-GlcNAc. In this case, O-GlcNAcylation induces relocalisation of CRTC2 into the nucleus, increasing the activity of CREB on gluconeogenic genes [52].

3.4. Pancreatic islet glucotoxicity

O-GlcNAc modification of proteins appears to play a major role in the biology of the endocrine pancreas. Recent work using an in vitro model of developing embryonic pancreas from E13.5 has shown that glucose, through the HBP, controls β-cell development, whereas modulation of this pathway had only modest effects on exocrine pancreas development [53]. In agreement with a role of the HBP in endocrine pancreas development and function, young (2 months) transgenic mice overexpressing GFAT specifically in pancreatic β-cells (Fig. 3), have increased insulin content in pancreatic islets [54]. It was also shown that islets isolated from these mice display increased sensitivity of insulin secretion to glucose stimulation [55]. However, in older transgenic animals (6–10 months), the resulting hyperinsulinemia was eventually associated with development of insulin resistance [54] and deterioration of the pancreatic function [55]. These observations strongly suggest that the HBP is involved in glucose sensing and insulin secretion by the β-cell. Histochemical and biochemical evidences also support the notion that O-GlcNAcylation plays an important role in pancreatic function. Thus, OGT is highly expressed in pancreatic islets [56], and immunoelectron microscopy showed OGT localisation around the secretory granules, suggesting a role in granular secretion [57]. In addition, a high content in O-GlcNAc modified proteins could be detected in rat β-cells by immunohistochemistry using anti-O-GlcNAc antibody [56]. Moreover, induction of transient hyperglycaemia by glucose infusion in rats resulted in a corresponding transient increase in O-GlcNAc protein levels in pancreatic β-cells, as detected by immunostaining with anti-O-GlcNAc antibody. In addition, pharmacological inhibition of GFAT in mouse islets [58], or reduction of OGT expression in MIN6 cells using siRNA, resulted in an inhibition of glucose-induced insulin secretion [59]. In agreement with a contribution of this pathway to the functional activity of the β-cell, Pdx1 and NeuroD1 – two major transcription factors involved in pancreatic development, β-cell differentiation and regulation of insulin gene expression – are regulated by O-GlcNAc [60,61]. Indeed, it was shown that O-GlcNAcylation of these transcription factors resulted in an increase in their DNA
binding activity on the promoter of the insulin gene [60,61].

Altogether, these data strongly support the notion that O-GlcNAc modification of proteins plays a major role in the functional activity of the endocrine pancreas under normal conditions. However, a number of studies indicate that excessive O-GlcNAc modification of proteins in pancreatic β-cells may also participate in alterations observed in diabetic conditions. Indeed, increases in OGt expression and O-GlcNAc levels in proteins were associated with deterioration of the glucose-stimulated insulin secretion in the pancreas of Goto-Kakizaki rats [59]. Increased OGt expression and O-GlcNAc levels were also observed in pancreas of rats with streptozotocin-induced diabetes [62]. Interestingly, streptozotocin, which has been largely used to induce experimental diabetes in animals, turned out to be an inhibitor of O-GlcNAcase [56]. It was therefore proposed that part of its effect on β-cell death relied on its ability to increase O-GlcNAc in pancreatic β-cells [63,64], although some authors have questioned this hypothesis [65,66].

Whereas the role of O-GlcNAc in streptozotocin-induced-pancreatic cell death remained debatable, several lines of evidence indicate that excessive flux through the HBP has deleterious effects on pancreatic β-cell survival. Indeed, an 8-hours intravenous infusion of glucosamine in rats induced significant pancreatic β-cell apoptosis [63]. In isolated human pancreatic islets, high-glucose concentrations induced apoptosis that can be reversed by pharmacological inhibition of the HBP [67]. Moreover, in RIN rat pancreatic β-cells, glucosamine treatment induced O-GlcNAcylation of the insulin receptor and of its substrates IRS1 and IRS2. This was associated with an inhibition of insulin signaling through the PI-3 kinase/Akt pathway, and a consecutive increase in apoptosis [67]. Finally, in rat INS1 and mouse βTC-6 pancreatic β-cell lines, O-GlcNAcylation of Akt1 correlated with decreased activity and increased apoptosis [68]. Altogether, these data suggest that excessive O-GlcNAcylation may participate in the toxic effect of glucose in pancreatic β-cells, by inhibiting the antiapoptotic PI-3kinase/Akt pathway.

One of the important endpoint of the PI-3kinase/Akt pathway is the transcription factor FoxO1, which is known to control proliferation, survival and apoptosis of pancreatic β-cells [69,70]. The recent discovery that the transcriptional activity of FoxO1 is increased by O-GlcNAcylation in liver and other cell types [33,51] suggests that O-GlcNAcylation of FoxO1 could also participate in glucotoxicity in pancreatic β-cells [11].

3.5. Vascular complications

Diabetic patients have an increased risk of cardiovascular disease. A close correlation exists between high plasma glucose levels and cardiovascular morbidity and mortality [71,72]. The vascular complications that develop in diabetic patients relate to pathogenesis of both macrovascular (increased incidence and severity of stroke and myocardial infarction) and microvascular (neuropathy, nephropathy, retinopathy and erectile dysfunction) systems. Endothelial dysfunction has been implicated in the development of both diabetic macrovascular and microvascular diseases. Hyperglycaemia is one of the most important features and a major risk factor to the pathogenesis of diabetic vascular complications [73–75].

3.5.1. O-GlcNAcylation and regulation of vascular tone by nitric oxide

The endothelial cell secretes vasoactive substances, hormones and cytoprotective biological factors. Nitric oxide (NO), the principal endothelium-dependent relaxing factor, is a vasodilator and anti-inflammatory molecule, and constitutes a key component of the vascular homeostasis. The fact that endothelial cells in diabetes fail to produce sufficient amount of NO and to induce vasorelaxation in response to the endothelium-dependent vasorelaxants (e.g. acetylcholine, bradykinin, shear stress, etc.) has been well documented both in animal models [63,76–78] and human studies [79–81]. An increasing number of reports implicate O-GlcNAc in the adverse effects of diabetes on NO production. eNOS, the enzyme responsible for NO production in endothelial cells, is activated upon phosphorylation on serine 1177 by Akt. Using cultured bovine aortic endothelial cells (BAECs) as well as aortae from diabetic rats, Du et al. showed that high glucose conditions resulted in increased O-GlcNAc modification on eNOS, associated with a decreased phosphorylation of the protein on the Akt site [82]. The effect of high glucose on eNOS activity and the changes in post-translational modifications were reversed by inhibition of GFAT using antisense oligonucleotides. Mutagenesis of serine 1177 into alanine markedly reduced the effect of high glucose on O-GlcNAcylation of eNOS, suggesting a reciprocal relationship (yin-yang) between eNOS phosphorylation and O-GlcNAcylation. Similar results were independently obtained in human coronary artery endothelial cells, in which phosphorylation of eNOS at Ser1177 by Akt was decreased by both high glucose and glucosamine [13]. Reversion of the effect of high glucose by GFAT inhibition with azaserine supported the role of increased HBP flux in this process [13]. Another group, studying eNOS in erectile function in rat penis, demonstrated increased O-GlcNAcylation of eNOS, associated with decreased phosphorylation on serine 1177 and reduced erectile response to shear stress or VEGF in diabetic rats, providing a potential mechanism for diabetes-associated erectile dysfunction [83].

In addition, recent evidence using rat artery segments indicate that O-GlcNAcylation induced by PUGNAc blunted vascular reactivity to the vasorelaxing effect of acetylcholine and augmented vasoconstriction in response to phenylephrine [84,85]. Importantly, these alterations in vascular reactivity were not observed in endothelium-denuded vessels, indicating that the effect of PUGNAc was mediated by endothelial cells [84]. O-GlcNAc-mediated impaired relaxation and enhanced vascular tone were again shown to be associated with decreased eNOS phosphorylation on serine 1177 [84,85]. These studies therefore provide an interesting link between O-GlcNAcylation and hypertension.

3.5.2. O-GlcNAcylation and atherosclerosis

NO production not only plays a pivotal role in the regulation of vascular tone but also inhibits platelet aggregation, leukocyte adhesion, vascular smooth muscle cell proliferation and
secretion of extracellular matrix (ECM) protein. Therefore, NO is a powerful inhibitor of the earlier phases of atherosclerosis and the mechanisms that lead to neointimal proliferation after vascular injury.

In human coronary artery endothelial cells, O-GlcNAc-induced inhibition of eNOS was associated with an increase in matrix metalloproteinase (MMP2 and MMP9) activity and expression, combined with decreased tissue inhibitor of metalloproteinase (TIMP) expression [13]. Such an imbalance between MMPs and TIMPs has been implicated in atherosclerosis-related complications in diabetes [86]. Therefore, O-GlcNAcylation of eNOS may contribute to the increased incidence of atherosclerosis in diabetic patients. In agreement with this notion, atherosclerotic plaques from diabetic patients revealed a marked increase in O-GlcNAc content compared to nonatherosclerotic vessels from nondiabetic subjects [13].

Beside the effects mediated by inhibition of NO production, O-GlcNAcylation also directly affects the expression of profibrotic and atherogenic factors. In cultured endothelial cells, hyperglycemia induced O-GlcNAcylation of the transcription factor Sp1, resulting in increased expression of Plasminogen Activator Inhibitor-1 (PAI-1, an inhibitor of fibrinolysis), and Transforming Growth Factor β (TGFβ, a profibrotic factor) that both have an important role in the formation of atherosclerotic lesions in obese and diabetic patients [87].

Thrombospondins are matricellular proteins that regulate cell–cell and cell–matrix interactions [88]. Thrombospondin-1 (TSP-1) is a potent antiangiogenic and proatherogenic protein found in early atherotic lesions and injured vascular wall [89]. In human aortic smooth muscle cells, high glucose concentrations, through activation of the HBP, increased the expression of TSP-1, and enhanced cell proliferation [89]. This HBP-induced effect on TSP-1 upregulation and vascular smooth muscle cell proliferation was abolished by inhibition of GFAT [89]. These results potentially suggest an additional link between hyperglycaemia, O-GlcNAcylation and atherosclerosis.

3.5.3. O-GlcNAcylation and angiogenesis

Tissue-specific abnormal angiogenesis (the formation of new blood vessels out of preexisting capillaries) contributes to the development and progression of diabetic vascular complications [74,90]. Clinical and animal reports have evidenced abnormal enhanced angiogenesis in the retina, leading to diabetic retinopathy [90]. At the same time, impaired angiogenesis in diabetes often leads to reduced wound healing, exacerbated peripheral limb ischemia, and cardiac mortality through reduced collateral vessel development [91–93].

Whereas a role for O-GlcNAc in abnormal angiogenesis in diabetic retinopathy has not been demonstrated, a recent work, using cultured human umbilical vein endothelial cells (HUVEC) as well as mouse aortic rings, have implicated the HBP in impaired angiogenesis. Elevated O-GlcNAc levels inhibited the potency of endothelial cells to migrate and form capillary-like tube, and this effect could be reversed by O-GlcNAcase overexpression [94]. Increased O-GlcNAc levels also markedly reduced vascular sprouting in mouse aortic rings after glucosamine treatment, as well as in aortic rings from streptozotocin-induced and high-fat diet-induced diabetic mice [94]. Impaired angiogenesis was mediated, at least in part, by O-GlcNAcylation of Akt, which decreased its activity and its phosphorylation at the Serine 473 site [94].

FoxO1 is also known to negatively regulate angiogenesis, by inhibiting endothelial cell proliferation and migration, and by reducing their capacity to form capillary tubes [95,96]. It can be speculated [11] that O-GlcNAc modification of FoxO1, by increasing its transcriptional activity [51], may also participate in diabetes associated vascular diseases.

3.6. Renal complications

One third of diabetic patients will develop diabetic nephropathy, a major chronic microvascular complication leading to a progressive decline in renal function characterized by decreased glomerular filtration rate and proteinuria. This pathology is associated with altered cellular signaling and structural alterations such as accumulation of ECM proteins. The crucial role of hyperglycaemia in diabetic nephropathy has been known since clinical studies (Diabetic Control and Complications Trial, UK Prospective Diabetes Studies) demonstrated the beneficial effect of glycaemia correction on microcomplication development [97]. The HBP and O-GlcNAc modifications have been causally involved in alterations associated with diabetic nephropathy and proposed as mediators of the adverse effects of hyperglycaemia.

Most of the observations supporting this idea have been made on mesangial cells, an important cell type in the renal glomeruli. Mesangial cells are smooth muscle-like pericytes that abut and surround the filtration capillaries within the glomerulus [98]. They play an important role in the development of diabetic nephropathy by undergoing hypertrophy and increasing secretion of matrix proteins resulting in glomerulosclerosis, one of the earliest events of the disease. Kolm-Litty et al. first reported that glucosamine is able to mimic the effects of high glucose concentrations on the inhibition of mesangial cell proliferation, induction of TGFβ expression, a prosclerotic cytokine implicated in diabetic nephropathy, and consecutive increase in ECM protein synthesis [99]. Moreover, the effects of high glucose were abolished by the GFAT inhibitor azaserine, thus supporting a role for the HBP as a mediator of high glucose effects [99]. In addition, GFAT overexpression has been shown to induce an increase in TGFβ expression and fibronectin synthesis [100]. The involvement of TGFβ in the HBP effects was confirmed by the observation that glucosamine action on fibronectin synthesis was inhibited by an anti-TGFβ antibody [101]. It was then suggested that HBP-induced ECM production might be mediated by protein kinases A and C. Indeed, high glucose and glucosamine have been shown to induce translocation of PKCα,β and ε in mesangial cells. High glucose effects on PKC translocation were inhibited by azaserine [102]. This idea was reinforced by the fact that glucosamine and TGFβ increased PKC and PKA activities as well as laminin and fibronectin production and that those effects were blocked by the use of inhibitors of these kinases [101,103].

A large number of studies have also demonstrated the role of the HBP in the increased expression of the fibrinolysis inhibitor
PAI-1, known to participate in nephropathy associated with diabetes. First, glucosamine and GFAT overexpression resulted in an increase in PAI-1 promoter activity in mesangial cells [104]. Moreover, activation of a PAI-1 promoter by high glucose was blocked by the use of DON, a GFAT inhibitor. The underlying mechanism appeared to involve activation of Sp1 based on the observation that glucosamine increased Sp1 DNA binding and that induction of PAI-1 promoter activity by glucosamine was markedly reduced by mutation of two putative Sp1 sites [105]. The same group then demonstrated the requirement for the PKC-β1 and δ signal transduction pathway in HBP-induced transcription by showing that glucosamine and high glucose stimulated PKC-β1 and δ activity, and that dominant negative PKC-β1 and δ completely blocked the induction of PAI-1 promoter by high glucose and glucosamine [106]. While the studies discussed above documented an involvement of the HBP, direct implication of O-GlcNAcylation in the regulation of PAI-1 expression by high glucose in glomerular mesangial cells was demonstrated more recently [107]. Indeed, limiting O-GlcNAc modification in mesangial cells using different molecular techniques (dominant negative of OGT, O-GlcNAcase overexpression or OGT knock-down by RNA interference) resulted in an inhibition of high glucose ability to increase Sp1 transcriptional activity, PAI-1 promoter activity as well as PAI-1 mRNA and protein levels [107]. These results demonstrated the role of Sp1 O-GlcNAc modification in high glucose-induced PAI-1 gene expression.

The HBP has also been implicated in inflammation pathway, especially VCAM-1 expression through NF-κB regulation, which is known to be involved in diabetic nephropathy development. In mesangial cells, GFAT overexpression both increased the activity of an NF-κB enhancer and activated VCAM-1 promoter, with further augmentation by high glucose and abrogation by inhibitors of the enzyme. In addition, the p65 subunit of NF-κB was O-GlcNAcylated upon treatment with high glucose or glucosamine, and this was associated with an increase in its DNA binding activity. These observations suggested that O-GlcNAc modification of NF-κB could play a role in the inflammation process taking place during the course of diabetic nephropathy [108].

Regarding mesangial cell proliferation, which is very much perturbed in diabetic nephropathy, it was shown that glucosamine treatment resulted in cell-cycle arrest, associated with increased expression of the cyclin-dependant kinase inhibitor p21Waf1/Cip1, as well as hypertrophy of mesangial cells [109]. This was later confirmed by Singh et al. who reported that high glucose and glucosamine induced cell-cycle arrest at G1 phase, hypertrophy and death of mesangial cells, associated with elevated proapoptotic caspase-3 activity and reactive oxygen species generation [110].

Interestingly, rhein, a compound of the Chinese medicine isolated from rhubarb, has been proven to be effective in treatment of diabetic nephropathy in db/db mouse model. Investigating its potential mechanism of action, the authors found that rhein was able to correct the diabetic phenotype (p21 expression and hypertrophy, fibronectin synthesis and TGFβ expression) of a mesangial cell line overexpressing the glucose transporter GLUT1. This effect was associated with an inhibition of the HBP (GFAT activity and UDP-GlcNAc levels) which supported the importance of this pathway in diabetic nephropathy development [111].

Renal proximal tubular cells are another cell type affected during diabetic nephropathy development. Like mesangial cells, they undergo hypertrophy eventually leading to interstitial fibrosis. It was reported that high glucose and glucosamine stimulated proximal tubular cell hypertrophy as well as angiotensinogen and renin mRNA expression, which are known to play a role in this phenomenon. High glucose but not glucosamine effects were blocked by GFAT inhibitors, and glucosamine effects appeared to be mediated via both p38 MAPK and PKC signal transduction pathways. These results indicated that high glucose action on angiotensinogen gene expression and induction of tubular cell hypertrophy might be mediated at least in part through activation of the HBP [112].

A few descriptive studies provided in vivo data suggesting the importance of the HBP and O-GlcNAcylation in diabetic nephropathy. GFAT protein and mRNA were shown to be expressed in glomerular cells of renal tissue from patients with diabetic nephropathy while they were undetectable in glomeruli of patients with non-diabetic renal disease, suggesting a possible induction of the HBP rate-limiting enzyme in diabetes [113]. Beside, a recent study reported an increase in O-GlcNAc modified proteins in both glomerular and tubular cells of kidneys from diabetic patients, based on an anti-O-GlcNAc immunostaining [114].

Therefore, as illustrated above, a large body of evidences now supports a role for the HBP and O-GlcNAcylation in alterations characteristic of diabetic nephropathy. However, transgenic animal models, especially overexpressing GFAT or OGT specifically in the kidney, are still required to fully sustain the implication of this pathway in the pathophysiology of diabetic nephropathy in vivo.

4. Concluding remarks

As we have seen, O-GlcNAcylation now appears to play a major role in glucotoxicity and participates in diabetic complications in various tissues. However, other mechanisms have been involved in the toxic effects of chronic hyperglycaemia, including increased production of reactive oxygen species, formation of advanced glycation end products, stimulation of PKC isoforms and activation of the polyol pathway. Interestingly, a unifying mechanism has been proposed [115], in which hyperglycaemia-induced increase in ROS production stimulates ADP-ribosylation and inhibition of Glyceraldehyde 3-Phosphate dehydrogenase. This would in turn results in accumulation of glycolysis intermediates that feed into the different pathways mentioned above. One of these intermediates, Fructose-6-Phosphate, will enter the hexosamine pathway, resulting in increase in UDP-GlcNAc concentrations and O-GlcNAcylation of proteins.

In addition to glucose, excess fatty acids also plays an important part in diabetic complications (lipotoxicity). Although beyond the scope of this review, it is interesting to mention
interrelations between fatty acids and the HBP. Infusion of lipid emulsion in rats resulted in a two-fold increase in UDP-GlcNAc content in skeletal muscle, associated with insulin resistance [116]. As already indicated (Fig. 2), fatty acid metabolism may feed the HBP by providing the acetyl-moiety of UDP-GlcNAc. In addition, fatty acids may act by stimulating the expression of enzymes involved in the HBP. Indeed, in cultured human myotubes, palmitate markedly increased the expression of GFAT mRNA and proteins [117], whereas in pancreatic β-cells, it stimulated the expression of glucosamine-phosphate N-acetyl transferase, another enzyme of the HBP [118]. Therefore, the concept of glucolipotoxicity might need to be revisited, in order to include modulation of the HBP by fatty acids.

Finally, it must be emphasised that the O-GlcNAcylation field is probably in its infancy. The presence of phosphate on proteins has been known since the beginning of the 20th century [119]. However, it is only in the middle of the 1950s, with the pioneer work of E.G. Krebs and E.H. Fisher on the regulation by phosphorylation of the activity of a metabolic enzyme (glycogen phosphorylase), that the importance of this post-translational modification began to be fully realized (Krebs and Fisher obtained, in 1992, the Nobel prize of Physiology/Medicine for their discoveries on the role of reversible phosphorylation of proteins as a crucial regulatory mechanism in biology [120]). It is now clearly appearing that protein O-GlcNAcylation, discovered much more recently, also regulates most cellular processes. The recent explosion of investigations in this field certainly indicates that important changes in our vision of the mechanisms of regulation of cell signaling are yet to come.

Conflict of interest statement

The authors have no conflict of interest.

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