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REVIEW

O-GlcNAcylation and oxidation of proteins: is signalling in the cardiovascular system becoming sweeter?

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ABSTRACT

O-GlcNAcylation is an unusual form of protein glycosylation, where a single-sugar [GlcNAc (*N*-acetylglucosamine)] is added (via β-attachment) to the hydroxyl moiety of serine and threonine residues of nuclear and cytoplasmic proteins. A complex and extensive interplay exists between O-GlcNAcylation and phosphorylation. Many phosphorylation sites are also known glycosylation sites, and this reciprocal occupancy may produce different activities or alter the stability in a target protein. The interplay between these two post-translational modifications is not always reciprocal, as some proteins can be concomitantly phosphorylated and O-GlcNAcylated, and the adjacent phosphorylation or O-GlcNAcylation can regulate the addition of either moiety. Increased cardiovascular production of ROS (reactive oxygen species), termed oxidative stress, has been consistently reported in various chronic diseases and in conditions where O-GlcNAcylation has been implicated as a contributing mechanism for the associated organ injury/protection (for example, diabetes, Alzheimer's disease, arterial hypertension, aging and ischaemia). In the present review, we will briefly comment on general aspects of O-GlcNAcylation and provide an overview of what has been reported for this post-translational modification in the cardiovascular system. We will then specifically address whether signalling molecules involved in redox signalling can be modified by O-GlcNAc (O-linked GlcNAc) and will discuss the critical interplay between O-GlcNAcylation and ROS generation. Experimental evidence indicates that the interactions between O-GlcNAcylation and oxidation of proteins are important not only for cell regulation in physiological conditions, but also under pathological states where the interplay may become dysfunctional and thereby exacerbate cellular injury.

Key words: cardiovascular system, diabetes, inflammation, O-linked *N*-acetylglucosamine (O-GlcNAc), oxidative stress, phosphorylation.

Abbreviations: AGE, advanced glycation end product; BEMAD, β-elimination followed by Michael addition with dithiothreitol; DOCA, deoxycorticosterone acetate; eNOS, endothelial NO synthase; ET-1, endothelin-1; FoxO1, forkhead box O1; GFAT, glutamine:fructose-6-phosphate amidotransferase; GlcNAc, *N*-acetylglucosamine; GPX1, glutathione peroxidase 1; HBP, hexosamine biosynthesis pathway; I/R, ischaemia/reperfusion; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; mPTP, mitochondrial permeability transition pore; NF-κB, nuclear factor κB; OGA, β-N-acetylglucosaminidase; O-GlcNAc, O-linked GlcNAc; O-GlcNAc-P, phosphorylated O-GlcNAc; OGT, O-GlcNAc transferase; PP1, protein phosphatase 1; PTM, post-translational modification; PUGNAc, *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate; RAGE, receptor for AGEs; ROS, reactive oxygen species; shRNA, short hairpin RNA; SOD, superoxide dismutase; TPR, tetratricopeptide repeat; VSMC, vascular smooth muscle cell.

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INTRODUCTION

A healthy vasculature is crucial to transport hormones, gases (oxygen and carbon dioxide), nutrients and metabolic wastes to and from every tissue in the body. Since adequate perfusion is critical to survival, blood distribution to the tissues/organs is under the control of tightly regulated mechanisms that ultimately modify the relaxation and contraction of VSMCs (vascular smooth muscle cells). Accordingly, vascular function is complexly regulated by a variety of signals: endocrine and paracrine substances, sympathetic nervous system activity, blood-borne and immune-cell-derived factors, and by the activity of the vascular endothelium. Not surprisingly, vascular dysfunction is linked to diverse disorders, including diabetes, arterial hypertension, the metabolic syndrome, chronic kidney disease, myocardial ischaemia, congestive heart failure, stroke, retinopathy, erectile dysfunction, cancers to name a few.

Diversity among signals that influence contraction/relaxation of VSMCs implies an assortment of signal transduction mechanisms (receptors and intracellular signalling pathways). Adding further complexity to the regulation of VSMCs is the fact that many receptors and intracellular signalling molecules are subject to PTMs (post-translational modifications), or the reversible addition of a small chemical group, which causes a change in activity or location of a signalling protein. Table 1 illustrates PTMs regarded as important in the regulation of protein activity, interaction with effectors, subcellular localization, stability, trafficking, translocation to lipid rafts, aggregation and other aspects of protein function. Among these PTMs, the reader's attention will be directed to glycosylation [1,2].

Glycosylation is the site-specific enzymatic addition of saccharides {from the Greek word sákkharon (sugar); also known in biochemistry as carbohydrates or hydrates of carbon due to the chemical empirical formula $[C_m(H_2O)_n\}$ to proteins and lipids [1–3]. Glycosylation has many functions in a cell: it allows correct folding of proteins (some proteins do not fold correctly unless they are glycosylated first), confers stability (some unglycosylated proteins are more rapidly degraded), allows cell–cell adhesion (e.g. surface glycoproteins are directly involved in the biological functions of lymphocytes), and modulates intracellular signalling pathways (glycosylation of proteins may enhance or inhibit enzymes' activities) [1–3].

There are many types of glycosylation: (i) phospho-linked, where the sugar is attached via the phosphate of a phospho-serine; (ii) C-linked, where the carbohydrate is added to a carbon on a tryptophan side chain; and (iii) the formation of a GPI (glycosylphosphatidylinositol) anchor (glypiation), where the sugar is linked to phosphoethanolamine, which in turn is attached to the terminal carboxy group of the protein. Furthermore, two other general classes of protein-bound glycans are represented by N- and O-linked glycans. In N-linked glycosylation, the carbohydrate is attached to a nitrogen of asparagine or arginine side chains. The modification of N-linked glycans within the Golgi does not follow a linear pathway. As a result, five different *N*-glycan linkages have been reported, of which GlcNAc (*N*-acetylglucosamine) to asparagine (GlcNAcβ1-Asn) is the most common [1,3]. Although there have been several published reports of nucleocytoplasmic or cytoplasmic *N*-glycans, there exists no definitive structural evidence that *N*-glycans actually occur on cytoplasmic or nuclear proteins nor on the cytoplasmic portions of membrane proteins [1]. In O-linked glycosylation, the carbohydrate is attached to the hydroxyl oxygen of serine, threonine, tyrosine, hydroxylysine or hydroxyproline side chains of nuclear and cytoplasmic proteins [1–3].

WHAT IS O-GlcNAcYLATION?

Cellular glycoproteins were initially thought to be targeted, after their synthesis, only to luminal or extracellular compartments. However, in 1984, Torres and Hart [4], who were interested in characterizing the role of cell-surface saccharides in the development and functions of lymphocytes, described a novel carbohydrate (GlcNAc)-peptide linkage, which was present on proteins localized in the cytosol and the cyto- and nucleo-plasmic faces of membranous organelles. In 1989, Kelly and Hart [5] described that *Drosophila* polytene chromosomes (i.e. polytene chromosome spreads prepared from the salivary glands of third instar stage *D. melanogaster* larvae) contained a surprisingly large amount of terminal GlcNAc residues along their lengths. Nearly all of the chromatin-associated GlcNAc moieties existed as single monosaccharide residues attached to proteins by an O-linkage [O-GlcNAc (O-linked GlcNAc)] [5]. In addition, in the late 1980s, the glycosyltransferase responsible for the addition of GlcNAc to proteins was found to be orientated with its active site in the cytoplasm, and the first proteins modified with O-GlcNAc were described [6].

These initial observations, which indicated a functional or biological significance for the O-linkage of GlcNAc to proteins, led to the term O-GlcNAcylation. Accordingly, O-GlcNAcylation is currently defined as an unusual form of protein glycosylation, where a single-sugar (O-GlcNAc) is added (β-attachment) to the hydroxy moiety of serine and threonine residues of nuclear and cytoplasmic proteins.

It is unusual in that it is found in nuclear and cytoplasmic proteins, representing the first reported example of glycosylated proteins found outside of the secretory channels. Unlike other peptide-linked

Table 1 Examples of PTMs of proteins

Modification	Description	Donor	Target	Reference(s)
Acetylation (an example of acylation)	Introduction of an acetyl group (-$COCH_3$) (the acyl derivative of acetic acid) catalysed by acetyltransferases	Acetyl-CoA	α-Amino group at the N-terminus (N-terminal acetylation) or ε-amino group on the side chain of lysine residues	[106,107]
Methylation (an example of alkylation)	Addition of an alkyl group (methyl, $-CH_3$) catalysed by methyltransferases	*S*-Adenosylmethionine (SAM)	Nitrogen and oxygen molecules (lysine residues, guanidino moiety of arginine, R-group amides or R-group carboxylates of glutamate and aspartate)	[108]
Phosphorylation	Addition of a phosphate group (PO_4^{3-}) by a kinase	ATP	Serine, threonine and tyrosine (O-linked), or histidine (N-linked)	[109]
Nitration	Addition of a nitro triatomic group (-NO_2)	NO	Tyrosine or cysteine residues to form nitrotyrosine	[110,111]
Nitrosylation or nitrosation*	Addition of an equivalent NO to a thiol, metal, amine or hydroxy aromatic group	NO diatomic group or nitrosyl group	Thiol, metal, amine or hydroxy aromatic group	
S-Nitrosation or S-nitrosylation†	Incorporation of the NO moiety into a sulfur atom to form an S-NO bond.	NO	Cysteine residue	
Sulfation	Addition of a sulphate (SO_4^{2-}) catalysed by tyrosylprotein sulfotransferase (TPST) in the Golgi apparatus	PAPS (3′-phosphoadenosyl-5′-phosphosulfate) = $2ATP + SO_4^{-2}$	Tyrosine residues	[112]
Prenylation (or isoprenylation)	Addition of an isoprenoid group [e.g. farnesol (farnesylation) or geranylgeraniol (geranylgeranylation)]	Isoprenoid compounds (15 carbon farnesyl groups and 20 carbon geranylgeranyl groups)	Cysteine residues at the C-terminus of proteins	[113]
S-Sulfhydration	Addition of hydrogen sulfide (H_2S)		Cysteine residue	[114,115]
S-Glutathionylation	Reversible formation of protein mixed disulfides (protein-SSG) with glutathione (GSH)	Glutathione (GSH)	Cysteine residue	[116]
Glycosylation‡	Addition of saccharides or a glycosyl group by glycosyltransferases	Nucleotide sugar	Nitrogen of asparagine or arginine side-chains (N-linked glycans); hydroxy oxygen of serine, threonine, tyrosine, hydroxylysine or hydroxyproline side-chains (*O*-linked glycans); oxygens on lipids such as ceramide	[1–3]

*The terms nitrosation and nitrosylation are distinguished by the addition of the NO diatomic group and nitrosyl group NO respectively. However, in NO species, both groups are the same and inclusion of the '-yl-' in terms describing the PTM is widespread.

†To trigger S-nitrosylation, NO is converted into dinitrogen trioxide (N_2O_3) with an electron acceptor such as oxygen, followed by partial dissociation into [$^+$ON–NO2$^-$]. As this reaction occurs, the nitrosonium (NO^+) moiety can interact with the nucleophile sulfur atom to form S-nitrosothiol (SNO) to the protein's thiol group.

‡The term glycation is used for the chemical or non-enzymatic linkage of a sugar molecule, such as fructose or glucose, to proteins or lipids. Glucose, for example, can slowly condense non-enzymatically with protein amino groups forming, initially, a Schiff base that may rearrange to form the Amadori product. The Amadori product subsequently degrades into α-oxoaldehyde compounds such as 1- and 3-deoxyglucosones, which can react with proteins to form cross-links, as well as chromo/fluorophoric adducts called Maillard products (or AGEs).

monosaccharides, the β-linked GlcNAc-Ser/Thr does not become further replaced by other sugars, remaining a single monosaccharide modification of the protein to which it is attached. O-GlcNAcylation is widely dispersed among eukaryotes, from protozoa to higher mammals. The amino acid consensus sequence or glycosylation motifs for the formation of O-GlcNAc bonds have not yet been found. However, information relating to the polypeptide domains that favour O-GlcNAc attachment has been obtained and seems to involve PEST [proline (P), glutamic acid (E), serine (S), and threonine (T)] sequences [7].

In the present review, we will briefly comment on general aspects of O-GlcNAcylation and provide an overview of what has been reported for this PTM in the cardiovascular system. We will then specifically address whether signalling molecules involved in phosphorylation and redox signalling can be modified by

O-GlcNAc and will discuss the physiological/pathological implications this may have.

A BRIEF OVERVIEW OF O-GlcNAcYLATION

Results from proteomic studies have suggested that more than 1500 proteins in the cell are modified by O-GlcNAc [8–11]. These proteins belong to almost every functional class of proteins, including transcription or translation factors, cytoskeletal proteins, nuclear pore proteins, RNA polymerase II, tumour suppressors, hormone receptors, phosphatases and kinases [8–11]. A database of O-GlcNAcylated proteins and sites, dbOGAP, was recently created and is based primarily on literature published since O-GlcNAcylation was first described in 1984. The database currently contains ~800 proteins with experimental O-GlcNAcylation information. The O-GlcNAcylated proteins are primarily nucleocytoplasmic, and include membrane- and non-membrane bounded organelle-associated proteins [12]. An O-GlcNAcylation site prediction system (O-GlcNAcScan) based on nearly 400 O-GlcNAcylation sites was also developed [13]. Both the database and the prediction system are publicly available at http://cbsb.lombardi.georgetown.edu/OGAP.html and http://cbsb.lombardi.georgetown.edu/filedown.php respectively [12,13].

The attachment of the single-sugar *β*-*N*-acetylglucosamine via an O-linkage to serine/threonine residues is controlled by two highly conserved enzymes OGT [O-GlcNAc transferase or UDP-NAc transferase (uridine diphospho-*N*-acetylglucosamine:polypeptide *β*-N-acetylglucosaminyl transferase] and OGA (*β*-N-acetylglucosaminidase or O-GlcNAcase). Whereas OGT catalyses the addition of O-GlcNAc to the hydroxy group of serine and threonine residues of a target protein using UDP-GlcNAc as the obligatory substrate, OGA catalyses the hydrolytic cleavage of O-GlcNAc from post-translationally-modified proteins [14–16] (Figure 1).

A single OGT gene is located on the X chromosome in humans and mice [17]. In some tissues, such as skeletal muscle, kidney and liver, three distinct isoforms of OGT have been identified, including two 110 kDa subunits and one 78 kDa subunit, which can assemble into multimers, and smaller mitochondrial isoforms [18]. Each variant contains a C-terminal catalytic domain, but differs in the number of TPRs (tetratricopeptide repeats) within its N-terminal domain. The TPRs serve as protein–protein interaction modules that appear to target OGT to accessory proteins and potential substrates, such as the related OIP106 (OGT-interacting protein 106) and PP1 (protein phosphatase 1) [19]. Phylogenetic analysis of eukaryotic OGTs indicates that plants have two distinct OGTs, SEC (secret agent)- and SPY (spindly)-like, which originated in prokaryotes and are involved in diverse

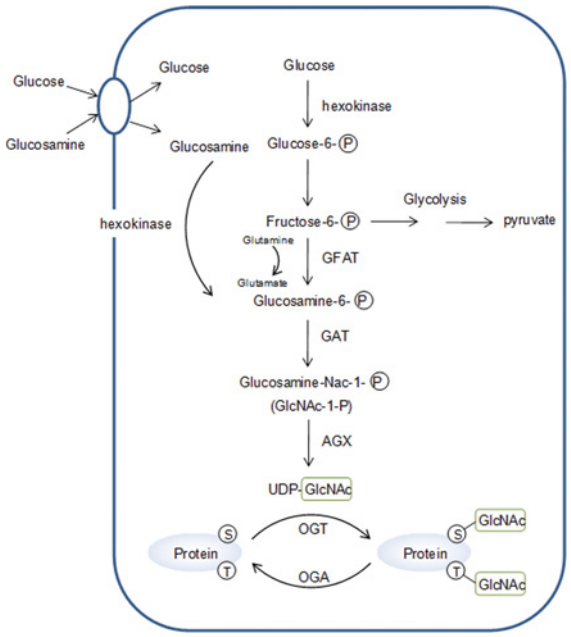

Figure 1 The HBP

After entering the cell via a glucose transporter and being converted into glucose 6-phosphate (glucose-6P) by a hexokinase and into fructose 6-phosphate (fructose-6P), glucose can either be used in the glycolytic pathway or the HBP. The HBP uses fructose 6-phosphate to form glucosamine 6-phosphate (glucosamine-6P), with glutamine serving as the donor of the amino group. The reaction is catalysed by the rate-limiting enzyme GFAT. Glucosamine 6-phosphate is rapidly acetylated through the action of acetyl-CoA:D-glucosamine-6-phosphate N-acetyltransferase (GAT) and isomerized to *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) and activated, via the action of UDP-GlcNAc pyrophosphorylase (AGX), to UDP-GlcNAc that serves as the donor of O-GlcNAc for OGT activity. Glucosamine can also enter the cell through the glucose transporter and is rapidly phosphorylated by hexokinase yielding glucosamine 6-phosphate, thereby bypassing the rate-limiting first step of the HBP. S, serine; T, threonine;

plant processes, including responses to hormones and environmental signals, circadian rhythms, development, intercellular transport and virus infection [20].

The donor substrate for OGT activity UDP-GlcNAc is a terminal product of the HBP (hexosamine biosynthesis pathway) (Figure 1). Flux through the HBP and UDP-GlcNAc levels change rapidly in response to many different nutrients, such as glucose, fatty acids and amino acids [21], altering the extent of O-GlcNAcylation of many proteins. Non-esterified 'free' fatty acids can increase HBP flux by inhibiting glycolysis, resulting in elevated fructose 6-phosphate levels. Acetyl-CoA, produced by fatty acid metabolism, serves as the donor for the acetylation of glucosamine in the formation of UDP-GlcNAc [21,22]. Exogenously, small amounts of glucosamine can dramatically increase UDP-GlcNAc pools in cells [23].

Since OGT activity is exquisitely sensitive to UDP-GlcNAc concentrations (Figure 1), O-GlcNAcylation

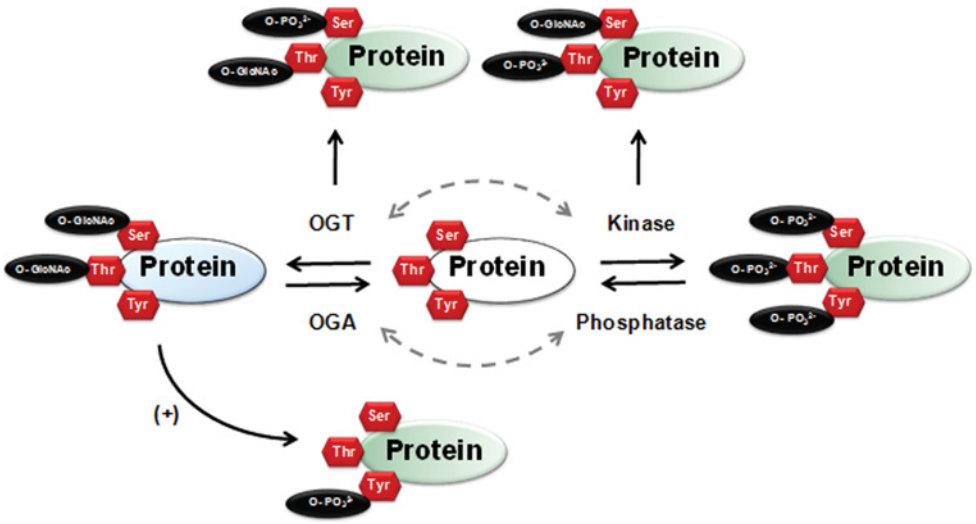

Figure 2 Interplay between O-GlcNAcylation and phosphorylation of proteins

Both phosphorylation and O-GlcNAcylation occur on serine/threonine (Ser/Thr) residues of proteins. In specific proteins, there is a competitive relationship between O-GlcNAc and O-phosphate for the same serine/threonine residues, although there can be adjacent or multiple occupancy for phosphorylation and O-GlcNAcylation on the same protein. The interplay between phosphorylation and O-GlcNAcylation creates molecular diversity by altering specific protein sites that regulate protein functions and signalling events. Tyr, tyrosine.

may act as a sensor for the general metabolic state of the cell. Consistent with this idea, O-GlcNAc has been intricately linked to cell survival induced by many forms of cell stress [24–26]. Interestingly, glucose deprivation also increases protein O-GlcNAcylation in some cancer cells [27–29]. The mechanisms of this paradoxical phenomenon seem to involve increased OGT expression and p38 MAPK (mitogen-activated protein kinase)-dependent OGT activity and decreased O-GlcNAc modification of OGT, as well as reduced O-GlcNAcase activity and decreased hexosamine flux-induced up-regulation of ncOGT (nucleocytoplasmic OGT) [27–29]. However, a recent study has shown that increased glycosylation induced by glucose deprivation is actually modification by N-GlcNAc$_2$, and suggests that repression of mature N-linked glycoproteins due to increased levels of N-GlcNAc$_2$-modified proteins represents a newly recognized pathway for effective use of sugar under stress and deprivation conditions [30].

O-GlcNAcase or OGA appears to use substrate catalysis involving the 2-acetamido group and contains an N-terminal glycosidase domain and a putative C-terminal histone acetyltransferase domain [31]. Two distinct isoforms of OGA have been described, a 130 kDa and a 75 kDa variant, which differ in their C-terminus. Whereas the 130 kDa or 'long OGA' contains a distinct N-terminal glycosidase domain and the C-terminal histone acetyltransferase domain, the 75 kDa or 'short OGA' lacks the C-terminal domain. One important functional aspect in the existence of these two splices is their differential sensitivity to previously described potent OGA inhibitors. For example, the short OGA exhibits comparative resistance to PUGNAc [*O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate] and NAG-thiazoline [1,2-dideoxy-2′-methyl-*a*-D-glucopyranoso-[2,1-*d*]-$\Delta^{2'}$-thiazoline], but is very sensitive to α-GlcNAc thiolsulfonate [11,15,31]. Inhibition of OGT and OGA represents an area of great interest in O-GlcNAcylation research, which is evident from the increasing number of studies addressing the molecular mechanisms of the enzymes for the addition and removal of O-GlcNAc [32–35].

O-GlcNAcYLATION AND PROTEIN PHOSPHORYLATION

The dynamic addition of O-GlcNAc to proteins has been implicated in modulating protein behaviour via one potential mechanism that includes a complex interplay between O-GlcNAcylation and phosphorylation. Many phosphorylation sites are also known glycosylation sites, and this reciprocal occupancy may produce different activities or alter stability in the target protein [36,37] (Figure 2). The competition between O-GlcNAcylation and phosphorylation for the same or neighbouring residues has been termed the 'Yin–Yang' hypothesis and has been reported in a variety of proteins [38].

The interplay between these two PTMs is not always reciprocal, since some proteins can be concomitantly phosphorylated and O-GlcNAcylated, and the adjacent phosphorylation or O-GlcNAcylation can regulate the

addition of either moiety. The cross-talk between O-GlcNAcylation and phosphorylation also exists among distantly located sites and they influence each other by regulating the activities or localization of other cycling enzymes. For example, OGT is directly activated by tyrosine phosphorylation and is itself O-GlcNAc-modified [38]. OGT also forms a stable and active complex with PP1β and PP1γ in rat brain [19]. The association between OGT and PP1 is particularly intriguing, as it may provide a direct mechanism to couple O-GlcNAc to the dephosphorylation of specific substrates.

A recent report has shown that rat brain assembly protein AP180, which is involved in the assembly of clathrin-coated vesicles in synaptic vesicle endocytosis, contains an O-GlcNAc-P (phosphorylated O-GlcNAc) within a highly conserved sequence (O-GlcNAc or O-GlcNAc-P, but not phosphorylation alone, was found at Thr^{310}) [39]. O-GlcNAcylation was thought to be a terminal modification, i.e. the O-GlcNAc was not found to be additionally modified. The existence of protein glycosyl phosphorylation (O-GlcNAc-P) adds further complexity to the phosphorylation–O-GlcNAcylation interplay.

Lastly, the interplay between O-GlcNAc modification and phosphorylation may not be limited to serine/threonine phosphorylation, but may also include tyrosine phosphorylation. On the basis of the higher prevalence of tyrosine phosphorylation among O-GlcNAc-modified proteins (~68 % compared with 2 % in non-O-GlcNAc-modified proteins), Mishra et al. [40] suggested that tyrosine phosphorylation plays a role in the interplay between O-GlcNAc modification and serine/threonine phosphorylation in proteins.

This clearly shows that the interplay between O-GlcNAcylation and phosphorylation is both complex and very extensive. As with any PTM, mapping the attachment sites is a prerequisite toward understanding the biological functions of O-GlcNAcylation [41,42]. The development of chemical tools coupled to mass spectrometry has greatly facilitated the localization of O-GlcNAc to short peptide sequences within proteins, and this combination can be used to determine exact glycosylation sites [42]. Since a direct observation of the O-GlcNAc moiety by mass spectrometry during CID (collision-induced dissociation) is difficult, as the glycosidic linkage is labile and readily cleaved [43], alternative and newer approaches, such as BEMAD (β-elimination followed by Michael addition with dithiothreitol), ETD (electron transfer dissociation) and ECD (electron capture dissociation), have been used [8,44–46]. In addition, oxogalactose–biotin tagging or azidogalactose–biotin tagging have been combined with BEMAD to identify specific glycosylation sites [16,43,47]. Furthermore, several chemical approaches to monitor changes in O-GlcNAcylation levels in response to cellular stimuli have been developed lately. A FRET (fluorescence resonance energy transfer)-based sensor has been recently developed for the detection of O-GlcNAc dynamics in living cells [48]. This approach provides an alternative to examine changes in the intracellular signalling pathways and the dynamics of O-GlcNAcylation on specific protein substrates and will help to clarify the cross-talk between O-GlcNAc and other PTMs. For further information on the complex interplay between O-GlcNAcylation and phosphorylation, please refer to the following comprehensive and excellent reviews [17,36,49,50].

O-GlcNAcYLATION AND THE CARDIOVASCULAR SYSTEM

A series of studies have consistently shown that activation of the pathways leading to O-GlcNAc formation improves cardiac cell survival and function after acute myocardial stress conditions [I/R (ischaemia/reperfusion) injury and trauma-haemorrhage], whereas inhibition of O-GlcNAc formation decreases myocardial cell survival [14,24,51–53]. This is in agreement with the suggestion that stress leads to a transient increase in O-GlcNAc levels that is associated with increased tolerance to stress [11,25,54]. The mechanisms associated with the beneficial or protective effects of O-GlcNAc on cardiac function will be discussed below and are also summarized in Figure 3.

On the positive or beneficial effects of O-GlcNAc

Administration of glucosamine, as well as an OGA inhibitor (PUGNAc, which increases O-GlcNAc levels), to a rat model of trauma-haemorrhage increased tissue levels of O-GlcNAc on proteins, improved cardiac function, and reduced circulating levels of inflammatory cytokines and activation of NF-κB (nuclear factor κB) [53]. In primary cultured cardiomyocytes, both glucosamine and overexpression of OGT attenuated LPS (lipopolysaccharide)-induced expression of inflammatory markers and activation of NF-κB [23], suggesting that the beneficial actions of O-GlcNAc involve anti-inflammatory effects.

O-GlcNAcylation also confers cytoprotection in the heart via attenuating the formation of the mPTP (mitochondrial permeability transition pore) and the subsequent loss of mitochondrial membrane potential. O-GlcNAcylation-induced attenuation of mPTP formation is associated with decreased Ca^{2+} overload and ROS (reactive oxygen species) generation [55,56].

Protective effects of O-GlcNAc have also been reported in other I/R models. Accordingly, coronary ligation to induce heart failure in mice increases cardiac OGT expression and levels of O-GlcNAc

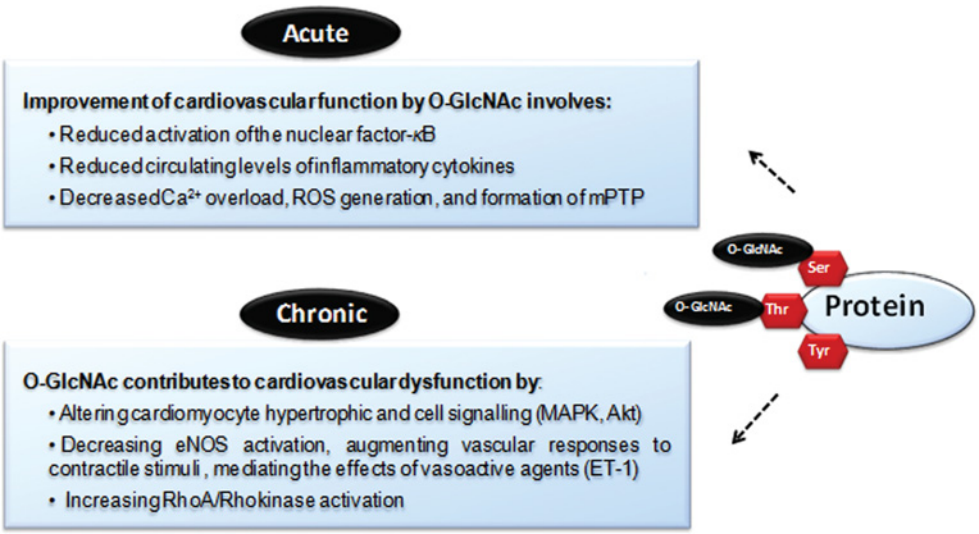

Figure 3 O-GlcNAc formation in the cardiovascular system produces both protective and harmful effects

The effects of O-GlcNAcylation in the cardiovascular system may depend on whether they are acute or chronic effects on the initial cellular metabolic/energetic/redox status of the cell and on the specific proteins that are O-GlcNAc-modified in each cell type/tissue/organ.

proteins in the surviving remote myocardium. Genetic deletion of cardiomyocytes OGT reduced cardiac O-GlcNAcylation of proteins and significantly exacerbated cardiac dysfunction [57]. Intraperitoneal glucosamine administration to rats that underwent middle cerebral artery occlusion reduced infarct volume, motor impairment and neurological deficits, and suppressed post-ischaemic microglial activation and LPS-induced up-regulation of pro-inflammatory mediators. The anti-inflammatory effects of glucosamine were mainly attributable to its ability to inhibit nuclear NF-κB activation [58]. Interestingly, glucosamine prevented LPS-induced p65 O-GlcNAcylation [58], which is somewhat puzzling since PTM of the NF-κB p65 subunit by O-GlcNAc (Thr^{322} and Thr^{352}) has been reported to decrease binding to IκBα (inhibitory κBα) and increase transcriptional NF-κB activity [59].

Acute administration of glucosamine or the OGA inhibitor PUGNAc also inhibited acute inflammatory and neointimal responses in a model of endoluminal arterial injury. O-GlcNAc-modified protein levels decreased markedly in injured arteries, but glucosamine and PUGNAc prevented the decrease in O-GlcNAc levels, as well as the expression of pro-inflammatory mediators and neutrophil/monocytes infiltration, and reduced neointima formation in injured arteries, indicating that O-GlcNAcylation in the vasculature may have anti-inflammatory and vasoprotective effects [60]. Accordingly, in endothelial cells, glucosamine decreased the expression of inflammatory markers and abrogated the phosphorylation of p38 MAPK and NF-κB [61,62], but, paradoxically, glucosamine was shown to have pro-oxidative properties [61].

The reports described above highlight that cardiac protein O-GlcNAcylation may serve as an autoprotective alarm or stress response. Although O-GlcNAcylation improves cardiac cell survival during acute stress (e.g. hypoxia, ischaemia and oxidative stress), limiting O-GlcNAcylation exacerbates cardiac cell damage (Figure 3). It should be noted, however, that the acute anti-inflammatory effects of elevated O-GlcNAc in the heart cannot be generalized to every other cell. For example, O-GlcNAc is crucial for T- and B-cell activation. O-GlcNAc modification, which increases shortly after activation of lymphoid cells, seems to be required for nuclear translocation of transcription factors. OGT is also required for the early activation of B-cells mediated by stimulation of the B-cell receptor. Both NF-κB and NFAT (nuclear factor of activated T-cells) are glycosylated with O-GlcNAc after direct binding to OGT [63,64].

Although acute increases in O-GlcNAc appear to be protective, O-GlcNAcylation seems to contribute to cardiac and vascular dysfunction in conditions of chronic stress, as will be discussed next.

On the negative or harmful effects of O-GlcNAc

Increased glucose metabolism via the HBP and associated increase in O-GlcNAc levels on proteins have been shown to contribute to the adverse effects of diabetes on the heart [65] (Figure 3). In diabetes models, O-GlcNAcylation leads to impaired cardiomyocyte hypertrophic and cell signalling pathways [65], and is associated with defects in cardiomyocyte mechanical

function (impaired relaxation) [66]. Negative effects of O-GlcNAcylation have also been reported in the vasculature [67–69].

In the vascular system, OGA has been shown to be expressed in both endothelial cells and smooth muscle cells of rat aortas [70]. OGT and GFAT (glutamine:fructose-6-phosphate amidotransferase) have also been identified in vascular cells and changes in the vascular expression of these enzymes have been reported in hyperglycaemic/diabetic, aging and hypertensive conditions [69–71]. Subsequent studies have demonstrated that diabetes, hyperglycaemia and arterial hypertension result in lower endothelial eNOS (endothelial NO synthase) protein activation, but increased O-GlcNAc modification (and possibly inactivation) of eNOS [67–69].

Our group has shown that increased levels of O-GlcNAcylation augments vascular responses to contractile stimuli [72] and that O-GlcNAc-modified proteins are increased in the vasculature of DOCA (deoxycorticosterone acetate) salt hypertensive rats [69]. In addition, ET-1 (endothelin-1), which plays a major role in vascular dysfunction associated with salt-sensitive forms of hypertension, directly activates O-GlcNAcylation of vascular proteins and this PTM mediates important vascular effects of the peptide [73,74]. O-GlcNAcylation seems to contribute to the vascular effects of ET-1 via activation of the RhoA/Rho-kinase pathway. Of importance, augmented contractile responses induced by increased O-GlcNAc levels in response to PUGNAc (an OGA inhibitor) are abolished by Rho-kinase inhibition (Y-27632) [15,74]. Glucosamine, which induces O-GlcNAcylation of proteins, also increases vessel contraction via augmentation of RhoA activity [75]. These studies indicate that O-GlcNAcylation negatively interferes with vascular function, mainly vascular reactivity, and that O-GlcNAc may play a direct role in mediating vascular dysfunction associated with arterial hypertension and diabetes.

Some confusion/surprise may arise when one initially looks at the O-GlcNAcylation literature on the cardiovascular system. It seems that O-GlcNAc is protective in the heart and harmful in the vasculature. However, there are many avenues that need to be considered before one can classify O-GlcNAcylation as 'good/protective' or 'bad/harmful'. First, acute compared with chronic effects of O-GlcNAcylation must be considered (Figure 3). It is possible that acute increases in cardiovascular O-GlcNAc-modified proteins may counteract stressor stimuli and have protective actions, whereas chronic cardiovascular O-GlcNAcylation may contribute to further cardiac/vascular dysfunction via modulation of other signalling pathways. Secondly, the initial cellular conditions when components of the O-GlcNAc system are manipulated (OGA/OGT/GFAT overexpression, deletion and pharmacological inhibition etc). Removing or adding O-GlcNAc to cells may produce opposite/differential effects depending on the initial metabolic/energetic/redox status of the cell. Thirdly, manipulations that globally increase O-GlcNAcylation may (and will) produce differential effects based on the specific proteins that are O-GlcNAc-modified in each cell type/tissue/organ.

It should be noted that it is not only in the cardiovascular system that, O-GlcNAcylation has been shown to have 'double dagger, Yin–Yang, paradoxical, friend–foe or positive–negative' actions. Quoting Marsh and Chatham [76]: "Our knowledge of the role(s) of O-GlcNAc on the regulation of the cardiovascular system is in its infancy". Consequently, much needs to be done/answered before we have a clear picture of the 'cardiovascular O-GlcNAcylation world'. Therefore future investigations are necessary in order to characterize the cardiovascular effects of O-GlcNAcylation and specific cardiac and vascular O-GlcNAcylated proteins (and to identify specific modification sites on these proteins), as well as to identify the mechanisms that regulate OGT and OGA activity in cardiac and vascular cells.

ROS AND O-GlcNAcYLATION

In this section, we will address whether signalling molecules involved in redox signalling can be modified by O-GlcNAc and the possible implications the interplay between O-GlcNAcylation and ROS generation may have. As will become apparent, experimental evidence indicates that the interactions between O-GlcNAcylation and oxidation of proteins are important not only for cell regulation in physiological conditions, but also under pathological states where the interplay may become dysfunctional and thereby exacerbate cellular injury.

Increased levels of ROS, defined as a disruption of redox signalling and termed oxidative stress, has been consistently reported in various chronic diseases and conditions, including those where O-GlcNAcylation has been implicated as a mechanistic contributor for the associated organ injury/protection (diabetes, arterial hypertension, Alzheimer's disease, aging and ischaemia) [77–80].

ROS participate in many intracellular signalling pathways leading to changes in gene transcription and protein synthesis, and consequently in cell function. In the cardiovascular system, ROS are produced in all cell types, including cardiac myocytes, endothelial cells, smooth muscle cells and cells in the adventitia, and are tightly regulated by antioxidants, including SOD (superoxide dismutase), catalase, thioredoxin, glutathione, vitamins and other small molecules [81,82]. However, under pathological conditions, ROS production is increased, leading to endothelial dysfunction, increased

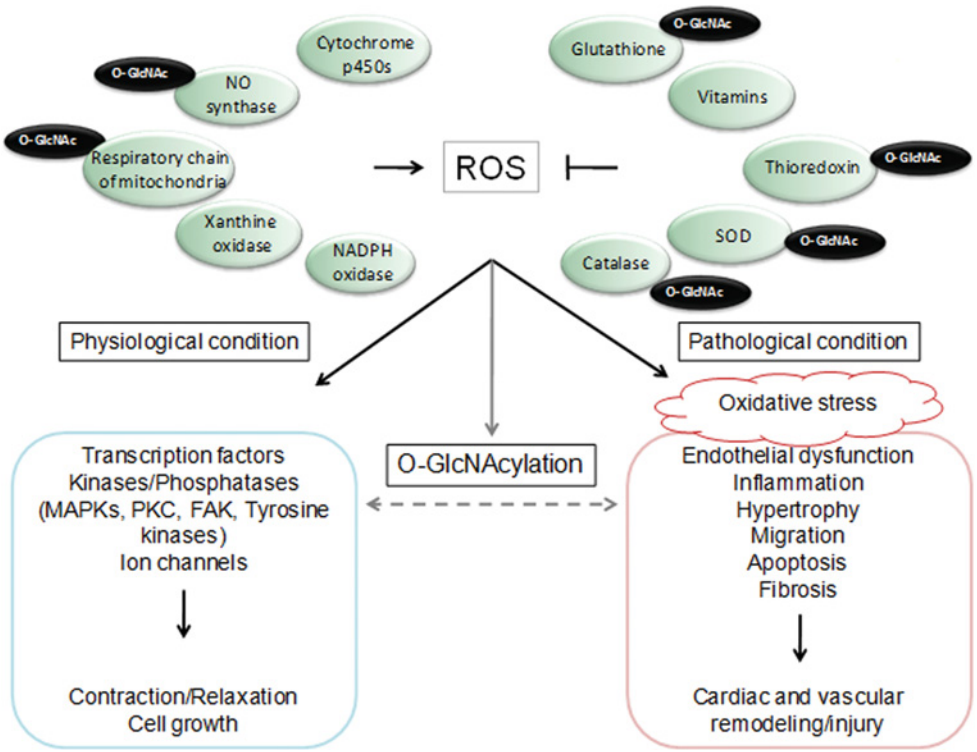

Figure 4 Interplay between ROS, phosphorylation and O-GlcNAcylation

Evidence indicates that ROS stimulates not only phosphorylation, but also O-GlcNAcylation of proteins. On the other hand, augmented O-GlcNAc induces ROS production and/or up-regulation of stress-related proteins. Increased production of ROS has been consistently reported in various chronic diseases and conditions, including those where O-GlcNAcylation has been implicated as a mechanistic contributor or protective agent. The O-GlcNAc symbol indicates proteins that have already been identified as O-GlcNAcylated, according to the dbOGAP. Numbers in parenthesis indicate the dbOGAP ID. Catalase (OG00108–OG00119); glutathione transferase (OG00341, OG00342, OG01049, OG01062 and OG003437); SOD (OG00848 and OG01057); thioredoxin (OG01067, OG010679, OG00648 and OG006487); NO synthase (OG00587, OG005878, OG00589, OG00590, OG005891 and OG005892); NADH dehydrogenase [ubiquinone] 1α subcomplex subunit 9 (OG00557–OG00561). FAK, focal adhesion protein.

contractility, VSMC growth, monocyte migration, lipid peroxidation, inflammation and other processes contributing to cardiovascular damage [77,81,82]. Although oxidative stress may not be the sole aetiology for these dysfunctional events, it amplifies vascular damage in the presence of other vasoactive factors [77,83].

Physiological or chemical stress normally induces signal transduction events that involve the activation and/or production of molecules and proteins that diminish the effects of the deleterious signalling [51]. Protein phosphorylation is typically the mechanism associated with these signal transduction pathways. However, in addition to modulating the activity of specific protein kinases and phosphatases [84–88], ROS also stimulate the HBP and, consequently, O-GlcNAcylation [89]. For instance, increased production of ROS by the mitochondria induces both changes in phosphorylation and O-GlcNAcylation of many proteins, including mitochondrial proteins, which would increase tolerance of mitochondria to stress [14,52].

The interplay between O-GlcNAc regulation of ROS will be discussed, i.e. ROS leading to activation of the HBP and activation of the HBP inducing ROS generation or oxidative stress (Figure 4). Most of the examples of the HBP inducing oxidative stress are associated with hyperglycaemia. This is consistent with the idea that increased ROS production activates the five major pathways involved in the pathogenesis of hyperglycaemic/diabetic complications: polyol pathway flux, increased formation of AGEs (advanced glycation end products), increased expression of RAGE (receptor for AGEs) and its activating ligands, activation of PKC (protein kinase C) isoforms and overactivity of the HBP (or augmented O-GlcNAcylation) [80].

Experimental evidence of ROS leading to the activation of the HBP pathway will be described below. Ngoh et al. [90] have shown that pharmacological induction of endoplasmic reticulum stress in neonatal rat cardiac myocytes augmented O-GlcNAc signalling. In addition, Jones et al. [52] have shown that, when cardiac

myocytes were submitted to different durations of H_2O_2 incubation, O-GlcNAc levels were increased early and gradually decreased after 45 min. Furthermore, oxidative stress associated with hyperglycaemia not only increases HBP flux, but also amplifies inflammation and impairs endothelial function [91]. Of interest, azaserine, a GFAT inhibitor, not only inhibits the HBP pathway, but it is also a strong antioxidant, and prevents inflammation and the impairment of endothelium-dependent relaxations associated with hyperglycaemia [91].

O-GlcNAc depletion in mesangial cells [by shRNA (short hairpin RNA) directed against OGT] prevented high-glucose-induced ROS formation. OGT shRNA also decreased high-glucose-stimulated p38 MAPK, JNK (c-Jun N-terminal kinase) and ASK1 (apoptosis signal-regulating kinase 1) activation, indicating that renal O-GlcNAcylation is coupled to pro-fibrotic p38 MAPK signalling by high glucose, possibly as a consequence of increased ROS [92]. The authors speculated that O-GlcNAc increases the phosphorylation of the NADPH oxidase subunit $p47^{phox}$, through p38 MAPK activation, and up-regulates the expression of the NADPH oxidase subunit Nox4 [92]. Reinforcing this view, a p38 MAPK inhibitor, SB203580, has been shown to block high-glucose-induced increases in ROS in mesangial cells [93].

ROS-induced O-GlcNAcylation has also been reported in cultured 3T3-L1 adipocytes. In these cells, urea induces ROS production, which increases modification of insulin signalling molecules by O-GlcNAc and reduces insulin-stimulated IRS (insulin receptor substrate), Akt phosphorylation and glucose transport. Similarly, uraemic mice have increased ROS production, modification of insulin signalling molecules by O-GlcNAc and become insulin-resistant and glucose-intolerant. Treatment with the SOD/catalase mimetic {MnTBAP [Mn(III) tetrakis (4-benzoic acid) porphyrin]} normalizes O-GlcNAc modification of insulin signalling molecules in visceral fat and corrects systemic insulin resistance and glucose intolerance [94].

In the opposite direction, activation of the HBP has been shown to induce ROS generation and oxidative stress. Accordingly, Singh et al. [95] have demonstrated that both high glucose and glucosamine induce mesangial cell ROS generation, cell-cycle arrest and apoptosis, and some of these effects are mediated by its metabolism via the HBP. In addition, in a microarray study using mouse oligochip arrays, glucosamine mimicked several gene expression patterns of high glucose and induced genes involved in oxidative stress, including the thioredoxin-interacting protein, an inhibitor of thioredoxin and antioxidative stress mechanisms. Glucosamine also down-regulated the expression of oxidative stress resistance genes, suggesting a possible mechanism for high glucose and glucosamine-induced oxidative stress in renal mesangial cells [96]. Furthermore, hyperglycaemia induces activation of GPX1 (glutathione peroxidase 1), an antioxidant enzyme essential for cell survival during oxidative stress. Activation of GPX1 is mediated by O-GlcNAcylation of this enzyme [97].

The regulation of FoxO1 (forkhead box O1) by O-GlcNAc is another example of the interlacing of O-GlcNAc modification with ROS. The transcription factor FoxO1 [FKHR (forkhead in rhabdosarcoma)], which regulates apoptosis, cell cycle, metabolism and oxidative stress, has recently been shown to be O-GlcNAcylated [14,98]. O-GlcNAc modification of FoxO1 leads to its activation [99], which in turn induces the transcription of oxidative stress response enzymes, such as catalase and Mn-SOD (manganese SOD). Furthermore, hyperglycaemia-induced increases in HBP flux and the subsequent increases in O-GlcNAcylation of the transcription factor Sp1 (specificity protein 1) in cultured endothelial cells have been linked to augmented mitochondrial superoxide production [100].

A recent report has shown that AGEs/RAGE activation increases I/R-induced endothelial injury by increasing oxidative/nitrative injury and subsequent nitrative inactivation of thioredoxin [101]. Interestingly, AGEs, or more specifically, the AGE carbon precursor methylglyoxal, have been shown to increase ROS formation, apoptosis, MAPK activation and nuclear O-GlcNAcylation in fetal human cardiac myocytes [102].

Other players may contribute to the link between O-GlcNAc modification and oxidative stress. A strong candidate is ET-1, which has been shown to induce oxidative stress [103,104] and to increase O-GlcNAcylation levels [73,74]. As discussed above, ET-1 augments vascular O-GlcNAcylation and this PTM contributes to the vascular changes produced by the peptide [15,73]. In addition, Callera et al. [103] have shown that ET-1 mediates oxidative stress in DOCA-salt hypertension. Interestingly, O-GlcNAc levels are increased in aortas from DOCA-salt hypertensive rats and treatment of these rats with an ET_A receptor antagonist prevented augmented O-GlcNAc levels [73], as well as decreasing vascular superoxide generation [105]. Whether ET-1-induced ROS generation contributes to the activation of the O-GlcNAc pathway or ET-1-induced O-GlcNAcylation leads to oxidative stress has not yet been investigated.

It is clear that there is a critical interplay between O-GlcNAcylation and ROS generation (and vice versa) in the cardiovascular system, and that this interplay is important not only for cell regulation in physiological conditions, but also under pathological states where ROS and O-GlcNAc may become dysfunctional and thereby exacerbate cellular injury. Figure 4 illustrates the interactions between ROS and O-GlcNAcylation, and also depicts pro-oxidative and antioxidant proteins that are regulated by O-GlcNAcylation (proteins that are either directly O-GlcNAcylated or whose

expression/activity is altered by changes in O-GlcNAc levels).

CONCLUSIONS

Our understanding of the O-GlcNAcylation process (enzymatic regulation, cellular targets and sites for O-GlcNAc addition, and modulation by other pathways), as well as of its functional importance and its contribution to (dys)regulation of the cardiovascular system under different circumstances (e.g. diabetes, ischaemia, trauma-haemorrhage, aging, arterial hypertension and the metabolic syndrome), is rapidly increasing.

It is also evident that the direct interactions between O-GlcNAcylation and phosphorylation and the fact that both PTMs can interfere with redox signalling (and vice versa) adds great complexity to our knowledge of the role(s) of O-GlcNAc on the regulation of the cardiovascular system.

Thus future investigations into protein O-GlcNAcylation and cardiovascular signalling should focus on the characterization of specific O-GlcNAcylated proteins, including the identification of the O-GlcNAc-modified sites. Studies addressing and identifying the factors involved in regulating OGT and OGA activity, such as redox signalling molecules and enzymes, are also needed. This will provide a greater understanding as to how O-GlcNAc exerts both positive and negative effects in the cardiovascular system and potentially provides an avenue for targeted interventions and therapies.

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