This Review is the last in a thematic series on **Novel Posttranslational Modifications of Proteins and Their Cardiovascular Significance**, which includes the following articles:

- The Emerging Characterization of Lysine Residue Deacetylation on The Modulation of Mitochondrial Function and Cardiovascular Biology [2009;105:830–841]
- Protein Acetylation in the Cardiorenal Axis: The Promise of Histone Deacetylase Inhibitors [2010;106:272–284]
- Protein S-Nitrosylation and Cardioprotection [2010;106:285–296]
- Sent to Destroy: The Ubiquitin Proteasome System Regulates Cell Signaling and Protein Quality Control in Cardiovascular Development and Disease [2010;106:463–478]
- S-Nitrosylation in Cardiovascular Signaling [2010;106:633–646]
- Sumoylation and Regulation of Cardiac Gene Expression [2010;107:19–29]

## O-GlcNAc Signaling in the Cardiovascular System

*Elizabeth Murphy, Guest Editor*

### O-GlcNAc Signaling in the Cardiovascular System

Gladys A. Ngoh,* Heberty T. Facundo,† Ayesha Zafir, Steven P. Jones

**Abstract:** Cardiovascular function is regulated at multiple levels. Some of the most important aspects of such regulation involve alterations in an ever-growing list of posttranslational modifications. One such modification orchestrates input from numerous metabolic cues to modify proteins and alter their localization and/or function. Known as the β-O-linkage of N-acetylglucosamine (ie, O-GlcNAc) to cellular proteins, this unique monosaccharide is involved in a diverse array of physiological and pathological functions. This review introduces readers to the general concepts related to O-GlcNAc, the regulation of this modification, and its role in primary pathophysiology. Much of the existing literature regarding the role of O-GlcNAcylation in disease addresses the protracted elevations in O-GlcNAcylation observed during diabetes. In this review, we focus on the emerging evidence of its involvement in the cardiovascular system. In particular, we highlight evidence of protein O-GlcNAcylation as an autoprotective alarm or stress response. We discuss recent literature supporting the idea that promoting O-GlcNAcylation improves cell survival during acute stress (eg, hypoxia, ischemia, oxidative stress), whereas limiting O-GlcNAcylation exacerbates cell damage in similar models. In addition to addressing the potential mechanisms of O-GlcNAc–mediated cardioprotection, we discuss technical issues related to studying protein O-GlcNAcylation in biological systems. The reader should gain an understanding of what protein O-GlcNAcylation is and that its roles in the acute and chronic disease settings appear distinct. (Circ Res. 2010;107:171-185.)

**Key Words:** myocardial ischemia ■ glucose ■ diabetes mellitus ■ mitochondria

Much has been written about glycolysis, β-oxidation, and the other major metabolic pathways in cells. Yet, there are several underinvestigated accessory glycolytic pathways whose importance in the cardiovascular system is now beginning to be appreciated. Eukaryotic glycosylation represents a highly varied and complex collection of biological pathways, which are too broad for serious discussion here. This review focuses on one unique form of glycosylation, and the reader should refer to definitive sources¹ for information on other forms of glycosylation. The hexosamine biosynthetic pathway (HBP) exemplifies one such accessory pathway for glucose metabolism. Based on evidence from cell lines,² the HBP consumes a small fraction of glucose and involves a series of enzyme-catalyzed reactions ending with the formation...
UDP-GlcNAc provides the glycoside precursor for glycoprotein synthesis, especially in cardiovascular disease. UDP-GlcNAc is synthesized from uridine diphosphate-N-acetylg glucosamine (UDP-GlcNAc). This pathway begins with the rate-limiting enzyme, glucosamine-6-phosphate acetyltransferase (GFAT) using glutamine. The next critical reaction involves the conversion of glucosamine-6-phosphate to N-acetylglucosamine-6-phosphate by the Emeg32 (glucosamine-6-phosphate acetyltransferase) and GFAT2 (glucosamine-6-phosphate acetyltransferase) using acetyl-CoA, a ubiquitous metabolic intermediate, links HBP to amino acid metabolism. Acetyl-CoA, a ubiquitous metabolic intermediate, links HBP to lipolysis/lipogenesis, glucose oxidation, and amino acid catabolism. Finally, the HBP requires ATP at the final step, ie, the conversion of N-acetylglucosamine-1-phosphate to UDP-GlcNAc by UDP-GlcNAc pyrophosphorylase. Because the precursors of UDP-GlcNAc are nutrient derived and potentially from other metabolic pathways, UDP-GlcNAc, and hence the O-GlcNAc posttranslational modification, might act as a nutrient/metabolic sensor.

Little is known about the regulation of HBP flux in the heart. Eukaryotic GFAT is highly conserved and regulated transcriptionally and posttranslationally by cAMP-dependent protein kinase and by UDP-GlcNAc feedback inhibition. GFAT exists in 2 isoforms, GFAT1 (highly expressed in the heart and CNS) and GFAT2 (highly expressed in the heart and CNS). Emeg32 (glucosamine-6-phosphate acetyltransferase) is critical for maintaining the proper intracellular concentration of UDP-GlcNAc, thus, it may indirectly regulate O-GlcNAcylation. Little is known about the regulation of HBP flux in the heart.

N-Glycosylation Versus O-GlcNAcylation Versus Phosphorylation

O-GlcNAc is a posttranslational modification of nuclear, cytoplasmic, and mitochondrial proteins.

Figure 1. Hexosamine biosynthetic pathway. Phosphorylated glucose enters either the glycogen synthetic pathway or is further converted to fructose-6-phosphate by glucose-6-phosphate isomerase. The majority of fructose-6-phosphate is channeled to glycolysis. Less than 5% of glucose uptake is ultimately channeled to a unique accessory pathway for glucose metabolism, the HBP. This pathway begins with the rate-limiting enzyme, GFAT, followed by acetylation of glucosamine-6-phosphate by Emeg32 (glucosamine-6-phosphate acetyltransferase) and UDP-GlcNAC-6-P (N-acetylglucosamine-6-phosphate). Next are 2 reversible reactions: the conversion of GlcNAC-6-P to GlcNAC-1-P by phosphatase-acetylglucosamine mutase and then the formation UDP-GlcNAC by UDP-GlcNAC pyrophosphorylase. This high-energy molecule serves as the monosaccharide donor for the posttranslational modification by O-GlcNAc transferase (OGT). O-GlcNAcase (OGA) removes O-GlcNAc modification from proteins.
Torres and Hart. In their study, they attempted to probe for glycans on surface proteins of lymphocytes using β-d-1 to 4-galactosylaminyl transferase. Most of the proteins labeled were intracellular proteins and the labels were incorporated on single GlcNAc residues rather than complex polysaccharide associated with cell surface proteins. O-GlcNAcylation is in many ways distinct from “classic” protein glycosylation. First, O-GlcNAc modified proteins are found mostly within the nucleus, cytoplasm, or mitochondria contrary to N-glycosylation, which predominates in cell surfaces, the lumen of membranous organelles, the endoplasmic reticulum, and Golgi apparatus. Second, O-GlcNAc is not elongated into complex structures or further modified with the exception of a nuclear pore protein, unlike the extraordinarily complex array of glycans found on extracellular glycoproteins. Third, O-GlcNAc rapidly cycles on and off proteins on a time scale similar to that of phosphorylation/dephosphorylation but unlike extracellular complex glycans, which are essentially static. Fourth, there is yet no obvious consensus sequence for the addition of O-GlcNAc to proteins, whereas N-glycosylation has Asn-X-Ser/Thr sequence (where X could be any amino acid other than proline or aspartic acid). Fifth, GlcNAc is added to proteins through an O-linkage on the hydroxyl group of Ser/Thr, whereas N-glycosylation, the monosaccharides are added through an N-linkage on the amide group of Asn.

O-GlcNAcylation is one of the most common posttranslational modifications and is similar to protein phosphorylation in that: Both O-GlcNAcylation and phosphorylation posttranslational modifications are found on serine and threonine residues, both modifications are dynamically added and removed from proteins in response to cellular signals, both alter the functions and/or associations of the modified protein. O-GlcNAc differs from protein phosphorylation in that only 2 enzymes catalyze the addition and removal of O-GlcNAc from proteins, whereas more than 600 genetically distinct kinases and phosphatases regulate the addition and removal of phosphorylation in mammalian cells. Even though many phosphorylation sites are also known glycosylation sites, the view that O-GlcNAc and phosphorylation exist in a “yin-yang,” or simply reciprocal relationship, likely represents an overly simplistic model.

O-GlcNAc and O-phosphate site-mapping studies suggest that there are several types of dynamic interplay between O-GlcNAc and O-phosphate. There is evidence of competitive occupancy at the same site, for example that which occurs in the transcription factor c-Myc, estrogen receptor-β, gynecoprotein SV-40 large T-antigen, and endothelial nitric oxide synthase; that is, a site is either O-GlcNAc modified, phosphorylated, or unmodified. In alternative occupancy occurring at adjacent sites, such as that observed in the tumor suppressor p53 and synapsin I, glycosylation can inhibit phosphorylation at adjacent sites by steric hindrance or modulation of protein structure. Other highly complex interactions also likely exist and do not fall into either of the former categories. Furthermore, the interplay between O-GlcNAc and O-phosphate is also underscored by the recent finding that OG T (uridine diphospho-N-acetylgalactosamine:polypeptide β-N-acetylgalactosaminyltransferase) transiently forms complexes containing the catalytic subunit of protein phosphatase 1c (PP1c), hence in some contexts there may exist a single enzyme complex for the addition of GlcNAc and removal of phosphate.

Enzymatic Regulation of O-GlcNAcylation
Modulation of protein O-GlcNAcylation is achieved by the concerted action of 2 highly evolutionarily conserved enzymes, a uridine diphospho-N-acetylgalactosamine: peptide β-N-acetylgalactosaminyl transferase (O-GlcNAc transferase; aka OGT) and β-β-N-acetylgalactosaminidase (O-GlcNAcase; also known as OGA, GCA, or mgea5). O-GlcNAc transferase (OGT) is a soluble, ubiquitously expressed, and highly conserved enzyme in all multi-cellular eukaryotic organisms involved with the addition of a single β-N-acetylgalactosamine (GlcNAc) moiety via an O-linkage to serine/threonine amino residues nuclear, cytoplasmic and mitochondrial proteins. OGT is expressed in all tissue types examined and most abundant in the glucose-sensing cells of the pancreas and in the brain. OGT is primarily located in the nucleus and has an optimum pH of ~6.32,33 OGT is encoded by a single copy X-linked gene in mammals, whereas plants have 2 OGT homologs, spy and secret agent.32,33,36,37 Even though OGT is coded by a single gene in mammals, alternative splicing of OGT mRNA leads to 3 isoforms: nucleocytoplasmic OGT (nOGT), mitochondrial OGT (mOGT), and short OGT (sOGT).32,33,38 These isoforms share an identical C-terminal catalytic domain but have distinct N-terminal domains contributing to their differential localization and unique targeting sequences. Structural, OGT contains an N-terminal tetratricopeptide repeat (TPR), a linker region and C-terminal catalytic domains.32,33 TPR domain consists of a 34 amino acid repeat varying from 3 to 12 involved in intersubunit interaction, protein-protein interaction, subcellular targeting, substrate recognition, cell cycle regulation, and transcriptional control.33,41–46 The linker region is the least conserved sequence of OGT. The catalytic domain of OGT is thought to have a UDP-GlcNAc binding site and is involved with the glycosylation of proteins. Posttranslational modification of OGT by tyrosine phosphorylation and O-GlcNAc modification, UDP-GlcNAc concentration, and protein-protein interaction are thought to regulate OGT activity.32,33,48,49

Recently, insulin signaling was shown to regulate OGT.50,51 In neuro-2a neuroblastoma cells, OGT mRNA and protein expression are regulated in an AMP-activated protein kinase-dependent manner, whereas OGT enzymatic activity is regulated in a p38 MAPK-dependent manner. Moreover, activated p38 has been shown to interact with OGT and recruit it to specific substrates, such as neurofibrilament H during glucose deprivation. Tissue specific OGT mutation causes disturbance in somatic cell function, whereas conventional OGT deletion is embryonic-lethal; hence O-GlcNAc is important for cellular viability.

O-GlcNAcase is a soluble, highly conserved enzyme, and expressed in all eukaryotic organisms involved with the removal of O-GlcNAc modification from proteins. O-GlcNAcase is primarily located in the cytoplasm with an optimum pH of 5.5 to 7 and coded for by a single gene. Two splice variants of O-GlcNAcase have been reported in rats both lacking O-GlcNAcase activity but retained HAT activity. The spliced variant detected in Goto–Kakizaki rats (~90 kDa) lacks exon
8, whereas the spliced variant in Sprague–Dawley (≈84 kDa) lacks both exons 8 and 9. Structurally, O-GlcNcase is a 917 amino acid protein with an N-terminal hexosaminidase and a C-terminal histone acetyltransferase domain (HAT).53–55 The N-terminal domain is similar to hyaluronidase and was originally identified as meningioma expressed antigen 5.53,55–57 Although there may be some activity against hyaluron in vitro, the preferred substrate for O-GlcNcase is O-GlcNAc.53,55 The C-terminal HAT domain can acetylate free histones and nucleosomal histone proteins.54 It is of interest to note that caspase 3 can cleave O-GlcNcase into HAT and hexosaminidase domains with no change in the activity of each domain.58 Protein-protein interaction and phosphorylation are also thought to regulate O-GlcNcase activity,53,58 though the data in this regard are limited. Interestingly, Hanover and colleagues59 have described a short form of OGA that seemingly lacks the HAT domain.

Cell Cycle
Cellular growth, division, and maturation are ordered processes and are tightly controlled by a number of different extracellular events and genetic programs. To this end, Slawson et al showed convincing evidence that O-glycosylation was modulated during the cell cycle, being lowest at M phase and highest at G1/S and G2/M.60 Most recently, it was demonstrated that OGT and O-GlcNcase interact transiently with the mitotic kinase Aurora B and the protein phosphatase 1.61 The strongest support for a functional link between O-GlcNcase and cell cycle is the observation that several proteins are modified by O-GlcNcase in a cell cycle dependent manner, including c-Myc,62 keratins,20 YY1,63 and vimentin.61 Also supporting this notion, OGT knockout cells became growth arrested.62 Interestingly, altering extracellular glucosamine levels has been implicated in growth arrest in some cancer cells,64 further strengthening the observation that O-GlcNac cycling is important for regulation of the cell cycle.

Transcriptional Regulation
Numerous proteins are responsible for the correct control and maintenance of transcription in the eukaryotic nucleus. Chromatin remodeling (stimulated by the activity of histone acetyltransferase) in response to stimuli, permits transcriptional machinery to initiate mRNA synthesis. Posttranslational modification of key proteins has distinct roles in controlling this process. Teleologically, it should not be surprising that O-GlcNac signaling can influence transcription because OGT is known to associate with histone deacetylase complexes.44 O-GlcNcase also contains a domain with reported histone acetyltransferases (HAT) activity in amino acids 583 to 917 and shares high homology with HAT in residues 772 to 899.54 Mutation in aspartic acid and phenylalanine residues ablates activity and correlates with evidence suggesting that O-GlcNAcase is capable of acetylating either nucleosomal histone proteins or free core histones.54

Experimental evidence supports O-GlcNacylation as an important posttranslational modification directly regulating transcription. In fact, several transcription factors have been identified to be regulated by O-GlcNac modification.65–68 O-GlcNacylation can either suppress or enhance transcription, depending on the promoter involved and other associated coactivator/repressor proteins. For example, OGT can mediate transcriptional repression after being recruited to promoter regions by association with the transcriptional corepressor mSin3A.69 Others have shown that the transcription factor STAT5A alters gene activation by preferentially binding to the coactivator of transcription, CREB-binding protein, when O-GlcNac–modified.69 O-GlcNac modification of Sp1 has multiple effects on the function of Sp1 as a transcription factor.68 Augmented O-GlcNac modification of Sp1 drives the transcription of plasminogen activator and extracellular matrix proteins, which have an important role in diabetic cardiovascular disease, whereas reduction of Sp1 O-GlcNacylation increased Sp1 proteasomal susceptibility.70 Several posttranslational modifications are necessary to control its activity, among these are 8 O-GlcNac modified sites.71 The O-GlcNacylation of Sp1 seems to have an exquisite logic regarding the modification site of the protein. O-GlcNacylation of the DNA-binding domain of the C terminus augments its activity.72,73 Similarly, inhibition of O-GlcNcase increased Sp1 activity, whereas overexpression of O-GlcNcase, RNAi against OGT, or a dominant negative form of OGT reduced the activity of Sp1.74 Conversely, if the O-GlcNac modification occurs in sites located in the N terminus, the result is an inhibition of its transactivation potential,72 inhibiting protein-protein interaction. The 11 TPR repeats of OGT (AA1–485) are the residues essential for Sp1 transcriptional repression and protein-protein interaction between OGT and mSin3A repressor.64 Interestingly, insulin elevates nuclear O-GlcNacylation of Sp1.75

The ability of OGT to promote O-GlcNac modification on RNA polymerase II on the same residues as phosphorylation,66 provides a clue regarding how the O-GlcNac modification can influence cellular transcriptional status. More specifically, O-GlcNacylation of RNA polymerase II occurs in the C terminus of the enzyme, which induces a conformational change, blocks phosphorylation on these residues, and potentially regulates gene expression.66 The O-GlcNacylation of RNA polymerase II is facilitated by a recruitment of OGT to transcriptional complexes by the OGT interacting protein OIP-106.76 Conversely, in vitro experiments have revealed that a single phosphate residue on the C terminus of RNA polymerase II blocks the activity of OGT on the enzyme.70

Transcriptional repression by OGT does not apply for all transcription factors. For example, the O-GlcNac modification on FOXO1 promotes promoter activation of some gluconeogenic genes.77,78 Similarly, O-GlcNacylation of the transcriptional coactivator CRTC2 induces nuclear translocation and also contributes for gluconeogenic gene transcription.79 CRTC2 is a coactivator for the cAMP response element binding protein (CREB) that has been reported to be O-GlcNac modified, producing transcriptional repression in vitro.67 Another interesting example is the transcription factor YY-1, which is incapable of binding to DNA when in complex with the retinoblastoma protein (pRb). O-GlcNacylation of YY-1 relieves the DNA binding inhibition by releasing the protein from a complex with the pRb protein and, consequently, activates transcription.63 As in many complex systems, questions still remain unanswered. For example, how does O-GlcNac accomplish the task of
activating some transcription factors and inhibiting others? What has to be determined is how O-GlcNAc “selects” which genes must be turned off or on.

Involvement in Diabetes and Insulin Signaling
Diabetes remains a primary risk factor for the development of heart disease. Numerous reports have implicated alterations in O-GlcNAc signaling in diabetic pathophysiology. From simple approaches like exposing cells to glucosamine or hyperglycemia, or, in a more advanced system such as using diet-induced or genetic animal models, data support the notion that O-GlcNAc may contribute to diabetes.80,81 Most recently, studies have described potential mechanisms relating high glucose78,79 and the enzyme OGT51 to insulin resistance (a hallmark of type II diabetes). Montminy’s group found that the transducer of regulated cAMP response element–binding protein 2 (TORK2 or CRTC2) is a substrate for OGT and is O-GlcNAcylated at Ser70 and Ser171, which are known phosphorylation sites.79 Phosphorylation of CRTC2 prevents its nuclear translocation via interaction with the chaperone protein 14:3:3.82 The O-glycosylation of CRTC2 impairs its phosphorylation and releases it from the complex with 14:3:3 protein79 (Figure 2). In addition to this effect, the O-GlcNAc modification of CRTC2 promotes activation of a conserved cyclic adenosine 3'-5' monophosphate (cAMP) response element (CRE) on the glucose-6-phosphatase (G6Pase) promoter,79 which is required for G6Pase transcription83 in response to glucose. So, it appears that a balance between O-GlcNAc and phosphorylation must exist to avoid disturbances in insulin signaling.

CRTC2 is not the only O-GlcNAc modified protein that augments expression of gluconeogenic genes after exposing cells to glucose. Housley and colleagues demonstrated that the transcription factor FOXO1 is also O-GlcNAc modified in diabetes, resulting in increased expression of gluconeogenic genes PEPCK and G6Pase. These authors also found that O-GlcNAc elevated expression of several antioxidant genes in association with elevated glucose production by hepatocytes.77,78

Recently, an innovative hypothesis regarding insulin signaling has emerged (see Figure 2). Yang et al, showed that insulin induces nuclear to cytoplasmic translocation of OGT, where OGT binds the lipid phosphatidylinositol-3,4,5-trisphosphate at the plasma membrane.51 This interaction is not constitutively active, but may underlie an important mechanism in insulin signaling. Such dynamic trafficking of OGT results in altered phosphorylation of key insulin signaling molecules and in attenuation of insulin signaling. Based on the fact that OGT shares some limited, regional homology with protein phosphatase 5, which exhibits affinity for lipids, they hypothesized the interaction of OGT with lipids. Moreover, the authors identified the regulatory domain, necessary to bind lipids, in the C terminus of OGT as being rich in lysine residues (K981, K982, K986, K989). The N terminus of OGT may contribute by making OGT specific to lipid classes. It was determined that OGT is recruited to the plasma membrane by nutrient excess, for example, impairs insulin signaling.51 Moreover, augmenting O-GlcNAc levels via OGT overexpression, or inhibiting O-GlcNAcase (via PUGNAc) also recapitulates the phenomenon of insulin resistance.51,79,84–86

Others have specifically investigated the potential contribution of O-GlcNAcylation because of the pathogenesis of diabetes/hyperglycemia. In the intact animal, Hu et al.87 were first able to link cardiac dysfunction in streptozotocin-induced diabetic mice to excessive O-GlcNAcylation. Work from McClain et al,81 showed that overexpressing OGT induces insulin resistance in myocytes. OGT upregulation has also been reported in other diabetic mouse models.81 Unfortunately, limited clinical insights exist regarding O-GlcNAc signaling. However, insights from some small studies relate cases of human diabetes to impairments in O-GlcNAc signaling. Namely, one study examining type II diabetes in Mexican-Americans found reduced O-GlcNAcase expression with the progression of the disease.88 It is then plausible that the O-GlcNAc network rearrangements (with increased OGT levels or decreased O-GlcNAcase, or both) are the major contributors to the development and progression of the disease. More recently, Hart’s laboratory has demonstrated
In a bid to characterize changes in the O-GlcNAc profile following short-term induction of oxidative stress, Jones et al showed that O-GlcNAc levels increased early on induction of oxidative stress and decreased by 45 minutes.108 Such decrement in O-GlcNAc levels corresponded to exacerbated mitochondrial dysfunction and cellular injury. How O-GlcNAc levels change during hypoxia–reoxygenation still remains unclear. However, data from Champattanachai et al showed that O-GlcNAc levels rise following 4 hours hypoxia and early reoxygenation,113 which was confirmed in a follow-up study by the same group.112 Whether such findings are consistent for all durations of hypoxia and reoxygenation remains to be seen. Finally, Ngoh et al recently showed that pharmacological induction of ER stress (with tunicamycin or brefeldin A) in NRCMs augmented O-GlcNAc signaling.103

Work in perfused, perfused rat hearts in Chatham’s laboratory revealed that simulated ischemia alone augments O-GlcNAc levels during the reflow phase.105 A follow-up study from the same group also showed that low-flow global ischemia augmented both UDP-HexNAc and O-GlcNAc levels. The increase in O-GlcNAc levels occurred early in the low-flow phase and then declined during reflow.110 Whether this is true for the intact myocardium remains to be tested. The differential response of O-GlcNAc levels to different models of isolated heart ischemia and in vivo myocardial ischemia may provide insights into the regulation of O-GlcNAc signaling in the hypoxic myocardium.

Severe injury such as trauma-hemorrhagic shock induces stress hormones, leading to a hypermetabolic state. An early response to severe injury is systemic hyperglycemia and enhanced peripheral glucose uptake.114 Even though the effects of hyperglycemia in trauma are still debatable, Mizock et al showed that stress-induced hyperglycemia, or the provision of additional glucose, could be beneficial by providing an adequate supply of glucose necessary for energy production in critical organs.114 Considering the protective effect of augmented O-GlcNAc signaling shown by Zachara et al,95 it is plausible that the increase in glucose uptake occurring during trauma-hemorrhage may boost flux through the HBP and consequently augment O-GlcNAc levels. Indeed, recent reports by Chatham’s group showed that trauma-hemorrhage models reduced O-GlcNAc signaling in rats.96–98

Inducing brief nonlethal episodes of ischemia and reperfusion to the heart before an episode of sustained lethal myocardial ischemia has the capacity to dramatically reduce myocardial injury. This phenomenon, termed ischemic preconditioning,115 is a transient, self-defense mechanism present in the heart and many other organs, including the kidney, liver, and brain. The ability of ischemic preconditioning to reduce myocardial infarct size is significant and reproducible, and, serves as the gold standard for studies of cardioprotection.116 Intense investigation of the mechanisms responsible for the protective effects of preconditioning has revealed numerous potential mediators and downstream effectors of preconditioning, but cause and effect relationships have not been fully delineated. However, several groups have successfully demonstrated that ischemic preconditioning enhances glucose uptake.117–119 Because the monosaccharide donor for O-GlcNAcylation of proteins, ie, UDP-GlcNAc, is derived
from an accessory pathway for glucose metabolism (HBP), it is possible that the increase in glucose uptake occurring during preconditioning may boost flux through the HBP and consequently augment O-GlcNAc levels. Indeed, a recent report showed that either early or delayed ischemic preconditioning can augment cardiac O-GlcNAc levels in vivo.109 Although it was not determined whether the changes in O-GlcNAcylation of proteins observed in preconditioning contributes to its protective effects, pharmacological augmentation of O-GlcNAc levels is sufficient to reduce infarct size in vivo.109 It would be interesting to determine whether ischemic preconditioning relies on O-GlcNAc signaling for cardioprotection, and also determine whether preconditioning of other organs like the brain and liver elevates O-GlcNAc signaling. Clearly, O-GlcNAc represents an acute stress signal in the heart. The next question is: Does nutrient/pharmacological/genetic alteration of O-GlcNAc signaling influence cell survival?

Hexosamine Biosynthetic Pathway Flux in Cardiomyocyte Survival

This section of the review focuses on approaches to alter O-GlcNAc signaling via manipulating HBP flux/activity. The rate-limiting step of the HBP requires glutamine, a nonessential amino acid abundant in muscle tissues, to form glucosamine-6-phosphate. Hence, glutamine contributes to the formation of UDP-GlcNAc, thereby driving the O-GlcNAc modification of proteins. In isolated perfused hearts, augmentation of flux through the HBP with glutamine or before hypoxia–reoxygenation increased O-GlcNAc levels reduced cardiac damage and preserved posthypoxic contractile function during the reflow phase.107 Conversely, perfusion of the hypoxic isolated heart with azaserine, blocked the HBP-mediated increase in O-GlcNAc levels, diminished functional recovery, and exacerbated posthypoxic tissue injury.105–107 Though the cardioprotective effect of glutamine had been reported by others before this study,120 the mechanism was not attributed to alterations in flux through HBP or O-GlcNAc signaling. However, results from an in vivo porcine model of myocardial ischemia-reperfusion injury did not demonstrate protection.121

Glucosamine contributes to the formation of UDP-GlcNAc, thereby driving the O-GlcNAc modification of proteins. Using an approach similar to the aforementioned glutamine studies, Chatham’s group perfused isolated rat hearts with glucosamine,105,107,110 which likely enters the HBP downstream of GFAT and depends on phosphorylation by hexokinase. Glucosamine treatment reduced injury resulting from calcium paradox and hypoxia–reoxygenation.105,107,110,111,112 In neonatal cardiac myocytes, Champattanachai et al showed that augmentation of O-GlcNAc levels with high glucose or glucosamine improved posthypoxic cellular viability and attenuated necrosis and apoptosis.113 Conversely, euglycemic cardiomyocytes or hyperglycemic cardiomyocytes treated with azaserine (ie, a GFAT inhibitor) were more sensitive to hypoxic stress.113 Interestingly, in vivo augmentation of O-GlcNAc levels using glucosamine after severe injury such as hemorrhagic shock has been shown to improve cardiac function and peripheral organ perfusion in rats. Taken together, these results provide evidence that the protective effect of glucosamine is associated with augmented O-GlcNAc signaling and can be translated to the in vivo environment. Clearly, approaches to boost hexosamine flux promote cardiomyocyte survival.

OGT in Cardiomyocyte Survival

Additional support for cytoprotection associated with global augmentation of O-GlcNAc levels can be found in studies using genetic and molecular approaches to evaluate the enzymes controlling the presence of O-GlcNAc on proteins. Studies involving pharmacological inhibition of O-GlcNAc transferase (OGT) have been limited, likely because the few inhibitors described have not been well characterized and may exhibit some level of toxicity. The most popular OGT inhibitor, alloxan, is a uracil and UDP-GlcNAc analog and may be an irreversible inhibitor of OGT.123 Nevertheless, use of such compounds has yielded consistent results. In one study, alloxan not only blocked the glucosamine-mediated increase in O-GlcNAc levels but also inhibited angiotensin II-induced increase in intracellular Ca2+ in NRCMs.104 Although results from these studies are cautiously interpreted because of the high concentration of alloxan used, its toxicity, its lack of specificity,124 and because of the recent revelation that alloxan may also inhibit O-GlcNAcase,125 they provide important insight into the necessity of OGT signaling in the context of elevated hexosamine biosynthesis. “Compound 4” and “compound 5,” were recently described to be potent OGT inhibitors.126,127 Ngoh et al showed that both compounds 4 and 5 (referred to as TT04 and TT40 in their article) reduced O-GlcNAc levels and exacerbated posthypoxic cardiomyocyte injury.102 Moreover, inhibition of OGT also exaggerated the posthypoxic collapse of mitochondrial membrane potential.102 Genetic manipulation of OGT has been shown to alter cardiomyocyte survival after hypoxia. Ngoh et al showed that adenoviral-mediated OGT overexpression (AdOGT) significantly elevated OGT protein expression, augmented O-GlcNAc levels, and reduced posthypoxic cardiac myocyte death, whereas OGT knockdown (via short interfering RNA) or knock out (via cre-lox recombination) reduced O-GlcNAc levels and exacerbated posthypoxic cell death.102 In addition, posthypoxic mitochondrial membrane potential was also better preserved in the AdOGT group compared with the posthypoxic control virus (AdGFP). This prosurvival role of OGT was supported by findings from Champattanachai et al showing that OGT overexpression attenuated H2O2-induced loss of mitochondrial membrane potential and hypoxia-induced apoptosis, whereas OGT knockdown (via siRNA) sensitized NRCMs to H2O2-induced loss of mitochondrial membrane potential and hypoxia-induced apoptosis.112 Thus, OGT seems essential in the constitutive, as well as inducible, abilities of the cell to withstand lethal stressors. In other words, OGT promotes cell survival during acute cardiomyocyte stress.

O-GlcNAcase in Cardiomyocyte Survival

Unlike OGT, there are several pharmacological inhibitors of O-GlcNAcase, including streptozotocin,128 PUGNAC,129 1,2-dideoxy-2-methyl-d-glucopyranosyl[2,1-d]-2-thiazoline-
PUGNAc, a GlcNAc analog, is the most widely studied inhibitor of O-GlcNAcase and prevents the binding of O-GlcNAcase to GlcNAc. Thus, PUGNAc prevents the removal of O-GlcNAc leading to a rapid increase in O-GlcNAc levels. Even though PUGNAc lacks the cytotoxic effects of STZ, it potentially inhibits other lysosomal hydrolases and shows limited specificity for O-GlcNAcase more than β-hexosaminidase.

In NRCMs, augmenting O-GlcNAc levels using PUGNAc attenuated posthypoxic oxidative stress-induced injury and inhibited both posthypoxic and H2O2-induced mitochondrial depolarization. Similar findings were observed in isolated perfused hearts where PUGNAc administration early in reperfusion improved cardiac functional recovery, reduced troponin release, and attenuated campl-mediated proteolysis of α-fodrin and Ca2+/calmodulin-dependent protein kinase II compared to untreated control. The cytoprotective effects of PUGNAc seen in NRCMs and isolated perfused hearts, can be replicated in an in vivo setting. Jones et al showed in an in vivo murine model of myocardial ischemia-reperfusion injury that PUGNAc reduced infarct size following acute myocardial ischemia reperfusion.

NAG-thiazoline inhibits O-GlcNAcase and has 1500-fold greater specificity for O-GlcNAcase over β-hexosaminidase than PUGNAc, but has not been as widely studied as PUGNAc and is not widely available. Recently, Champattanachai et al showed that NBuGT can attenuate cardiac myocyte death following hypoxia and oxidative stress. Few studies have addressed the role of O-GlcNAcase inhibition in cellular injury beyond the use of PUGNAc or NBuGT. As with inhibitors used in the OGT work above, concern remains regarding the off-target effects of the putative inhibitors of O-GlcNAcase. Thus, the use of other approaches, such as RNA interference or adenoviruses, could assuage concerns of some of the off-target effects of the aforementioned pharmacological approaches. Indeed, Ngo et al recently showed that siRNA knockdown of O-GlcNAcase augmented O-GlcNAc levels, preserved posthypoxic cardiomyocyte membrane potential, and mitigated cellular injury, whereas overexpression of O-GlcNAc reduced O-GlcNAc levels, sensitized NRCMs to loss of mitochondrial membrane potential, and exacerbated cellular injury. In short, O-GlcNAcase activity antagonizes cell survival during acute cardiomyocyte stress.

Mechanisms of O-GlcNAc Signaling in Cytoprotection

Although the specific mechanisms underlying the cytoprotection associated with O-GlcNAc signaling remain to be determined, several putative mechanisms have been advanced to explain augmented stress tolerance. O-GlcNAc signaling likely involves numerous intracellular targets, which may contribute to varying degrees during myocardial preservation. Progress in the area of target protein identification has been restricted because of the limitations of the tools necessary for the identification of O-GlcNAc targets. Zachara et al showed that increased O-GlcNAc signaling activated transcription of heat shock proteins HSP40 and HSP70. Because the cardioprotective effects of glucosamine observed in isolated perfused hearts occurred early, there may be mechanisms other than de novo protein synthesis contributing to O-GlcNAc-mediated cytoprotection. Recent studies from the Jones laboratory revealed that modulating O-GlcNAc levels may alter O-GlcNAc modification on (at least) the mitochondrial voltage-dependent anion channel (VDAC), which may represent a unique mechanism of cytoprotection. Activation of mPTP formation is a critical step in mitochondrial mediated death pathway, and, although the molecular identity of the mitochondrial permeability transition pore (mPTP) remains debatable, VDAC has been widely recognized as a putative component, or at least a modulator of mPTP. In these studies, treatment of mice with PUGNAc (an O-GlcNAcase inhibitor), increased O-GlcNAc modification of VDAC and produced resistance of isolated, adult cardiac mitochondria to calcium-induced mitochondrial swelling. Conversely, treating mice with compound 4 (a putative OGT inhibitor) reduced O-GlcNAc modification of VDAC and sensitized isolated, adult cardiac mitochondria to mPTP formation. Augmented O-GlcNAc levels via OGT overexpression or O-GlcNAcase inhibition (with PUGNAc or siRNA) preserved posthypoxic mitochondrial membrane potential.

Conversely, treating mice with compound 4 (a putative OGT inhibitor) reduced O-GlcNAc modification of VDAC and sensitized isolated, adult cardiac mitochondria to mPTP formation. Augmented O-GlcNAc levels via OGT overexpression or O-GlcNAcase inhibition (with PUGNAc or siRNA) preserved posthypoxic mitochondrial membrane potential and cytochrome c release in NRCMs.

O-GlcNAc levels affect posthypoxic mitochondrial Ca2+ overload in NRCMs. Overexpression of O-GlcNAcase exacerbated hypoxia-induced Ca2+ overload, whereas inhibition of O-GlcNAcase mitigated hypoxia-induced Ca2+ overload. Several studies have implicated Ca2+ overload as a key contributor to mitochondrial permeability transition leading to ischemia-reperfusion injury and inhibiting the rise in mitochondrial [Ca2+] has been shown to confer cardioprotection following acute myocardial ischemia. Therefore, it is possible that blocking mitochondrial Ca2+ overload may be an upstream action of O-GlcNAc signaling to prevent mPTP formation in addition to direct effects on mPTP components (Figure 3). Finally, augmented O-GlcNAc levels have been shown to increase mitochondrial Bel-2 in NRCMs subjected to hypoxia–reoxygenation. Because Bel-2 is thought to inhibit mPTP formation by interacting with VDAC, it is possible that augmented O-GlcNAc levels would activate Bel-2 translocation to the mitochondria, increasing the interaction of Bel-2 with VDAC and subsequently blocking mPTP formation and the release of death factor from the mitochondria. Thus, there is a potential mechanistic link among a likely target of O-GlcNAc signaling, mitochondrial preservation, and cell viability.

Using glucosamine to boost O-GlcNAc levels, others have shown activation of p38 MAPK and reduce calpain proteolytic activity, reduced ischemic contracture, and attenuated reperfusion induced arrhythmias in isolated perfused hearts. Such effects could be related to alterations in calcium handling and/or heat shock protein activation. Considering the evidence for O-GlcNAc-mediated regulation of the ubiquitin-proteasome system, modulation of UPS activity could represent yet another target in the portfolio of O-GlcNAc-mediated cytoprotection. Indeed, just as the targets of O-GlcNAc modification are
numerous, so too are the potential mechanisms responsible for cytoprotection. Regardless of the mechanisms (and there are likely multiple targets), the salient feature is that acute, global changes in cellular O-GlcNAcylation reflect a pro-adaptive stress response.

**O-GlcNAc Signaling and Vascular Injury/Inflammation**

Arterial hypertension is a multifactorial condition considered a major risk factor for cardiovascular disease. Hypertension is characterized by abnormal vascular reactivity, impaired endothelium-dependent relaxation, and enhanced sensitivity to vasoconstrictors. Several proteins involved in vascular function have been identified to be O-GlcNAc modified, such as eNOS and protein kinase B (PKB/Akt). Even though it is well established that O-GlcNAc is critical for cellular function, very few studies have addressed the vascular effects of O-GlcNAcylation. Recently, Lima et al showed that the aorta and mesentery of deoxycorticosterone acetate-salt hypertensive rats have augmented O-GlcNAc levels compared to control. Such elevated O-GlcNAc signaling was associated with increased reactivity to constrictor stimuli, phenylephrine, and impaired endothelium-dependent vasodilatation to acetylcholine. Whether the change in O-GlcNAc signaling observed with DOCA hypertension is true for all forms of hypertension remains to be tested, though this same group has continued to extend their findings.

Inflammation contributes to the pathogenesis of numerous cardiovascular diseases. Even though our understanding of O-GlcNAc signaling in most of cardiovascular pathophysiology remains limited, several recent studies indicate its potential impact extends beyond the cardiomyocyte. Oparil’s group tested the hypothesis that in vivo arterial injury may be affected by alterations in O-GlcNAc signaling. Using ovariolectomized rats, balloon injury of the carotid artery produced the expected inflammation and vascular pathology in this model. However, treatment with either glucosamine (enhances HBP flux) or PUGNAc (which inhibits O-GlcNAcase and increases O-GlcNAc levels) reduced leukocyte infiltration, inhibited tumor necrosis factor (TNF)-α–stimulated chemokine and adhesion molecule (ICAM-1 and VCAM-1) expression, IκB-α phosphorylation and nuclear factor (NF)-κB activation. These data indicate antiinflammatory effects of augmented O-GlcNAc signaling in their model. Conversely, Tostes’ group has found a potential role for O-GlcNAcylated in the pathophysiology of hypertension, implying a complex interplay in the vasculature.

Severe injury such as trauma-hemorrhagic shock has been shown to alter O-GlcNAc signaling and that enhanced O-GlcNAc signaling improved functional recovery in the heart following hemorrhagic shock. In series of in vivo studies from Chatham’s group, glucosamine administration during resuscitation significantly attenuated hemorrhagic shock induced increase in circulating TNF-α and interleukin (IL)-6 levels, ICAM expression, IκB-α phosphorylation, NF-κB expression, and NF-κB DNA-binding activity in rat heart. The same group also confirmed this finding with an O-GlcNAcase inhibitor (PUGNAc). In addition, intravenous administration of PUGNAc 30 minutes after the onset of resuscitation following trauma-hemorrhage in rats showed attenuated circulating TNF-α and IL-6 levels supporting the protective effect of O-GlcNAc signaling on stress-mediated inflammation.

**Technical Limitations/Suggestions**

Since its discovery more than 2 decades ago, several analytic procedures have been evaluated to identify O-GlcNAc modified proteins. Because of its substoichiometric concentration, labile nature, lack of charge, and small mass, identification of O-GlcNAc modification has been difficult. The initial tool used enzymatic labeling of terminal O-GlcNAc residues with radioactive uridine diphospho-galactose (UDP[3H]Gal) using galactosyltransferase. Because O-linkage of GlcNAc to a protein is resistant to peptide/N-glycosidase F (PNGase F), nonspecifically UDP[3H]Gal tagged N-linked oligosaccharides are cleaved by PNGase F treatment. The main limitation of this technique is that O-GlcNAc is not very accessible to galactosyltransferase, thereby limiting its utility in many instances.

Others have also used succinylated wheat germ agglutinin (sWGA) to identify O-GlcNAc–modified proteins.

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**Figure 3. Mitochondria and O-GlcNAc signaling.** Myocardial ischemia induces mitochondrial Ca\(^{2+}\) overload and ROS generation, with subsequent mitochondrial permeability transition pore formation (mPTP). Formation of mPTP causes loss of mitochondrial trans-inner membrane potential, mitochondrial swelling, rupture of mitochondrial membrane, and cytochrome c release. Augmentation of O-GlcNAc levels before myocardial ischemia attenuates ischemia-induced Ca\(^{2+}\) overload, ROS generation, and subsequent mPTP formation. Augmented O-GlcNAc signaling also mitigates mPTP formation by possibly augmenting O-GlcNAcylation of VDAC and/or Bcl-2 interaction with VDAC. This is an example of one potential mechanism; there are likely several. Moreover, augmented O-GlcNAc levels diminish mPTP-mediated mitochondrial swelling, loss of mitochondrial membrane potential, and cytochrome c release.
sWGA binds to any terminal GlcNAc residue; hence this technique is not particularly selective. The specificity of this technique may be improved using PNGase F to remove N-acetylglucosamine, which competes for binding with the antibody. The blot on the right is the same membrane and primary antibody (CTD110.6), without GlcNAc. In addition, the lysate loaded in lane 6 is the result of a parallel aliquot of lane 5 incubated with O-GlcNAcase (in vitro), showing the loss of immunoreactivity and validating the signal is O-GlcNAc. Such simple measures can confirm the fidelity of the O-GlcNAc signal via Western blots.

The development of monoclonal antibodies that react with O-GlcNAc in the context of protein structure has significantly increased the efficiency of identifying O-GlcNAc–modified proteins. CTD 110.6 antibody was raised against an O-GlcNAc–modified peptide from the large subunit RNA polymerase II, whereas RL2 antibody was raised against O-GlcNAc–modified peptide from nuclear pore proteins. O-GlcNAc immunoblotting shows multiple bands because it is a posttranslational modification of numerous proteins. The blot on the left is the result of a CTD110.6 antibody coincubated with N-acetylglucosamine, which competes for binding with the antibody. The blot on the right is the same membrane and primary antibody (CTD110.6), without GlcNAc. In addition, the lysate loaded in lane 6 is the result of a parallel aliquot of lane 5 incubated with O-GlcNAcase (in vitro), showing the loss of immunoreactivity and validating the signal is O-GlcNAc. Such simple measures can confirm the fidelity of the O-GlcNAc signal via Western blots.

Several chemical approaches have also been developed to analyze O-GlcNAc residues. Initial attempts at O-GlcNAc site mapping were time consuming and complicated by numerous HPLC peptide purifications and manual Edman degradation reactions. Moreover, the low stoichiometry of the O-GlcNAc modification required a higher starting concentration of purified protein. Because of this, a combination of alkaline β-elimination, collision-induced dissociation (CID), and electrospray ionization mass spectrometry have been used by some experts. Even though alkaline β-elimination reduced the CID energy needed to ionize and fragment the peptide for sequencing by reducing the size of the glycopeptide and CID fragment while preserving O-GlcNAc modification, it causes significant peptide degradation. Another more recent approach, mild β-elimination followed by Michael addition with dithiothreitol (BEMAD), tags the α,β-unsaturated carbonyl (the product of β-elimination) with a nucleophilic tag stabilizing the O-linkage during collision-induced dissociation. Tagging allowed for site identification by LC-MS/MS, making BEMAD useful for mass spectrometry. Not only does the BEMAD method allow for simultaneous study of O-GlcNAc and O-phosphate quantitatively, it also allows the enrichment of either posttranslational modification in the study of normal versus diseased states.

Recently, Bertozzi’s group reported that OGT and O-GlcNAcase could tolerate analogs of their natural substrates. OGT incorporated can incorporate an azide modified GlcNAc (GlcNazi) into protein targets. Once labeled, these GlcNazi-modified proteins can be covalently derivatized with various biochemical probes at the site of protein glycosylation using Staudinger ligation. This strategy could identify O-GlcNAc–modified proteins, as well as map protein target sites that bear O-GlcNAc modification. Because UDP-GlcNAc is incorporated into several classes of glycoconjugates, specificity must be demonstrated with properly controlled experiments when cells are labeled metabolically. Similar, more specific and improved techniques, tagging-via-substrate and Click-chemistry have been described. These techniques are highly sensitive and especially useful for lower molecular weight proteins. Despite the development of such techniques, the lack of a recognizable consensus motif somewhat complicates the analyses of O-GlcNAc function and limits predictive capabilities. Currently, it appears that amino acids modified by O-GlcNAc often are surrounded by serine/threonine residues, with a proline often 3 amino acids to the N-terminal side of the modification. However, there are numerous exceptions to such guidelines. Should a clear consensus sequence be established, this field would experience even more growth than its present rate.

Conclusions

Most aspects of cardiovascular biology remain untapped for the potential involvement of O-GlcNAc signaling. The current status of O-GlcNAc in cardiac biology represents an exciting time for discovery. Indeed, multiple phenomena will likely be identified as regulated by O-GlcNAc signaling in the immediate future. Moreover, our limited understanding of diabetes and its impact on metabolism in the cardiovascular system will remain important areas of investigation. Based on...
existing evidence, it appears that O-GlcNAc signaling participates in the pathophysiology of diabetes. Although significant advances in O-GlcNAc proteomics have occurred in the last 5 years, a long road of technological innovation and dissemination remains.

For the uninitiated, many questions likely remain, which may relate to consensus sequence (not clearly identified), the promoters for OGT/O-GlcNacase (not reported), and the biophysical impact of O-GlcNAcylation on proteins (no uniform answer). The existing literature suggests that O-GlcNAcylation is a metabolic sensor, but this review emphasizes an emerging role for O-GlcNAcylation as a robust stress response. Of course, such a possibility is not mutually exclusive with the current “metabolic sensor” function of O-GlcNAc. Although readers (and reviewers) want to know exactly what the targets of O-GlcNAcylation are in each specific context, it is our opinion that the absence of such information should not be the sole barrier to publication or funding. After all, we have collectively accepted targeted mutagenesis studies of Ser/Thr residues to be definitive evidence of the importance of specific kinase targets. Were we inadvertently precluding the possibility of O-GlcNAcylation? Should we collectively reevaluate some of our conclusions regarding phosphorylation of Ser/Thr in this new context?

What is clear is that in addition to its purported functions as a metabolic sensor, O-GlcNAcylation of proteins apparently can also reflect cellular stress in the cardiovascular system.

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Disclosures
None.

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