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Review

Protein *O*-GlcNAcylation: a new signaling paradigm for the cardiovascular system

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Laczy B, Hill BG, Wang K, Paterson AJ, White CR, Xing D, Chen Y, Darley-Usmar V, Oparil S, Chatham JC. Protein *O*-GlcNAcylation: a new signaling paradigm for the cardiovascular system. *Am J Physiol Heart Circ Physiol* 296: H13–H28, 2009. First published November 21, 2008; doi:10.1152/ajpheart.01056.2008.—The posttranslational modification of serine and threonine residues of nuclear and cytoplasmic proteins by the *O*-linked attachment of the monosaccharide β-*N*-acetylglucosamine (*O*-GlcNAc) is a highly dynamic and ubiquitous protein modification. Protein *O*-GlcNAcylation is rapidly emerging as a key regulator of critical biological processes including nuclear transport, translation and transcription, signal transduction, cytoskeletal reorganization, proteasomal degradation, and apoptosis. Increased levels of *O*-GlcNAc have been implicated as a pathogenic contributor to glucose toxicity and insulin resistance, which are both major hallmarks of diabetes mellitus and diabetes-related cardiovascular complications. Conversely, there is a growing body of data demonstrating that the acute activation of *O*-GlcNAc levels is an endogenous stress response designed to enhance cell survival. Reports on the effect of altered *O*-GlcNAc levels on the heart and cardiovascular system have been growing rapidly over the past few years and have implicated a role for *O*-GlcNAc in contributing to the adverse effects of diabetes on cardiovascular function as well as mediating the response to ischemic injury. Here, we summarize our present understanding of protein *O*-GlcNAcylation and its effect on the regulation of cardiovascular function. We examine the pathways regulating protein *O*-GlcNAcylation and discuss, in more detail, our understanding of the role of *O*-GlcNAc in both mediating the adverse effects of diabetes as well as its role in mediating cellular protective mechanisms in the cardiovascular system. In addition, we also explore the parallels between *O*-GlcNAc signaling and redox signaling, as an alternative paradigm for understanding the role of *O*-GlcNAcylation in regulating cell function.

hexosamine biosynthesis; protein *O*-glycosylation; β-*N*-acetylglucosamine transferase; diabetes mellitus

POSTTRANSLATIONAL MODIFICATION (PTM) of proteins is a common mechanism for the modulation of protein function, location, and turnover. Although protein phosphorylation is probably the most widely studied form of PTM, there are many other PTMs, including acylation, ubiquitylation, methylation, acetylation, thiolation, nitration, and glycosylation (107). The focus of this review is protein glycosylation, specifically, *O*-glycosylation of nuclear and cytoplasmic proteins. Classical protein glycosylation occurs in the endoplasmic reticulum and Golgi, leading to the formation of stable and complex elongated oligosaccharide structures via both *N*-linkage on asparagine and *O*-linkage on the hydroxy amino acids serine and threonine in addition to hydroxyproline, hydroxylysine, and tyrosine residues of proteins that become secreted or membrane component glycoproteins (189). In contrast, glycosylation of nuclear and cytoplasmic proteins is a rapid and dynamic modification of serine or threonine residues by the *O*-linked attachment of the monosaccharide β-*N*-acetylglucosamine (*O*-GlcNAc) (97, 195); this process is referred to as protein *O*-GlcNAcylation to contrast it with traditional *N*- and *O*-glycosylation within the secretory pathways.

The modification of serine/threonine residues by *O*-GlcNAc was first identified by Torres and Hart in 1984 (195). *O*-GlcNAcylation is a highly dynamic and ubiquitous PTM that plays a role in altering the function (121), activity (59), subcellular localization (57, 73, 219), and stability of target proteins (84, 229). *O*-GlcNAcylation is often considered analogous to phosphorylation (88) in that it is a tightly regulated enzyme-catalyzed process that leads to the modification of specific serine/threonine residues, which, in many cases, are

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also subject to phosphorylation. However, there are also some distinct differences between these two types of PTMs. For example, in contrast to the hundreds of kinases and phosphatases that regulate protein phosphorylation, to date, only one enzyme has been identified that catalyzes the formation of *O*-GlcNAc and one enzyme that catalyzes its removal, which raises questions regarding the requirements of the biological regulation of the specificity of *O*-GlcNAcylation. In this review, we also explore the possibility that the *O*-GlcNAc pathway has much in common with redox cell signaling pathways that arise from the modification of specific reactive cysteine residues on proteins.

Protein *O*-GlcNAcylation is rapidly emerging as a key regulator of critical biological processes, such as nuclear transport (77), translation and transcription (30), signal transduction (139, 183, 225, 226), cytoskeletal reorganization (42, 87), proteasomal degradation (83, 227), and apoptosis (138, 208). Much of our present knowledge regarding the role of *O*-GlcNAcylation on cellular function is in the context of chronic diseases, including senescence (62, 64, 177), cancer (27, 47, 182), and neurodegenerative disorders such as Alzheimer's disease (45, 85, 139, 208). Sustained increases of *O*-GlcNAc have been implicated as a pathogenic contributor to glucose toxicity and insulin resistance (15, 33), which are major hallmarks of diabetes mellitus and diabetes-related vascular complications. However, in contrast to these adverse effects, there is a growing body of data supporting the beneficial role of *O*-GlcNAc in mediating cellular protection designed to enhance cell survival. Reports on the effect of altered *O*-GlcNAc levels on the heart and cardiovascular system have been growing rapidly over the past few years and have implicated a role for *O*-GlcNAc in mediating the response to ischemic injury as well as contributing to the adverse effects of diabetes on cardiac and vascular function.

The goal of this review is to summarize our present understanding of protein *O*-GlcNAcylation and its effect on the regulation of cardiovascular function. We provide information regarding the pathways regulating protein *O*-GlcNAcylation and discuss, in more detail, our understanding of the role of *O*-GlcNAc in both mediating the adverse effects of diabetes as well as its role in mediating cellular protective mechanisms in the cardiovascular system. In addition, we also examine the parallels between *O*-GlcNAc signaling and redox signaling as an alternative paradigm for understanding the role of *O*-GlcNAcylation in regulating cell function.

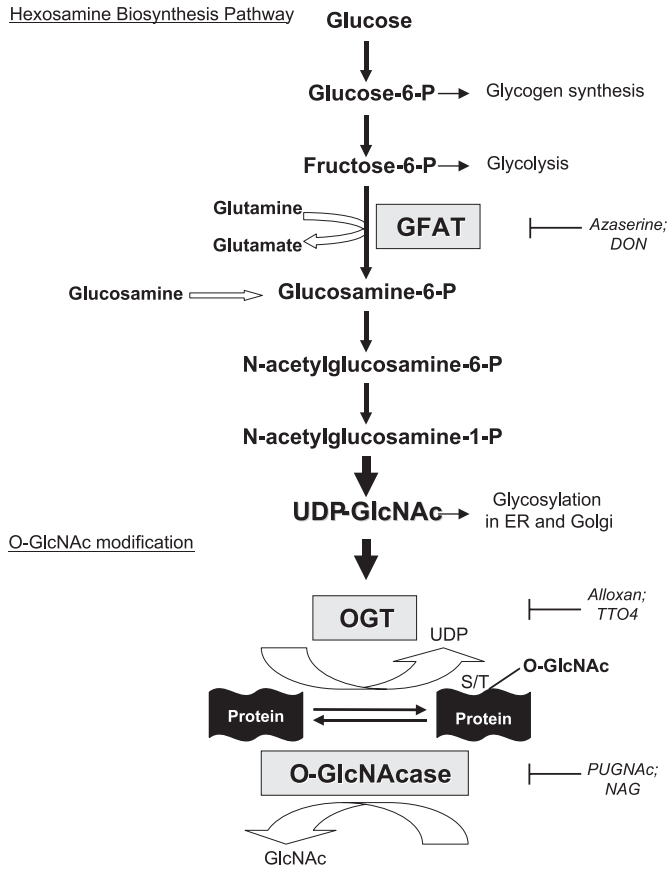

Fig. 1. The hexosamine biosynthesis pathway (HBP) and protein *O*-GlcNAcylation. Glucose imported into cells is rapidly phosphorylated to glucose-6-phosphate (glucose-6-P) and converted to fructose-6-phosphate (fructose-6-P), which is metabolized to glucosamine-6-phosphate by L-glutamine-D-fructose 6-phosphate amidotransferase (GFAT), resulting in the synthesis of UDP-*N*-acetylglucosamine (UDP-GlcNAc). GFAT can be inhibited by the glutamine analogs 6-diazo-5-oxo-L-norleucine (DON) and *O*-diazoacetyl-L-serine (azaserine). Flux through the HBP can be increased with glucosamine, which bypasses GFAT. UDP-GlcNAc is a sugar donor for classical glycosylation reactions in the endoplasmic reticulum (ER) and Golgi apparatus and is also the obligatory substrate for uridine-diphospho-*N*-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyltransferase (OGT), leading to the formation of *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc)-modified proteins. β-*N*-acetylglucosaminidase (*O*-GlcNAcase) catalyzes the removal of *O*-GlcNAc from proteins. The level of *O*-GlcNAc on proteins can be blocked by inhibiting OGT with the uridine analog alloxan or with 2[(4-chlorophenyl)imino]tetrahydro-4-oxo-3-[2-tricyclo(3.3.1.13.7)dec-1-ylethel] (TTO4), whereas *O*-GlcNAcylation of proteins can be rapidly increased by inhibiting *O*-GlcNAcase with *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc) or with 1,2 dideoxy-2-methyl-D-glucopyranoso(2,1-D)-2-thiazoline (NAG-thiazoline). S/T, serine/threonine.

The Hexosamine Biosynthesis Pathway

It is estimated from in vitro cell culture studies that between 2% and 5% of total glucose entering the cell is metabolized via the hexosamine biosynthesis pathway (HBP; Fig. 1) (148). Glucose entry into the HBP is regulated by L-glutamine-D-fructose 6-phosphate amidotransferase (GFAT), which converts fructose-6-phosphate to glucosamine-6-phosphate with glutamine as the amine donor (118). Glucosamine-6-phosphate is then metabolized via various hexosamine intermediates, leading to the synthesis of UDP-GlcNAc. UDP-GlcNAc provides glycosidic precursors for the synthesis of glycoproteins, glycolipids, and proteoglycans; it is also the essential sugar nucleotide donor for the formation of *O*-GlcNAc-modified proteins (126). Flux through the HBP can be increased with the addition of exogenous glucosamine, which enters cells via the glucose transporter system and is phosphorylated to glucosamine-6-phosphate by hexokinase, thus bypassing GFAT and leading to a rapid increase in UDP-GlcNAc levels (149).

Our understanding of the regulation of GFAT activity is surprisingly limited. GFAT is highly conserved among species, and the crystal structure of GFAT of *Escherichia coli* has been solved (190). Whereas bacterial GFAT is regulated at the gene expression level, eukaryotic GFAT is regulated both transcriptionally and posttranscriptionally. Since glutamine is the essential amine donor, GFAT activity is glutamine dependent and can be inhibited by the glutamine analog 6-diazo-5-oxo-

L-norleucine (DON) and *O*-diazoacetyl-L-serine (azaserine) (153). The enzyme exists in two isoforms, GFAT1 and GFAT2, which are transcribed from separate genes. The GFAT1 gene is localized to chromosome 2p13-p1, whereas the GFAT2 gene maps to chromosome 5q34-q35 in humans; this latter gene is a candidate for a type 1 diabetes susceptibility gene (167). Northern blot analysis showed different tissue distributions between GFAT1 and GFAT2. GFAT1 was more highly expressed in the pancreas, placenta, and testis than GFAT2, whereas GFAT2 was more abundantly expressed in the heart and central nervous system (167). A splice variant of GFAT1 is selectively expressed in striated muscle and has an increased sensitivity to UDP-GlcNAc feedback inhibition than that of GFAT1 (41). Two polymorphisms of GFAT have been identified in humans, one of which was associated with an increased risk for obesity (204). This is consistent with observations that GFAT activity is increased in muscles from diabetic subjects (223) and that overexpression of GFAT in skeletal muscle and adipose tissue resulted in insulin resistance (32, 91). Despite the similar abundance in GFAT expression between muscles from lean and *ob*/*ob* mice, increased GFAT activity was found in muscles from *ob*/*ob* mice (16). Different mechanisms of GFAT1 and GFAT2 gene expression and activity raise the possibility that they have different roles and involvement in diabetic pathophysiology.

In the heart, both aging (62) and pressure-overload hypertrophy (224) led to significant increases in GFAT2 mRNA expression with little or no change in GFAT1 mRNA levels, which, in both cases, was associated with increased UDP-GlcNAc levels, consistent with an increase in HBP flux. These studies would suggest, therefore, that GFAT expression and HBP flux in the heart are subject to regulation in response to chronic stress. Acute ischemic stress also increases UDP-GlcNAc (65, 124), but it is not known whether this is substrate driven or due to changes in GFAT activity.

Both GFAT1 and GFAT2 are subject to phosphorylation by cAMP-dependent protein kinase. However, phosphorylation of GFAT1 decreases its activity in vitro (22), whereas phosphorylation of GFAT2 leads to increased activity (101). Beside this isoform-specific phosphorylation, a novel phosphorylation site was identified in human GFAT1 in vivo. This Ser^{243} site was specifically phosphorylated in vitro by AMP-activated protein kinase (AMPK) and Ca^{2+}/calmodulin-dependent kinase (CaMK) II, which resulted in increased enzymatic activity (132). The importance of changes in GFAT activity via phosphorylations by different kinases remains unclear. The fact that AMPK phosphorylates GFAT1 under physiological conditions, and AMPK activity is stimulated by increased HBP flux in adipocytes leading to increased fatty acid oxidation (143), it is likely that GFAT represents a key regulatory point between HBP and AMPK signaling. Unfortunately, our present knowledge regarding GFAT activity and its phosphorylation state in the heart under normal and diseased circumstances are very limited due to, at least in part, the lack of available GFAT antibodies.

Regulation of Protein O-GlcNAcylation

The attachment of a single β-*N*-acetylglucosamine moiety via an *O*-linkage to specific serine/threonine residues of nuclear and cytoplasmic proteins is catalyzed by *O*-GlcNAc transferase (OGT; uridine diphospho-*N*-acetylglucosamine: polypeptide β-*N*-acetylglucosaminyltransferase; Fig. 1) using UDP-GlcNAc as the obligatory substrate (79, 81). The global extent of *O*-GlcNAc modification has been reported to be tightly dependent on the flux through HBP, since OGT catalytic activity is highly sensitive to changes in UDP-GlcNAc concentrations (120). The level of *O*-GlcNAc on nuclear and cytoplasmic proteins is also regulated by β-*N*-acetylglucosaminidase (*O*-GlcNAcase), which catalyzes the removal of the sugar moiety from proteins (48, 68).

A single OGT gene is located on the X chromosome in humans and mice (86, 119, 164). The observations that tissue-specific OGT mutants caused disturbances in somatic cell function (166) and that OGT deletion in mice is embryonically lethal (180) emphasize the physiological importance of *O*-GlcNAc for cell viability. OGT is highly conserved in organisms from *Caenorhabditis elegans* to humans and appears to be ubiquitously expressed (141). In some tissues, such as the muscle, kidney, and liver, OGT exists as a heterotrimer consisting of two 110-kDa subunits and one 78-kDa subunits (79); in other tissues, it is a homotrimer of three 110-kDa subunits (119). The 110-kDa subunit of OGT has two main domains; the COOH-terminus has a catalytic domain related to glycogen phosphorylase (214) that is altered by tyrosine phosphorylation (119), suggesting that the enzyme may be controlled by signaling cascades. This domain is also a target for *O*-GlcNAc modification (119), indicating a possible feedback regulatory element on OGT activity. Insulin increases both tyrosine phosphorylation and *O*-GlcNAcylation of OGT, leading to increased activity and transient translocation from the nucleus to cytoplasm (210, 219). It has also been shown that in neuroblastoma cells, OGT is regulated by p38 MAPK. Activated p38 has been shown to interact with OGT and recruit it to specific substrates, such as neurofilament H upon glucose deprivation (25).

The NH_2-terminal domain contains multiple tetratricopeptide repeats (TPRs) that mediate the protein-protein interactions involved in the recognition and activation of specific protein targets (120, 142). The crystal structure of a bacterial homolog of OGT, xcOGT, has recently been reported, demonstrating important insights into how OGT recognizes its protein substrates (29, 151). The three-dimensional structure shows how binding of partners along the TPR domain provides a mechanism to prevent or enhance the binding of specific proteins for *O*-GlcNAc modification. These studies also provide a critical molecular framework for improving our understanding of OGT specificity as well as for the development of novel OGT inhibitors.

In addition to the nucleocytoplasmic isoform (ncOGT), two other variants are transcribed from a single gene through alternative splicing, each with identical COOH-terminal catalytic domains and distinct NH_2-termini contributing to the different localization and unique targeting sequences (86, 140, 164). The short isoform (sOGT), similar to the larger ncOGT isoform, is distributed throughout the cell, whereas the mitochondrial variant (mOGT), consisting of 103-kDa subunits, is localized in the inner membrane of mitochondria and contains the mitochondrial targeting information (140). The initial report of mOGT suggested that it had limited catalytic activity and was unable to effect *O*-GlcNAcylation of mitochondrial proteins (139). More recently, a number of mitochondrial proteins have been identified as targets for *O*-GlcNAc modifi-

cation, including the voltage-dependant anion channel (VDAC), one of the putative components of the mitochondrial permeability transition pore (mPTP) (108).

O-GlcNAcase is also highly conserved in mammals, is expressed in all tissue types examined, and has a similar tissue distribution to OGT (68). In contrast to OGT, which is reported to be localized primarily to the nucleus, the active form of *O*-GlcNAcase is predominantly localized to the cytoplasm (90%) (68, 205). The potential implications for the markedly different intracellular distributions of OGT and *O*-GlcNAcase remain to be determined. However, the fact that OGT distribution changes in response to insulin stimulation (210, 219) and that OGT and *O*-GlcNAcase form a transient complex during mitosis (184) suggests that the differential localization may play a critical role in regulating the targeting and processing of *O*-GlcNAcylated proteins.

O-GlcNAcase activity is specific for *N*-acetyl-β-D-glucosaminides and, unlike lysosomal hexosaminidases, has an pH optimum near neutral (48, 68). *O*-GlcNAcase is a 106-kDa heterodimer complex containing a 54-kDa α-subunit and a 51-kDa β-subunit (48). The catalytic domain of *O*-GlcNAcase is in the NH_2-terminus (193), whereas the COOH-terminus has histone acetyl-transferase (HAT) activity in vitro (194). *O*-GlcNAcase contains a caspase-3 cleavage site; thus, it may be regulated during apoptosis (18). However, this specific cleavage site may have other regulatory roles on *O*-GlcNAcase (e.g., stabilization, localization, or targeting), because cleavage per se does not adversely affect its enzymatic activity (18, 194, 205). The human *O*-GlcNAcase gene, which maps to the 10q24 chromosomal region, was originally identified as meningioma-expressed antigen 5 (MGEA5) because of its association with human meningiomas (92). This gene locus has also been linked to Alzheimer's disease (10). Interestingly, a single-nucleotide polymorphism in the MGEA5 gene correlates to the susceptibility for type 2 diabetes in the Mexican-American population (127).

Two splice variants of *O*-GlcNAcase have been reported in rats. One splice variant, which was detected in Goto-Kakizaki rats, has a molecular weight of ~ 90kDa and lacks exon 8; consequently, it does not have functional *O*-GlcNAcase activity but retains HAT activity. The other splice variant, identified in Sprague-Dawley rats, has a molecular weight of ~ 84 kDa and lacks both exon 8 and exon 9; it also does not have *O*-GlcNAcase activity but retains HAT activity (194).

The mechanism(s) by which OGT and *O*-GlcNAcase dynamically regulate *O*-GlcNAc cycling under normal physiological conditions or in response to acute or chronic stimuli remains poorly understood. Interestingly, OGT forms a complex with the catalytic subunit of protein phosphatase I (206), suggesting that a single enzyme complex may both add *O*-GlcNAc and remove phosphate. OGT can also form a complex with *O*-GlcNAcase, which has been referred to as the *O*-GlcNAczyme (211), thereby allowing a rapid cycling of *O*-GlcNAc on proteins. OGT and *O*-GlcNAcase can associate with both kinases and phosphatases, and this transient protein complex can contribute to the regulation of mitosis in HeLa cells (184). Such studies provide further support for the concept of a complex and reciprocal relationship between phosphorylation and *O*-GlcNAcylation, as shown in Fig. 2.

Pharmacological studies of the rate of *O*-GlcNAc formation and removal have been limited due to the lack of specific high-affinity inhibitors. Alloxan, a uridine analog, has been used to inhibit OGT (117); however, its utility is very limited due its toxicity and lack of specificity (128). Recently novel and potent OGT inhibitors have been reported (75, 76), which may lead to more feasible and specific approaches to the study of OGT activity; however, there are relatively few reports on their use in biological systems. One report has demonstrated the effectiveness of two such inhibitors in decreasing *O*-GlcNAc levels in isolated neonatal cardiomyocytes (162). The most widely used pharmacological approach to modulate *O*-GlcNAc levels has been *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc), an analog of GlcNAc that is an effective and relatively specific competitive inhibitor of *O*-GlcNAcase (80, 181). By inhibiting *O*-GlcNAcase, PUGNAc slows or prevents the removal of *O*-GlcNAc, leading to a relatively rapid increase in *O*-GlcNAc levels. The effectiveness of PUGNAc in increasing *O*-GlcNAc has been demonstrated in a variety of biological systems, including perfused hearts and isolated cardiomyocytes (21, 108, 135). There is some concern that PUGNAc may inhibit lysosomal hexosaminidases. The new *O*-GlcNAcase inhibitor 1,2 dideoxy-2′-methyl-D-glucopyranoso(2,1-D)-2′-thiazoline (NAG-thiazoline) and its derivatives have been found to be more effective and more selective than PUGNAc (145, 212). For example, 1,2 dideoxy-2′-propyl-D-glucopyranoso(2,1-D)-2′-thiazoline has been shown to have a 3,100-fold selectivity toward *O*-GlcNAcase over hexosaminidases (145). Recently, synthesized GlcNAcstatin has been reported to inhibit *O*-GlcNAcase in the picomolar range and to have a 100,000-fold selectivity over lysosomal hexosaminidases; thus, it may be

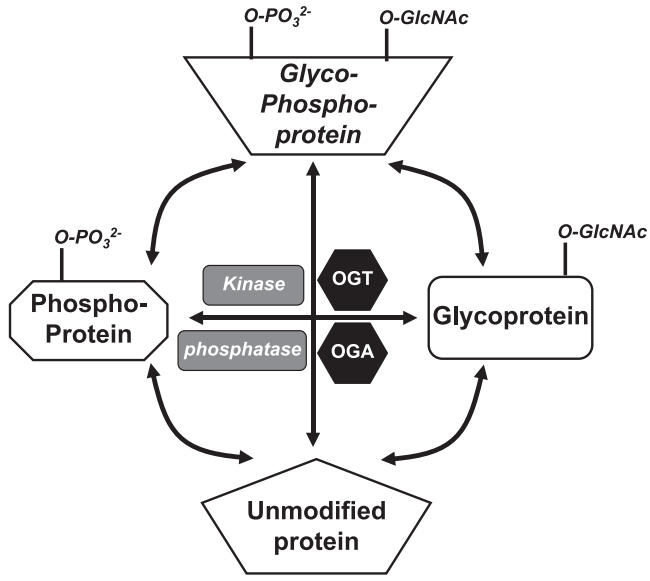

Fig. 2. The interaction between *O*-GlcNAcylation and *O*-phosphorylation. Analogous to phosphorylation, *O*-GlcNAcylation is a dynamic posttranslational modification occurring on serine/threonine residues of proteins. For a subset of cellular proteins, there is a competitive relationship between *O*-GlcNAc 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 combination of *O*-phosphate and *O*-GlcNAc modifications creates molecular diversity by altering specific protein sites that are involved in signaling events. Thus, this complex interplay between phosphorylation and *O*-GlcNAcylation can dynamically regulate protein functions and modulate critical signaling pathways. OGA, β-*N*-acetylglucosaminidase. [Modified from Zachara and Hart (226).]

more selective and potent than both PUGNAc and NAG-thiazolines (49). It should be noted that streptozotocin (STZ) has been used as an *O*-GlcNAcase inhibitor (116, 137); however, there is some controversy regarding this issue, since STZ treatment did not inhibit *O*-GlcNAcase activity in vitro (145).

O-GlcNAcylation and Protein Function

Several hundred distinct proteins from diverse functional groups, including nuclear pore proteins, transcription factors, nuclear hormone receptors, signal transduction molecules, kinases, phosphatases, metabolic enzymes, chaperones, cytoskeletal proteins, and actin regulatory proteins, are known to be modified by *O*-GlcNAc (139, 209, 225, 226). Interestingly, some serine/threonine residues that are targets for *O*-GlcNAc on proteins such as endothelial nitric oxide (NO) synthase (eNOS) (53), estrogen receptor-β (24), the COOH-terminal domain of RNA polymerase II (111), and c-Myc (109) can also be phosphorylated. The competition between *O*-GlcNAcylation and phosphorylation for the same residues has been termed the "yin-yang phenomenon"; however, it should be emphasized that the interplay between these two PTMs is not always reciprocal. For example, some proteins, such as p53 (218) and vimentin (202), can be concomitantly phosphorylated and *O*-GlcNAcylated, and the adjacent phosphorylation or *O*-GlcNAcylation can regulate the addition of either moiety. Other proteins, such as Sp1 (104), insulin receptor (IR) substrate (IRS)-1 (219), and glycogen synthase kinase (GSK)-3 (202), have multiple and distinct phosphorylation/*O*-GlcNAcylation sites on the same protein (Fig. 2). Interestingly, inhibition of GSK-3 resulted in increased *O*-GlcNAcylation of 10 proteins, including heat shock protein (HSP)27 and vimentin, but decreased *O*-GlcNAcylation of 19 proteins, including the transcription factor host-cell factor-1 (202). Conversely, increasing global *O*-GlcNAc levels by *O*-GlcNAcase inhbition resulted in a decrease in phosphorylation at ~33% of sites and an increase in phosphorylation at ~18% of sites (201). These studies clearly demonstrate nonreciprocal interplay between *O*-GlcNAcylation and phosphorylation and indicate the possibility for cooperativity between these PTMs.

Alterations in *O*-GlcNAcylation can affect critical signaling events; increased *O*-GlcNAcylation has been implicated in modulating a number of regulatory kinase cascades, including PKB/Akt, PKC, AMPK, p38 MAPK, and NF-κB (14, 53, 65, 71, 113, 115, 143, 144, 215, 230). However, in contrast to the hundreds of kinases and phosphatases regulating phosphorylation (147), *O*-GlcNAc turnover is mediated by only two enzymes: OGT and *O*-GlcNAcase. While questions remain regarding how *O*-GlcNAcylation of specific proteins is regulated, there is growing evidence that these enzymes transiently associate with binding partners to form holoenzyme complexes that have unique properties and specificities (184).

O-GlcNAcylation and Endothelial-Vascular Dysfunction

Hyperglycemia is a major contributing factor to the increased risk for both microvascular and macrovascular disease associated with diabetes (40, 158, 159, 198). In the development of diabetic vascular complications, the adverse effects of hyperglycemia have been attributed to a number of different pathways (44, 179) including increased oxidative stress (12, 13), increased flux through the polyol pathway (12, 13), increased advanced glycation end-product (AGE) formation (2), increased PKC activity (36), and increased HBP flux and protein *O*-GlcNAcylation (15, 33). Increased flux through the HBP may occur simply as a consequence of elevated extracellular glucose; alternatively, it has been suggested that increased mitochondrial ROS inhibit GAPDH, decreasing glycolytic flux and thereby increasing glucose entry into the HBP (52, 54).

The constitutive production of NO by eNOS plays a pivotal role in the regulation of vascular tone and remodeling, the inhibition of platelet aggregation, leukocyte adhesion, vascular smooth muscle cell (VSMC) proliferation, and the synthesis and secretion of extracellular matrix proteins (222). In cultured bovine aortic endothelial cells (BAECs), hyperglycemia increased mitochondrial superoxide production in association with an increase in *O*-GlcNAc on eNOS and a reciprocal decrease in phosphorylation at Ser^{1177}, the site responsible for the activation of the enzyme (53). *O*-GlcNAcylation of eNOS was blocked by GFAT antisense oligonucleotides, confirming the role of increased HBP flux. Similarly, aortas from diabetic rats also exhibited decreased eNOS activity and altered eNOS modification at this Akt phosphorylation site with no changes in total eNOS expression (53). This demonstrates that *O*-GlcNAcylation can modulate the function of catalytic proteins without altering transcriptional regulation.

In human coronary artery endothelial cells, phosphorylation of eNOS at Ser^{1177} by Akt was decreased by both high glucose and glucosamine, and the effect of high glucose was reversed by inhibiting GFAT with azaserine (59), supporting the role of increased HBP flux in mediating these events. Moreover, both high glucose and glucosamine increased *O*-GlcNAcylation of IRS-1, IRS-2, and p85 proteins and decreased insulin-stimulated eNOS activity and Ser^{1177} phosphorylation. This *O*-GlcNAc-induced decrease in eNOS activity was associated with an increase in matrix metalloproteinase (MMP) activity and expression combined with decreased tissue inhibitor of metalloproteinase (TIMP) expression. Such an imbalance between MMPs and TIMPs has been implicated in atherosclerosis-related complications in diabetes (67), such as plaque instability or restenosis after angioplasty (186). Increased protein *O*-GlcNAc levels also activated the mitogenic signaling pathway (59), suggesting that *O*-GlcNAcylation not only impairs the protective effects of NO but also promotes vascular cell proliferation (156). This is supported by the observation that elevated *O*-GlcNAc levels have been observed in the endothelial layer, VSMCs, and macrophages of human carotid atherosclerotic plaques from type 2 diabetic patients compared with nondiabetic healthy subjects (59).

In isolated rat thoracic aortic rings, increasing *O*-GlcNAc formation using PUGNAc blunted vascular reactivity to acetylcholine and augmented vasoconstriction in response to phenylephrine (PE) (133). *O*-GlcNAc-mediated enhanced vascular tone resulted from increased α-adrenergic reactivity and impaired relaxation via decreased eNOS (Ser^{1177}) phosphorylation (133). BAECs treated with higher physiological concentrations of the fatty acid oleate showed increased overall *O*-GlcNAcylation, which was associated with decreased eNOS and PGI_2 synthase activity (51). The latter is both an endogenous vasodilator and inhibitor of platelet aggregation and VSMC proliferation (179).

Increased plasminogen activator inhibitor (PAI)-1 expression has been strongly implicated in the development of the

diabetic vascular dysfunction (34, 179). High glucose stimulated the expression of PAI-1 in VSMCs by activating two Sp1-binding sites at the promoter region of the PAI-1 gene (23). In BAECs, hyperglycemia was shown to increase HBP activity and *O*-GlcNAc levels on Sp1 transcription factor, leading to increased PAI-1 expression; however, when the two Sp1-binding sites were mutated, PAI-1 expression was not altered (54). High glucose, glucosamine, and overexpression of GFAT in mesangial cells enhanced the PAI-1 activation via Sp1 sites as well, providing further evidence for the role of *O*-GlcNAc in the regulation of PAI-1 expression (70, 72, 105). It has been well characterized that Sp1 has multiple sites that are subject to *O*-GlcNAc modification (220). Furthermore, increased *O*-GlcNAcylation of Sp1 enhanced expression of transforming growth factor (TGF)-β_1 (54, 105, 114), a potent inducer of extracellular matrix protein synthesis that leads to both VSMC and fibroblast proliferation and plays a major role in the progression of diabetic nephropathy (110).

Tissue-specific abnormal angiogenesis contributes to the development and progression of diabetic vascular complications (44). Activation of the HBP and increasing *O*-GlcNAc formation with high glucose, glucosamine, or PUGNAc increased the synthesis of thrombospondin (TSP)-1, a potent antiangiogenic and proatherogenic protein, and enhanced the proliferation of cultured human aortic smooth muscle cells (173). Inhibition of GFAT prevented the high glucose-induced upregulation of TSP-1 and inhibited VSMC proliferation (173). In vitro, the migration and capillary-like tube formation of endothelial cells was inhibited through elevated *O*-GlcNAc levels, and this could be reversed by inducing *O*-GlcNAcase overexpression (144). Increased *O*-GlcNAc levels also markedly reduced vascular sprouting in mouse aortic rings after glucosamine treatment as well as in aortic rings from STZ-induced and high-fat diet-induced diabetic mice (144). Impaired angiogenesis was mediated, at least in part, by *O*-GlcNAcylation of Akt, which decreased its activity and phosphorylation at the Ser^{473} site (144).

High glucose also induced *O*-GlcNAc modification of Sp3 and upregulated angiopoietin-2 (Ang-2) gene expression in microvascular endothelial cells (221). This resulted in the increased expression of ICAM-1 and VCAM-1 and sensitization of microvascular endothelial cells to the proinflammatory effects of TNF-α (221). Interestingly, both Sp1 and Sp3 transcription factors, which are targets for *O*-GlcNAcylation (220, 221), are also involved in regulating the endothelial cell-specific expression of VEGF receptor (89). Increased VEGF is thought to be responsible for the increased vascular proliferation and permeability in diabetic retinopathy (19). From these observations, it is apparent that *O*-GlcNAcylation has a role in altering the angiogenic response in different types of vascular cells (i.e., stimulation in microvessels and inhibition in macrovessels of the heart), contributing to the vascular complications of diabetes and influencing the endothelial inflammatory responses by regulating Ang-2 expression.

Thus, increased HBP flux and the resulting increased protein *O*-GlcNAc levels in endothelial cells and VSMCs contribute to the complex pathogenesis of diabetic vascular dysfunction. This occurs directly by regulating protein gene expression (e.g., PAI-1, TGF-β_1, TSP-1, and Ang-2), altering the function of key molecules (e.g., eNOS and Akt), interfering with critical signaling pathways [e.g., IR/IRS/phosphatidylinositol 3-kinase (PI3K)/Akt and the MAPK cascade] or indirectly by stimulating the expression and activity of proinflammatory cytokines (e.g., TNF-α), vasoactive substances (e.g., endothelin-1), growth factors (e.g., VEGF), and adhesion molecules (e.g., ICAM-1 and VCAM-1). This combination of *O*-GlcNAc-mediated events leading to impaired vascular reactivity and increased smooth muscle cell proliferation, atherogenesis, and plaque instability as well as abnormal or impaired angiogenesis could be considered the "perfect storm" of vascular abnormalities.

O-GlcNAcylation and Cardiomyocyte Dysfunction

Diabetes, excitation-contraction coupling, and Ca^{2+} handling. While many of the adverse effects of diabetes can be attributed to micro- and macrovascular disease, extensive clinical and experimental evidence demonstrates that diabetes also leads to abnormalities at the level of the cardiomyocyte independent of vascular defects, thereby increasing the risk for cardiac dysfunction and heart failure (9, 90). The mechanisms underlying cardiomyocyte dysfunction in diabetes are multiple, and, as with the increase in vascular disease, hyperglycemia is one of the major contributing factors leading to the activation of alternative pathways and oxidative stress (11, 90, 172). AGEs (170), PKC activation (38), and increased polyol pathway activity as well as alterations in cardiac energy metabolism (3, 37) clearly contribute to diabetes-induced cardiac dysfunction. In addition, accumulating evidence suggests that increased protein *O*-GlcNAcylation may also mediate the adverse effects of diabetes on the heart.

Diabetes leads to abnormal cardiomyocyte excitation-contraction (E-C) coupling, including prolonged action potential duration, slowed cytosolic Ca^{2+} removal, and prolonged myocyte relaxation (37, 123). In isolated ventricular myocytes, these mechanical dysfunctions can be observed as early as a few days after STZ-induced diabetes (175) and after long-term diet-induced insulin resistance (56, 213). Similar phenotypic changes were found after 1-day treatment of normal adult cardiomyocytes with high glucose (39, 55, 176). These early alterations in E-C coupling were associated with depressed sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) activity without changes in the expression of SERCA and the sarcolemmal Na^+/Ca^{2+} exchanger or expression and phosphorylation state of phospholamban (PLB) (55). However, in chronic models of diabetes, altered expression of genes involved in the regulation of E-C coupling also occurs (1, 8, 26, 123). The potential roles of the HBP and protein *O*-GlcNAcylation in mediating the effects of high glucose were supported by the observation that glucosamine mimicked the effects of high glucose on cardiomyocyte E-C coupling (176). In type 2 diabetic rats, the transition from normoglycemia to hyperglycemia has been associated with impaired Ca^{2+} transient decay and relaxation mechanics (64), similar to that previously reported in acute STZ-induced diabetes (175). This impaired cardiomyocyte relaxation was also associated with increased UDP-GlcNAc levels and increased levels of *O*-GlcNAc-modified proteins compared with nondiabetic controls (64).

Increased *O*-GlcNAc levels have been linked more directly to cardiomyocyte dysfunction by the demonstration that high glucose, glucosamine, and increased OGT expression all resulted in increased *O*-GlcNAc levels and prolonged Ca^{2+}

transient decays in neonatal cardiomyocytes (25). SERCA mRNA and protein expression were decreased in neonatal cells, an effect not observed in adult cardiomyocytes (46). *O*-GlcNAcase overexpression reversed the effects of increased *O*-GlcNAcylation, improving both Ca^{2+} handling and SERCA expression, whereas OGT overexpression and high glucose increased nuclear *O*-GlcNAcylation of Sp1. It was proposed that this might contribute to reduced expression of SERCA because the SERCA promoter contains multiple Sp1-binding sites required for adequate expression. Furthermore, expression of MEF-2a, a transcription factor important for cardiomyocyte function and maturation, was also depressed but was not itself subject to *O*-GlcNAc modification.

Subsequently, the same group demonstrated that after prolonged STZ-induced diabetes in mice, hearts showed increased cardiac *O*-GlcNAc levels and cardiac dysfunction, both of which could be reversed by increased *O*-GlcNAcase expression (100). Associated with *O*-GlcNAcase overexpression, SERCA protein levels and PLB phosphorylation were increased, whereas total PLB content was decreased, providing further support for a link between *O*-GlcNAc and cardiomyocyte Ca^{2+} handling. Disturbances in sarcoplasmic reticulum (SR) function are well-recognized phenomenon in the pathogenesis of heart failure (4, 35, 154, 171). Changes in SR function associated with heart failure include reduced activity or expression of SERCA; altered PLB expression and phosphorylation; and decreased rate of Ca^{2+} uptake by the SR leading to prolonged Ca^{2+} transients. These alterations in SR function are similar to those seen in response to diabetes, which, as described above, may be mediated via increased *O*-GlcNAcylation. This raises the possibility that changes in *O*-GlcNAcylation of SR proteins may play a role in the development and progression of heart failure.

Impaired myocardial contractility may also play a role in the adverse effects of diabetes via altered myofilament Ca^{2+} sensitivity (95), slower cross-bridge cycling (103), and decreased expression or activity of contractile proteins, such as α-actin and myosin ATPase (43). *O*-GlcNAcylation in hearts from type 2 diabetic rats has been shown to be primarily associated with high-molecular-weight proteins (i.e., >205 kDa) (64). Cardiomyocytes contain many high-molecular-weight proteins including cytoskeletal and contractile proteins such as titin, dystrophin, and myosin (98). In rodents, both type 1 and type 2 diabetes induce a shift in myosin heavy chain (MHC) isoform distribution by reducing fast α-MHC expression levels and increasing slow β-MHC expression levels, which is consistent with myocardial contractile dysfunction (43, 46, 82, 131).

Key contractile proteins such as MHC, actin, myosin light chain (MLC)-1, and MLC-2 have also been shown to be *O*-GlcNAc modified in both slow and fast skeletal muscle (93), but it has been suggested that *O*-GlcNAc levels are greater in slow than fast fibers (28). Moreover, incubation of skeletal muscle fibers with glucosamine resulted in decreased Ca^{2+} sensitivity and Ca^{2+} activity, possibly via inhibition of protein-protein interactions by *O*-GlcNAc modification (93). It has also been proposed that *O*-GlcNAc modification of MLC-2 may counteract the effect of phosphorylation leading to increased Ca^{2+} sensitivity of muscle fibers and myofilament assembly (93). Thus, an imbalance between phosphorylation/*O*-GlcNAcylation may alter actin-myosin interactions and myosin assembly. Importantly, specific sites of *O*-GlcNAc modification have been established on cardiac myofilament proteins, including MHC (Ser^3), α-cardiac actin (Ser^2), and MLC-1 (Thr^2) (69). It has also been demonstrated that exposure of rat skinned cardiac trabeculae to GlcNAc markedly depressed the Ca^{2+} sensitivity and affinity of myofilaments (69). These findings support the notion that *O*-GlcNAcylation on specific cardiac proteins has an important role in regulating myocardial contractile function and that increased *O*-GlcNAc levels on these proteins may contribute to the adverse effects of diabetes on the myocardial contractile apparatus.

Several target proteins of cardiac PKC isoforms, such as cardiac troponin I (cTnI), the L-type Ca^{2+} channel, SERCA, and PLB, are directly involved in E-C coupling and Ca^{2+} homeostasis in cardiomyocytes (146, 152). Increased HBP flux due to either high glucose or glucosamine has been shown to enhance the activity of specific PKC isoforms (e.g., PKC-β1, PKC-δ, and PKC-ε) (71); whether this is directly mediated by *O*-GlcNAcylation of PKC has not been demonstrated. Interestingly, CaMK IV has been shown to phosphorylate and activate OGT both in vivo and in vitro (188). Furthermore, membrane depolarization promoted OGT activation and elevated overall protein *O*-GlcNAcylation, and this effect was blunted by inhibition of either L-type voltage-gated Ca^{2+} channels or CaMK IV (188). While these observations were from nonmyocyte cell culture studies, they suggest that OGT activity can be directly regulated by Ca^{2+} influx and CaMK-dependent phosphorylation. It remains to be determined whether this is true in cardiomyocytes.

In the intact heart, increased HBP flux induced by either short-term STZ-induced diabetes or after acute glucosamine treatment blunted the positive inotropic responses to the α-adrenergic agonist PE, whereas the response to β-adrenergic stimulation was unaffected (168). In ventricular myocytes, increased *O*-GlcNAcylation attenuated the increase in cytosolic Ca^{2+} levels induced by PE and other IP_3-generating agonists such as angiotensin II (157, 169). In myoblasts, the bradykinin-mediated increase in intracellular Ca^{2+}, which, like PE, is mediated by phospholipase C (PLC) activation, was also attenuated by high glucose, glucosamine, or PUGNAc-induced protein *O*-GlcNAcylation, including *O*-GlcNAcylation of PLC-β_1 (112). The authors proposed that increased *O*-GlcNAc levels on PLC-β_1 decreased its activity, thereby attenuating the response to bradykinin (112). Since PLC activation is critical to G protein-coupled signaling, *O*-GlcNAc-induced attenuation of PLC activity could have broad implications for the adverse effects of diabetes on the heart.

Cardiomyocyte apoptosis and hypertrophy. Pathological cardiomyocyte growth is regulated by a number of different signaling pathways (50, 94), including the PI3K/Akt and p38/MAPK branches of the insulin signaling pathway, the calcineurin-nuclear factor of activated T cell (NFAT) pathway, cyclin-dependent kinases, and activation of PKC isoforms. Several key elements of these kinase cascades and transcription factors have been reported to be regulated either directly or indirectly by *O*-GlcNAc modification, with important functional consequences (209, 219, 225). For example, impaired Akt activity by *O*-GlcNAc can alter hypertrophic responses and increase apoptosis (200), whereas regulation of FoxO transcriptional activity by *O*-GlcNAc may both alter the expression of regulatory genes for glucose and lipid metabolism (99, 122) and affect cell proliferation and apoptosis (155).

Cardiomyocyte hypertrophy in response to G protein-coupled receptor activation by angiotensin II or PE is mediated, at least in part, by an increase in cytosolic Ca^{2+} levels via the capacitive Ca^{2+} entry pathway (169). This results in the activation of calcineurin and subsequent nuclear translocation of the transcription factor NFAT (102). High glucose and glucosamine prevented both angiotensin II- and PE-mediated increases in capacitative Ca^{2+} entry and blocked the hypertrophic response; the effect of high glucose was reversed by inhibiting GFAT with azaserine (169), suggesting that increased HBP flux mediated these effects. The angiotensin II-induced increase in intracellular Ca^{2+} was inhibited by glucosamine and PUGNAc, both of which were associated with increased protein *O*-GlcNAc levels (157).

In cell culture studies, increased levels of *O*-GlcNAc interfered with cell growth and division through altered cyclin expression (185). Although cardiomyocytes are generally considered to be terminally differentiated, D-type cyclins are critical regulators of cardiac hypertrophy (17). Thus, it may be postulated that increased cardiomyocyte *O*-GlcNAc levels could impair cyclin-mediated hypertrophic signaling pathways, thereby contributing to the adverse response to hypertrophic stimuli. It remains to be determined whether increases in *O*-GlcNAc levels attenuate the hypertrophic response in vivo. If this is the case, impaired hypertrophic signaling could be detrimental during the initial adaptive response to increased hemodynamic demand, thereby accelerating the progression to failure. This mechanism could account for the increased risk for heart failure seen in diabetic patients with hypertension or after myocardial infarction (9, 90, 187).

In insulin-responsive tissues, both high glucose and glucosamine have been reported to increase angiotensinogen gene expression (66), providing a link between *O*-GlcNAc and enhanced angiotensin II formation. This may contribute to the elevated angiotensin II levels observed in diabetes that have been associated with increased oxidative damage and enhanced cardiomyocyte apoptosis and necrosis (61). It has also been shown that in adult cardiomyocytes, high glucose-induced apoptosis was mediated via increased angiotensin II synthesis, activation of p38 MAPK, and increased *O*-GlcNAcylation of the transcriptional factor p53 (60). Inhibition of p53 *O*-GlcNAcylation attenuated the activation of both p38 MAPK and p53, decreased angiotensin II generation, and reduced myocyte apoptosis (60). In human cardiac myocytes, high glucose also enhanced apoptosis via caspase-3 activation accompanied by temporal activation of p38 MAPK and nuclear *O*-GlcNAcylation (130).

Increased myocardial fibrosis contributes to cardiac dysfunction in the setting of cardiac hypertrophy (6) and in the diabetic heart, possibly via increased renin-angiotensin system activation and angiotensin II levels (31), which may be augmented by increased *O*-GlcNAcylation. There is also evidence linking *O*-GlcNAcylation to increased fibroblast activation. High glucose has been shown to increase cardiac myofibroblast proliferation, collagen synthesis, fibronectin, and TGF-β_1 gene expression (5, 160, 192). In mesangial cells, activation of the HBP mimicked the effects of high glucose, increasing TGF-β expression and bioactivity, leading to enhanced production of matrix components such as fibronectin (105, 114). Thus, although to date there are no direct studies linking increased HBP flux and *O*-GlcNAcylation to cardiac fibroblast activation, there is strong circumstantial evidence to suggest that this might occur and could contribute to the increased fibrosis and impaired diastolic function that is characteristic of diabetes.

Protein O-GlcNAcylation and Cardiovascular Protection

Much of our understanding of the role of the HBP and *O*-GlcNAcylation is in the context of disease states such as diabetes (15, 33), cancer (27, 47, 182), and neurodegenerative disorders such as Alzheimer's disease (45, 85, 139, 208). However, since OGT is essential for cell viability (180) and is highly conserved from an evolutional perspective (141), its presence must convey some survival advantage to cells and organisms. A growing body of data demonstrates that activation of pathways leading to increased *O*-GlcNAc formation enhances tolerance to stress and improves cell survival. Overall *O*-GlcNAc levels have been shown to be dramatically increased in response to multiple experimental stress stimuli in several mammalian cell lines (228). Importantly, when the *O*-GlcNAc response was prevented by decreasing OGT expression, cell viability decreased, whereas augmentation of *O*-GlcNAc levels with PUGNAc increased cell survival (228). It has also been reported that acute heat stress increased *O*-GlcNAc levels and enhanced cell survival, whereas inhibition of GFAT decreased *O*-GlcNAc levels and increased cell death (186a).

In neonatal cardiomyocytes, hypoxia-reoxygenation causes a transient increase in *O*-GlcNAc levels, and augmentation of this response by elevating flux through HBP with high glucose, glucosamine, or PUGNAc improves cell viability and decreases necrosis and apoptosis (21). Conversely, attenuation of this response with glucose removal or GFAT or OGT inhibition reduces cell survival (21). Overexpression of OGT mimicked the effect of glucosamine in protecting against ischemic and H_2O_2-induced injury, thus demonstrating a direct role for OGT in mediating cardiomyocyte survival (20). Conversely, decreased OGT expression attenuated both the basal and ischemia-induced increase in *O*-GlcNAc levels as well as markedly increased the sensitivity of cardiomyocytes to hypoxia-reoxygenation injury, increasing necrosis and apoptosis (20). Others have also demonstrated that transcriptionally silenced OGT significantly reduced OGT expression and *O*-GlcNAc levels and exacerbated posthypoxic injury in cardiac myocytes (162, 163). These data directly support the importance of OGT and *O*-GlcNAc in mediating cardiomyocyte survival.

In the isolated perfused heart, ischemia alone increased both UDP-GlcNAc and overall *O*-GlcNAc levels (65, 124), indicating that this endogenous stress-activated pathway is active in the heart. Activation of the HBP with either glucosamine or glutamine before the induction of ischemia significantly increased cardiac *O*-GlcNAc levels, improved contractile function, and decreased tissue injury after reperfusion (134, 136). Perfusion with azaserine (GFAT inhibitor) and alloxan (OGT inhibitor) prevented the increase in *O*-GlcNAc levels, decreased functional recovery, and enhanced tissue injury after ischemia-reperfusion (134, 136). Moreover, treatment with PUGNAc during early reperfusion also improved functional recovery and attenuated tissue injury assessed by cTnI release (135). There was a significant correlation between increased *O*-GlcNAc levels and lower cTnI release (135). In vivo administration of PUGNAc has been shown to reduce infarct size

after ischemia-reperfusion in mice (108). Furthermore, in vivo augmentation of *O*-GlcNAc levels after hemorrhagic shock using either glucosamine or PUGNAc significantly improved cardiac function and improved peripheral organ perfusion in rats (217, 231, 232). These results provide strong evidence that the protective effect seen associated with increasing *O*-GlcNAc levels at the cellular and isolated organ levels can be translated to the in vivo environment.

Acute activation of *O*-GlcNAc formation has also been shown to be protective in the setting of endoluminal vascular injury in vivo (216). Pretreatment with either glucosamine or PUGNAc increased *O*-GlcNAc-modified protein levels in balloon-injured rat carotid arteries compared with vehicle-treated controls. Increased *O*-GlcNAc levels in injured arteries of glucosamine- and PUGNAc-treated animals were associated with attenuated expression of proinflammatory mediators, decreased neutrophil and monocyte infiltration, and a 50% reduction in neointima formation compared with vehicle controls (216). This is the first demonstration of anti-inflammatory and vasoprotective effects of *O*-GlcNAc protein modification in injured arteries.

Several putative mechanisms have been put forward to explain the increased tolerance to stress associated with increased *O*-GlcNAc levels. Increasing *O*-GlcNAc levels have been associated with increased transcription of HSP40 and HSP70 levels (228), and the latter is a known target for *O*-GlcNAc modification (199, 207). Whereas we found no change in HSP70 expression in cardiomyocytes after glucosamine treatment (21), PUGNAc treatment augmented HSP70 levels in response to oxidative stress (108). In the isolated perfused heart, cardioprotection is conferred after a very brief (5 min) exposure to glucosamine (65, 136), suggesting that *O*-GlcNAc-related protection does not necessarily depend on de novo protein synthesis. Activation of *O*-GlcNAc formation attenuates mPTP opening, a critical step in the initiation of apoptosis and cell death (20, 108, 161, 162). Glucosamine, OGT overexpression, and *O*-GlcNAcase inhibition all attenuate H_2O_2-induced loss of mitochondrial membrane potential and cyctochrome *c* release (20). We found that this was associated with increased mitochondrial Bcl-2 levels; Bcl-2 inhibits mPTP opening, possibly by a direct interaction with VDAC, one of the putative components of mPTP (196, 197). Interestingly, cardiac mitochondria isolated from PUGNAc-treated mice and also OGT-overexpressing neonatal rat cardiomyocytes are resistant to Ca^{2+}-induced mPTP formation, whereas inhibition of OGT increased the sensitivity to Ca^{2+}-induced mitochondrial swelling (108, 161, 162). One of the *O*-GlcNAc-modified proteins identified as a potential *O*-GlcNAc target is VDAC, and it has been hypothesized this modification of VDAC may preserve mitochondrial integrity by interfering with mPTP formation (108).

A number of other pathways may also contribute to *O*-GlcNAc-mediated cardioprotection. For example, increasing *O*-GlcNAc levels with glucosamine attenuated the ischemia-induced increase in p38 MAPK phosphorylation, decreased ischemic contracture, and reduced incidence of reperfusion-induced arrhythmias (65). Paradoxically, at the end of reperfusion, phosphorylated p38 levels were increased in response to glucosamine treatment. This could lead to the activation of prosurvival pathways through downstream effectors, such as αB-crystallin and HSP27, both of which have been shown to play a role in ischemic protection (58, 96, 129, 150, 174) and are also targets for *O*-GlcNAc modification (178, 208, 209). Preliminary studies have also shown that ischemia-reperfusion alters the level of *O*-GlcNAc modification of glycogen phosphorylase b, mitochondrial aconitase 2, and the cytoskeletal protein vinculin (124). It remains to be determined whether changes in *O*-GlcNAcylation of these proteins alter the functional response to ischemia-reperfusion injury. Increased levels of *O*-GlcNAc have also been reported to inhibit protein degradation (83, 225), most likely due to inhibition of the proteasome (229), and this could contribute to enhanced cell survival. It is also possible that cardioprotection is mediated via inhibition of Ca^{2+} overload on reperfusion (135).

Inflammation plays an important role in the pathogenesis of many forms of cardiovascular disease, and a number of studies have demonstrated that acute increases in *O*-GlcNAc attenuate the inflammatory response induced by tissue injury and stress (165, 215–217, 231, 232). Glucosamine treatment during resuscitation significantly attenuates the trauma-hemorrhage-induced increase in ICAM-1 expression, IκB-α phosphorylation, NF-κB expression, and NF-κB DNA-binding activity in the heart (231), suggesting that elevated *O*-GlcNAc levels may contribute to the downregulation of the NF-κB pathway. Glucosamine treatment in rat aortic smooth muscle cells enhances *O*-GlcNAc levels and inhibits TNF-α-stimulated chemokine and adhesion molecule (ICAM-1 and VCAM-1) expression, IκB-α phosphorylation, and NF-κB activation (215). In isolated cardiomyocytes, both glucosamine and OGT overexpression increase *O*-GlcNAc levels, attenuate LPS-induced TNF-α and ICAM-1 expression, and decrease IκB-α phosphorylation and nuclear NF-κB levels (230). Conversely, knockdown of OGT decreases *O*-GlcNAc levels and enhances the LPS-induced increase in IκB-α phosphorylation (230). These results indicate that modulation of cellular *O*-GlcNAc signaling events alters the response to activation of NF-κB signaling and suggest that attenuation of NF-κB signaling may contribute to the protection associated with increased protein *O*-GlcNAc levels in vivo.

Low-grade inflammation has been closely associated with both insulin resistance and the progression of type 2 diabetes as indicated by increased levels of cell adhesion molecules (e.g., VCAM-1, ICAM-1, and E-selectin), interleukins (e.g., TNF-α), oxidative stress, and activation and translocation of NF-κB (74, 191, 203). Downregulation of the inhibitor of NF-κB kinase-β, a serine/threonine kinase involved in the phosphorylation of IκB, prevented the TNF-α-induced insulin resistance in skeletal muscle (7). Since augmentation of *O*-GlcNAc levels attenuated the activation of these inflammatory mediators in response to acute cardiovascular injury (215, 216, 231), it is possible that the increase in *O*-GlcNAc levels seen in diabetes may be beneficial by attenuating the proinflammatory response. However, it is important to note that the anti-inflammatory effect is associated with acute activation of *O*-GlcNAc; in contrast, in diabetes, there is a sustained increase in *O*-GlcNAc levels. Indeed, it has been reported that a sustained increase in HBP flux, leading to *O*-GlcNAcylation of the p65 subunit of NF-κB stimulated NF-κB-dependent promoter activation, a proinflammatory rather than an anti-inflammatory effect (106).

Thus, acute activation of pathways leading to *O*-GlcNAc formation mediates short-term protection in the cardiovascular system. This may occur via transcriptionally independent mechanisms, such as attenuation of Ca^{2+}-mediated stress re-

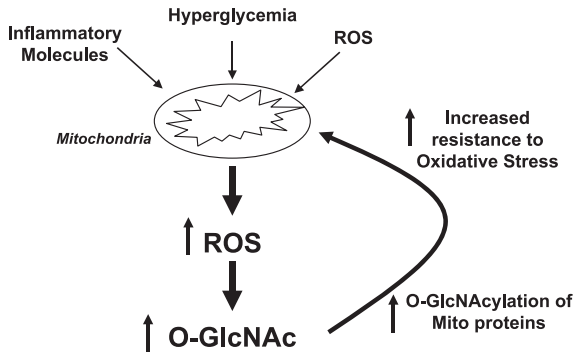

Fig. 3. Relationship between mitochondrial oxidative stress and *O*-GlcNAcylation. Increased production of mitochondrial ROS induced either by inflammatory mediators, hyperglycemic condition (e.g., diabetic milieu), or oxidant stress leads to an increase in *O*-GlcNAc levels. Increased *O*-GlcNAcylation of mitochondrial proteins (e.g., the voltage-dependent anion channel) in turn protects cells against lethal damage by increasing mitochondrial stability and tolerance in response to oxidative stress stimuli.

sponses or preservation of mitochondrial integrity. Longer-term cardiovascular protection may be facilitated via increased transcription of prosurvival factors such as HSPs or attenuation of inflammation-mediated responses. To better understand the mechanisms underlying the cardiovascular protection associated with *O*-GlcNAc will require further research to determine the impact of stress on the regulation of *O*-GlcNAc turnover as well as to identify the proteins that are subjects of *O*-GlcNAc modification in response to stress.

Cross-Talk Between Redox and O-GlcNAc Signaling

The generation of ROS is an inevitable byproduct of mitochondrial metabolism and also occurs in response to activation of oxidative enzymes such as NADPH oxidase and xanthine oxidase as well as in response to a wide array of stress stimuli. It is now widely recognized that rather than just leading to nonspecific modifications of lipids and proteins, ROS and reactive nitrogen species play an important role as signal transducers and are integral in regulating redox cell signaling pathways (78, 125). An emerging concept in the field of redox biology is the concept of "redox tone" (78, 125), which suggests that cell signaling, metabolism, and cell survival decisions are linked to the antioxidant and thiol status of cells. Interestingly, several links between *O*-GlcNAc protein modification and oxidative stress suggest that *O*-GlcNAc signaling may represent an important redox-sensing pathway that could play integral roles in the response to oxidant stress and modulate redox tone.

It is well established that numerous stress stimuli including inflammatory molecules, hyperglycemia, and ROS themselves lead to an increase in mitochondrial ROS production (78). One consequence of this is increased flux through the HBP and an increase in *O*-GlcNAc synthesis (53, 54), which, in turn, leads to an increase in *O*-GlcNAcylation of mitochondrial proteins and increased tolerance of mitochondria to stress (108, 162). Furthermore, it has been suggested that inhibition of GAPDH by mitochondrial superoxide was an important factor leading to increased *O*-GlcNAcylation (53, 54). However, given our relatively limited understanding of the regulation of GFAT, OGT, and *O*-GlcNAcase, it is also possible that their activities may be directly modified in response to ROS. Nevertheless, hyperglycemia-induced increases in HBP flux and the subsequent increases in *O*-GlcNAcylation of the transcription factor Sp1 and eNOS in cultured endothelial cells have been linked to increased mitochondrial superoxide production (53, 54). Furthermore, recent studies have shown that augmentation of *O*-GlcNAc levels in cardiomyocytes attenuates H_2O_2-induced loss of mitochondrial membrane potential (20, 108, 162) and that this may be due to *O*-GlcNAcylation of mitochondrial proteins such as VDAC. In settings where *O*-GlcNAcylation was shown to mediate cell survival (228), the stresses used to increase *O*-GlcNAc levels, such as UV, cobalt chloride, ethanol, and heat shock, are all associated with increased ROS production. Taken together, these studies suggest that ROS and mitochondria may contribute to the regulation of *O*-GlcNAc synthesis, which could, in turn, modulate the response to oxidative stress, as shown in Fig. 3.

NO is one of the most widely recognized reactive species involved in redox signaling, and NOS is subject to both phosphorylation and *O*-GlcNAcylation, indicating a potential

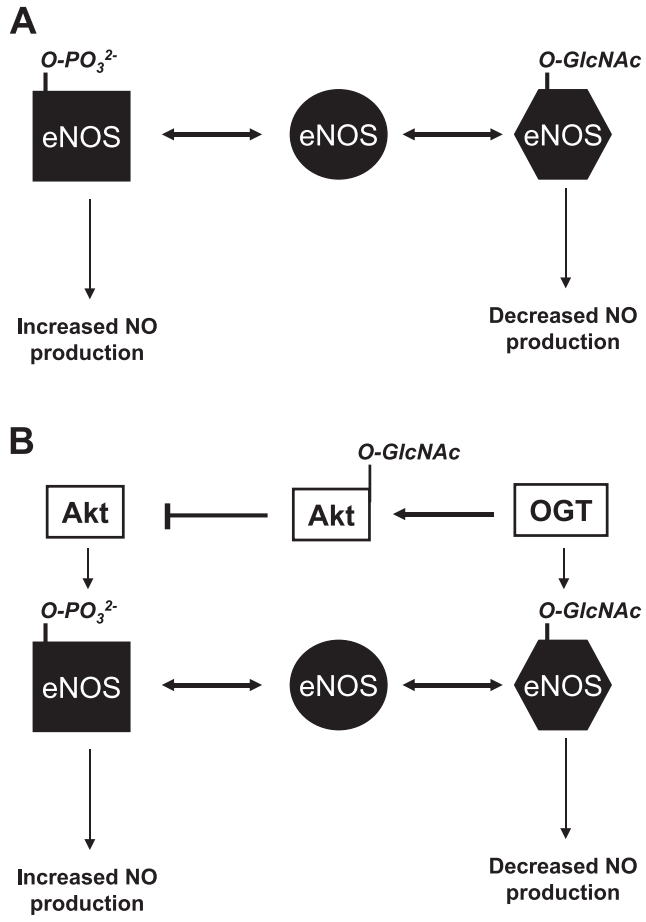

Fig. 4. Cross-talk between *O*-GlcNAc, phosphorylation, and redox signaling. *A*: phosphorylation of endothelial nitric oxide (NO) synthase (eNOS) at Ser^{1177} results in increased eNOS activity and NO production, whereas *O*-GlcNAcylation of the same site leads to decreased enzyme activity and NO production. *B*: theoretically, *O*-GlcNAc-dependent reactions are not limited to interactions with specific proteins but rather act to regulate an entire pathway. For example, *O*-GlcNAc modification of eNOS decreases its activity and NO production. At the same site, Akt-mediated phosphorylation and activation of eNOS leads to increased NO-production. However, Akt is also subject to *O*-GlcNAcylation, which reduces its activity, thereby inhibiting eNOS phosphorylation and NO production.

for cross-talk between redox and *O*-GlcNAc signaling (Fig. 4*A*). eNOS is activated by Akt-mediated phosphorylation, thereby increasing NO production. This Akt phosphorylation site (Ser^{1177}) is also subject to *O*-GlcNAcylation, leading to lower activity and decreased NO production (53). Thus, the modulation of NO production by *O*-GlcNAc is one example of the convergence of redox signaling with *O*-GlcNAc protein modification.

The intersection of redox and *O*-GlcNAc signaling may apply not only to a specific protein such as eNOS but could also be involved in the regulation of an entire signaling pathway. For example, in the insulin signaling pathway, a number of components, including IR-β, IRS-1/2, PI3K, Akt, and GSK-3β, have all been reported to be *O*-GlcNAcylated, and the result for each is lower activity and an attenuated response to insulin (63). Insulin signaling is also regulated by NO, the production of which could be regulated by direct *O*-GlcNAcylation of NOS or upstream activators, as described above. Thus, increasing flux through OGT is envisaged to decrease NO production not only by direct modification of NOS but also by reducing NOS phosphorylation via *O*-GlcNAcylation of the IR complex and Akt. Finally, the transcription factor FoxO1, which functions as a nutrient and stress sensor and plays a regulatory role in diabetes, has been recently shown to be *O*-GlcNAcylated (99). Regulation of FoxO1 by *O*-GlcNAc exemplifies the interlacing of *O*-GlcNAc modification with redox tone; *O*-GlcNAc modification activates FoxO1, which can, in turn, activate the transcription of oxidative stress response enzymes such as catalase and MnSOD (99). Collectively, these studies underscore a definitive role of *O*-GlcNAc in modulating redox biology and suggest that *O*-GlcNAc can regulate redox tone and mediate responses to oxidative stress.

Conclusions

O-GlcNAc modification of proteins is emerging as a new signaling mechanism that regulates cell function and plays a particularly critical role in mediating the response of cells to stress. The function of the majority of cell types that comprise the cardiovascular system, including endothelial cells, smooth muscle cells, and cardiomyocytes, is influenced by changes in *O*-GlcNAc levels. However, a number of fundamental issues remain to be understood. In the heart, *O*-GlcNAc levels respond rapidly (i.e., within minutes) to activation of the HBP with glucosamine or inhibition of *O*-GlcNAcase with PUGNAc (135, 136). This indicates that the *O*-GlcNAc "on/off" cycle is a highly dynamic process that contributes to acute regulation of cell function in response to both physiological and pathophysiological stimuli. However, our knowledge regarding the regulation of *O*-GlcNAc synthesis and degradation in the heart is remarkably limited. For example, the increase in *O*-GlcNAc that occurs in response to ischemia (65, 124) could be due to an increase in flux through the HBP driven in part by increased glycogenolysis, increased GFAT activity, or both. Alternatively, given the presence of such an active *O*-GlcNAc cycle, ischemia could indirectly or directly modulate *O*-GlcNAc enzymes, such that activation of OGT and/or inhibition of *O*-GlcNAcase could lead to a rapid increase in *O*-GlcNAc levels independent of changes in the HBP. The potential interaction between *O*-GlcNAcylation, ROS, and mitochondria is particularly intriguing and suggests that *O*-GlcNAc signaling may play a role in intracellular redox sensing and, conversely, that ROS may be involved in regulating cellular *O*-GlcNAc levels. Whatever the case may be, *O*-GlcNAcylation is undoubtedly an integral component of the complex signaling network involved in regulating cellular response to both physiological and pathophysiological stimuli, thereby playing a critical role in mediating cell survival. Our challenge now is to elucidate more fully how *O*-GlcNAc signaling works to develop treatments for cardiovascular disease.

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