O-GlcNAcylation: a novel pathway contributing to the effects of endothelin in the vasculature

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Department of Physiology, Medical College of Georgia, Augusta, Georgia; Department of Pharmacology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil; Department of Surgery, Medical College of Georgia, Augusta, Georgia; and Department of Pharmacology, School of Medicine of Ribeirao Preto, University of Sao Paulo, Sao Paulo, Brazil

Lima VV, Giachini FR, Hardy DM, Webb RC, Tostes RC. O-GlcNAcylation: a novel pathway contributing to the effects of endothelin in the vasculature. Am J Physiol Regul Integr Comp Physiol 300: R236–R250, 2011. First published November 10, 2010; doi:10.1152/ajpregu.00230.2010.—Glycosylation with O-linked β-N-acetylglucosamine (O-GlcNAc) or O-GlcNAcylation on serine and threonine residues of nuclear and cytoplasmic proteins is a posttranslational modification that alters the function of numerous proteins important in vascular function, including kinases, phosphatases, transcription factors, and cytoskeletal proteins. O-GlcNAcylation is an innovative way to think about vascular signaling events both in physiological conditions and in disease states. This posttranslational modification interferes with vascular processes, mainly vascular reactivity, in conditions where endothelin-1 (ET-1) levels are augmented (e.g., salt-sensitive hypertension, ischemia/reperfusion, and stroke). ET-1 plays a crucial role in the vascular function of most organ systems, both in physiological and pathophysiological conditions. Recognition of ET-1 by the ET\(_A\), and ET\(_B\) receptors activates intracellular signaling pathways and cascades that result in rapid and long-term alterations in vascular activity and function. Components of these ET-1-activated signaling pathways (e.g., mitogen-activated protein kinases, protein kinase C, RhoA/Rho kinase) are also targets for O-GlcNAcylation. Recent experimental evidence suggests that ET-1 directly activates O-GlcNAcylation, and this posttranslational modification mediates important vascular effects of the peptide. This review focuses on ET-1-activated signaling pathways that can be modified by O-GlcNAcylation. A brief description of the O-GlcNAcylation biology is presented, and its role on vascular function is addressed. ET-1-induced O-GlcNAcylation and its implications for vascular function are then discussed. Finally, the interplay between O-GlcNAcylation and O-phosphorylation is addressed.

endothelin receptor; vascular signaling; O-GlcNAc modification

GLYCOSYLATION IS THE SITE-specific enzymatic addition of saccharides to proteins and lipids. There are many types of glycosylation, but great interest has been directed to O-GlcNAcylation, or glycosylation with O-linked β-N-acetylglucosamine or O-linked 2-acetamido-2-deoxy-o-glycopyranose (54, 55, 169). In this unusual form of protein glycosylation, a single sugar [β-N-acetylglucosamine (O-GlcNAc)] is added to serine and threonine residues of nuclear or cytoplasmic proteins (54, 55, 169).

Considering that almost every functional class of proteins is subject to O-GlcNAcylation (55, 177), this specific posttranslational modification has been implicated in several biological functions, including transcription or translation, stress responses, and energy metabolism. Proteins with an important role in vascular function are also targets for O-GlcNAcylation. Examples include endothelial nitric oxide (NO) synthase (eNOS), sarcoplasmic reticulum calcium (Ca\(^{2+}\))-ATPase (SERCA), phospholipase C (PLC), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and proteins involved in cytoskeleton regulation and microtubule assembly (22, 55, 177), indicating that this posttranslational modification may play an important role in vascular (dys)function. Accordingly, augmented levels of O-GlcNAc in the vasculature increases vascular reactivity to constrictor stimuli (91) and decreases endothe-lium-dependent vasodilation, which is associated with O-GlcNAc modification of eNOS (90, 91). In addition, augmented levels of O-GlcNAc have been found in the vasculature of mineralocorticoid hypertensive animals (93) and may play a role on the vascular abnormalities in salt-sensitive hypertension. On the other hand, Xing et al. (173) have shown that O-GlcNAc modification of proteins may inhibit acute inflammatory and neointimal responses to endoluminal arterial injury, suggesting that O-GlcNAcylation may display cardioprotective and anti-inflammatory effects in arteries subjected to acute endoluminal injury.

It is well recognized that the endothelin (ET) system plays an important role in vascular dysfunction in many diseases, including arterial hypertension, stroke/cerebral vasospasm, renal failure, and diabetes. ET-1 binds the ET\(_A\) and ET\(_B\) receptors and activates intracellular signaling pathways that result in rapid and long-term alterations in cell activity and function. In the vasculature, ET-1 induces vasoconstriction through its binding to the ET\(_A\) and ET\(_B\) receptors from vascular smooth muscle cell, and also activates transcriptional factors responsible for the coordinated increase in many cytokines and enzymes, thus enhancing inflammation, oxidative stress, fibrosis, and tissue damage (65, 139, 140, 155). All of these are important in the regulation of vascular tone, vascular injury, and remodeling.

ET receptor activation leads to diverse cellular responses through interaction with pertussis toxin-sensitive and insensitive pathways, indicating that multiple G proteins are involved (60, 61, 109). Consequently, ET-1 binding to ET receptors on vascular smooth muscle cells stimulates signaling cascades that include PKC, mitogen-activated protein kinases (MAPKs), and RhoA/Rho kinase with subsequent effects on intracellular Ca\(^{2+}\) and calmodulin-dependent pathways as well as on Ca\(^{2+}\)-independent pathways (12, 16, 47) (Fig. 1). Most recently, it has been shown that ET-1 also produces O-GlcNAcylation of vascular proteins, and this modification mediates some of the vascular effects produced by the peptide (92).

In this mini-review we will discuss how this relatively novel posttranslational modification of proteins, O-GlcNAcylation, may impact vascular function and ET-1 actions. The readers will be presented with a short description of the O-GlcNAcylation biology and a brief view of classical aspects of the ET-system. They will be referred to excellent reviews on these specific topics (6, 8, 11, 12, 30, 31, 34, 37, 67, 79, 81, 115, 122, 131, 141, 142, 154, 160, 166).

**O-GlcNAcylation**

**Biology of the O-GlcNAc modification.** The cycling of O-GlcNAc on serine or threonine residues of target proteins is controlled by two highly conserved enzymes, O-GlcNAc transferase [OGT or uridine diphospho-N-acetyl glucosamine; polypeptide β-N-acetylglucosaminyl transferase; uridine-diphosphate (UDP)-N-acferase] and β-N-acetylglucosaminidase (OGA or O-GlcNAcase). Whereas OGT catalyses the addition of O-GlcNAc to the hydroxyl group of serine or threonine residues of a target protein, OGA catalyses the hydrolytic cleavage of O-GlcNAc from posttranslationally modified proteins (55, 97, 167).

The OGT enzyme is a soluble protein that is found in the cytosol, nucleus, and mitochondria rather than in the endoplasmic reticulum or Golgi (97). Three distinct isoforms of OGT have been identified (70, 83, 86, 97). They contain a COOH-terminal catalytic domain, and different numbers of tetra-tripeptide repeats within its NH\(_2\)-terminal domain. The tetra-tripeptide repeats serve as protein-protein interaction modules that appear to target OGT to accessory proteins and potential substrates, such as the related OGT interacting protein (OIP106) and protein phosphatase-1 (68, 168). The association between OGT and protein phosphatase-1 is particularly intriguing because it may provide a direct mechanism to couple O-GlcNAc to dephosphorylation of specific substrates.

Overall catalytic activity of OGT is controlled by the concentration of its donor substrate, UDP-GlcNAc or uridine-

![Fig. 1. Signaling pathways activated by endothelin-1 (ET-1) in vascular smooth muscle cells (VSMC). Recognition of ET-1 by the ET\(_A\) and ET\(_B\) receptors in VSMCs activates intracellular signaling pathways and cascades that result in rapid alterations in cell activity and function and initiates transcriptional responses. Highlighted in the figure is the activation of PKC, MAPKs, and RhoA/Rho kinase signaling pathways, with subsequent effects on intracellular Ca\(^{2+}\) and calmodulin-dependent pathways as well as Ca\(^{2+}\)-independent pathways. IP\(_3\), inositol 1,4,5-trisphosphate; SRC, src; P, phosphorylation; Pyk2, proline-rich tyrosine kinase-2; GDI, GDP dissociation inhibitors; MEK, MAPK kinase; MLC, myosin light chain; IP\(_3\)R, IP\(_3\) receptor; SR, sarcoplasmic reticulum.](http://ajpregu.physiology.org/)

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diphosphate-N-acetylglucosamine. UDP-GlcNAc is highly sensitive to flux in nutrients and energy, mainly through the hexosamine biosynthetic pathway (HBP) (14, 100). Increased flux through the HBP, either through increased glucose uptake or glucosamine treatment, increases the production of UDP-GlcNAc and stimulates O-GlcNAc modification of proteins (96) (Fig. 2).

Glutamine fructose-6-phosphate transferase is the rate-limiting enzyme of the HBP, converting fructose 6-phosphate to glucosamine 6-phosphate, with glutamine as the amine donor. Because OGT activity is exquisitely sensitive to UDP-GlcNAc concentrations (52), O-GlcNAcylation may act as a sensor for the general metabolic state of the cell. Consistent with this idea, O-GlcNAc has been intricately linked to cell survival (111, 113) induced by many forms of cell stress (19, 178).

OGA is also found in the nucleus and cytosol (167). OGA contains an NH2-terminal glycosidase domain and a putative COOH-terminal histone acetyltransferase domain (153).

To date, two distinct isoforms of OGA have been described: a 130-kDa and a 75-kDa variant, which differ in their COOH terminus. Whereas the 130-kDa or long OGA contains a distinct NH2-terminal glycosidase domain and the COOH-terminal histone acetyltransferase domain, the 75-kDa or short OGA lacks the COOH-terminal domain. One important functional aspect in the existence of these two splices is their differential sensitivity to previously described potent OGA inhibitors. For example, the short OGA exhibits comparative resistance to PUGNAc and NAG-thiazoline, but is very sensitive to α-GlcNAc thiosulfonate (27, 77).

NH2-OGA is also glycosylated by OGT (86, 170). The OGA catalytic site contains a deep pocket that accommodates the glycosylated protein (85). The potential histone acetyltransferase activity of OGA may provide an interesting mechanism for coupling deglycosylation of nuclear proteins to transcriptional activation. As with OGT, OGA has been shown to interact with specific proteins, including protein phosphatase-2B (167).

O-GlcNAcylation and Vascular Function

Over the past decade, a surge of discoveries in O-GlcNAcylation has revealed new roles for this modification in the cardiovascular system. However, one of the central challenges, specifically to the vasculature, will be to understand the unique molecular and cellular O-GlcNAc signaling as it relates to function. In this sense, recent studies have demonstrated that many proteins important in the cardiovascular system are targets for O-GlcNAcylation (44). So far, few studies have addressed the impact of augmented O-GlcNAc levels on vascular function or whether chronically elevated levels of O-GlcNAc play a role in vascular dysfunction.

Considering that proteins, such as eNOS, PKC, SERCA, PLC, and PI3K, which directly modulate vascular tone, are all targets for O-GlcNAcylation (44, 55, 96, 177) (Fig. 3), we hypothesized that this posttranslational modification plays an important role in vascular reactivity. Accordingly, we have reported that eNOS and PKC are targets for O-GlcNAcylation in the vasculature (53, 90, 91, 93, 127) and that increased modification of vascular proteins by O-GlcNAc augments reactivity to a contractile stimulus (93). Our studies indicate that O-GlcNAcylation is indeed important to vascular function and may be an additional mechanism that controls vascular reactivity.

Fig. 2. Hexosamine biosynthetic pathway (HBP). Enzymes involved in the synthesis of uridine-diphosphate-O-GlcNAcylation (UDP-O-GlcNAc) from glucose and glucosamine and the O-GlcNAc modification of proteins are shown. Depicted in the figure is the interplay between O-GlcNAc and O-phosphorylation.
The dynamic nature of O-GlcNAc is a unique characteristic that distinguishes it from other forms of glycosylation. This feature has important implications for the regulation of protein structure and function and for the interplay with other post-translational modifications. We will briefly focus on a few specific pathways (PKC, MAPK, and RhoA/Rho kinase) that are important to vascular function and discuss the implications of their interaction with O-GlcNAc (Fig. 3). These signaling pathways are all activated by ET-1 and have been closely implicated in vascular dysfunction associated with augmented levels and activity of this peptide. A brief overview of classical aspects of the ET system will be provided before we discuss O-GlcNAc modification of these signaling pathways activated by ET-1.

**ET System**

Classical biology of the ET system. The discovery of ET 21 years ago (174) led to a virtual explosion of information on the actions of ET peptides. ET-1 plays a crucial role in the vascular function of most organ systems including the lung, kidneys, heart, and brain, both in physiological and disease conditions. In addition, the diverse presence of ET in physiological systems, as well as the physio(patho)logical changes observed with manipulations of the ET system, has implicated ET-1 in multiple diseases, including hypertension, stroke/cerebral vasospasm, renal failure, diabetes, inflammation, fibrosis, scleroderma, pain, sexual dysfunction, and glaucoma.

The ET family comprises three 21-amino acid isopeptides (ET-1, ET-2, and ET-3), which are structurally similar to the snake venom sarafotoxins. A fourth isoform (ET-4) has been reported in the rat and mouse as the analog to human ET-2. ETs are derived from gene products of 212-amino acids, or prepro-ETs, which are cleaved to big ETs. The pro-polypeptides are processed by ET-converting enzymes, or membrane-bound zinc metalloproteases, generating the biologically active ETs and a COOH-terminal fragment (66, 174).

The ET isopeptides, which are differentiated by their ET receptor-binding selectivity and characteristics, bind to two cell surface G protein-coupled receptors: ET subtype A (ETA) and ET subtype B (ETB) receptors. While ET-1 and ET-2 bind both ETA and ETB receptor subtypes with equal affinity, ET-3 has much higher affinity for ETB receptors. A third receptor subtype, ETC, has been cloned from *Xenopus laevis* oocytes, but a homologue was not identified in mammalian tissues (4, 33, 133). ET receptors exhibit a broad tissue distribution and are expressed in the vasculature, kidneys, heart, brain, liver, lungs, and skin, reinforcing the importance and wide-ranging biological activities of the ET system. Within the vasculature, ETA and ETB receptors, located on vascular smooth muscle cells, mediate the vasoconstrictor effects of ET-1, the major vascular isoform and of most importance in the cardiovascular system (58). ETB receptors are also found on vascular endothelial cells, where their activation results in vasodilation mediated mainly by NO (35, 157).

ETA receptors are functionally coupled to Gq/11 protein to activate PLC, and to Gi protein to inhibit adenylyl cyclase (128). Binding of ET-1 to the ETA receptor induces a conformational change in the receptor that allows guanosine triphosphate (GTP) binding to the α-subunit of the receptor associated-trimeric Gq/11 protein. Activation of Gq/11-α results in dissociation from the βγ-complex and the initiation of downstream G protein signaling. ETA receptor-mediated activation of Gq/11 protein and PLC results in phosphatidylinositol 4,5-bisphosphate breakdown and the generation of inositol 1,4,5-trisphosphate and diacylglycerol (DAG). Inositol 1,4,5-trisphosphate acts on specific receptors on the intracellular Ca^{2+} stores and stimulates Ca^{2+} release (109). ET-1 also activates plasma...
membrane Ca\textsuperscript{2+} channels and stimulates Ca\textsuperscript{2+} influx (23, 147). ET-1-induced increase in DAG stimulates PKC activity (73, 106, 146). PKC and Ca\textsuperscript{2+}/calmodulin-dependent protein kinase activate receptor and nonreceptor tyrosine kinases, such as Src and proline-rich tyrosine kinase-2. ETA receptor stimulation also activates phospholipase D with generation of DAG, phospholipase A\textsubscript{2} with release of arachidonic acid, the Na\textsuperscript+/H\textsuperscript{+} exchanger, Src family tyrosine kinases, MAPKs, PI3K, and RhoA/Rho kinase (16, 32, 138, 175). Additional information on these pathways will be presented in the next topic (Signaling Pathways Activated by ET-1 That Can Be Modified by O-GlcNAc). Of interest, ETA and ET\textsubscript{B} receptors have also been described in nuclear membranes from human aortic vascular smooth muscle cells (Syto\textsubscript{11}) (7). Nuclear ET\textsubscript{B} receptor activation induces an increase in nuclear Ca\textsuperscript{2+}, which relies on both extracellular Ca\textsuperscript{2+} influx and intracellular Ca\textsuperscript{2+} release. It has been speculated that nuclear ET receptors may contribute to the long-lasting effects of ET-1 (7). Novel aspects on ET receptor pharmacology can be found in two very elegant articles by Dr. Stephanie Watts (166) and Dr. Erika Boesen (11). These authors focused on the interactions between ET receptors (formation of heterodimers and homodimers, which may explain some puzzling results from receptor-binding and functional studies) (11, 166), the possibility of differential signaling or biased agonists (that different agonists can elicit different signal transduction events through the same receptor), and interactions of components of the ET system with other molecules, e.g., the dual angiotensin II-ET receptor and the Parkin-associated ET receptor-like receptor (where the ET receptor B-like protein receptor 1 functions as a substrate for Parkin, a polypeptide with similarities to ubiquitin protein ligase E3) (166).

With a plasma half-life of 1 min, ET-1 is removed from the circulation through receptor- and non-receptor-mediated mechanisms (46). ET\textsubscript{B} receptors have a major role in the clearance of circulating ET-1. Binding of ET-1 to ET\textsubscript{B} receptors induces ligand-receptor complex internalization and intracellular degradation accounting for the majority of ET-1 clearance, particularly in the pulmonary circulation (39). Although ET-1 has a short half-life, this peptide has both short- and long-term effects in the vasculature, including contraction, cell growth, proliferation, differentiation, inflammation, and oxidative stress generation (47).

The rapid discovery and success of ET receptor antagonists and ET-converting enzyme inhibitors in preventing or attenuating important signs and symptoms of disease in various experimental models involving the cardiovascular, nervous, respiratory, gastrointestinal, urinary, and reproductive systems have led to human studies. Numerous clinical studies and successful trials have already resulted in FDA approval of selective and nonselective ET receptor antagonists for therapeutic treatment of pulmonary hypertension and, more recently, treatment of digital ulcers seen in scleroderma, for example. Great attention has been directed to signaling pathways activated by the interaction of ET-1 with its receptors since new potential therapies to treat vascular dysfunction associated with multiple diseases may arise from such investigational approach.

**Signaling Pathways Activated by ET-1 That Can Be Modified by O-GlcNAc**

**PKC pathway.** PKC, originally described as a Ca\textsuperscript{2+}-activated phospholipid-dependent protein kinase, is involved in signaling pathways that regulate cell growth, apoptosis, ion channel activities, and actin cytoskeleton (13). The PKC family constitutes a group of multifunctional Ser/Thr protein kinases that are classified into three groups: the classic PKCs (PKC\textgreek{a}, PKC\textgreek{b}I, PKC\textgreek{b}II, PKC\textgreek{y}), the novel PKCs (PKC\textgreek{b}, PKC\textgreek{e}, PKC\textgreek{η}, PKC\textgreek{μ}, PKC\textgreek{θ}), and the atypical PKCs (PKC\textgreek{ζ}, PKC\textgreek{λ}) (134).

Functional studies have demonstrated that interaction of PKC with its protein substrate triggers activation of a cascade of kinases that ultimately stimulate vascular contraction. PKC phosphorylates CPI-17, which in turn inhibits myosin light chain (MLC) phosphatase, increases MLC phosphorylation and enhances vascular smooth muscle contraction. PKC also phosphorylates the actin-binding protein calponin, and thereby reverses its inhibition of actin-activated myosin ATPase, allowing more actin to interact with myosin and increases vascular contraction (13, 134, 171). PKC activation not only mediates ET-1-induced vascular contraction (2, 16, 123) and regulates ET-1 receptors expression (112), but is intimately linked to ET-1-mediated regulation of hypertrophy, growth, proliferation, and survival in vascular smooth muscle cells (12). PKC activation (PKC-δ), via phosphorylation of STAT3, also mediates ET-1-induced decreases in arterial eNOS protein levels and NO generation (150) as well as ET-1-induced vascular formation of superoxide anion (103).

Many PKC isoforms influence vascular function. Accordingly, PKC-\textgreek{α} enhances Ca\textsuperscript{2+}-dependent vascular smooth muscle contraction (76, 95, 134), the Ca\textsuperscript{2+}-independent PKC-\textgreek{ε} increases myofilament sensitivity to Ca\textsuperscript{2+} in vascular smooth muscle cells (162); PKC-\textgreek{δ}, mainly associated with the cytoskeleton, plays a role in vascular remodeling (74); and PKC-\textgreek{ζ}, localized in the nucleus, induces vascular growth (95, 134). Therefore, abnormal regulation of PKC isoforms may interfere with important vascular processes. In this sense, increased PKC activity has been implicated in the development of cardiovascular diseases, such as arterial hypertension.

In the following section, O-GlcNAc modification of PKC isoforms, which can interfere with vascular processes regulated by these enzymes, will be discussed.

**PKCS AND O-GLCNAC.** It is important to determine whether components of vascular signaling pathways, such as PKC, normally activated by vasoactive agents, such as ET-1, can interfere with O-GlcNAcylation and vice-versa. Accordingly, early studies indicated that activation of PKC or cAMP-dependent protein kinase significantly decreased overall O-GlcNAcylation in neuronal cytoskeletal proteins (49). Conversely, inhibition of PKC, cAMP-dependent protein kinase, cyclin-dependent protein kinases, or S6 kinase increased overall O-GlcNAc levels in fractions from these cells. Stimulation of the transactivation of Spi, which is O-GlcNAcylation dependent, can be blocked by molecular and pharmacological inhibition of PKC (42). In cerebellar neurons from early postnatal mice, activation of cAMP-dependent protein kinase or PKC results in reduced levels of O-GlcNAc specifically in the fraction of cytoskeletal and cytoskeleton-associated pro-
tein inhibitors, whereas inhibition of the same kinases results in increased levels of O-GlcNAc (50).

In the reverse direction, all PKC isoforms expressed in rat hepatocytes are dynamically modified by O-GlcNAc. O-GlcNAcylation of PKC-α negatively correlates with enzyme activity (129). Increased O-GlcNAc modification in a human astroglial cell line, in response to glucosamine (which increases the production of glucosamine 6-phosphate and stimulates O-GlcNAc modification of proteins) or PUGNac (which blocks OGA activity, mimicking the enzyme-stabilized transition state), results in a decrease in membrane-associated PKC-ε and PKC-α, but not PKC-κ, indicating that increased levels of the O-GlcNAc modification regulates specific PKC isoforms (104).

Data from our laboratory, obtained by immunoprecipitation assays with vascular segments, show that different PKC isoforms, including PKC-α, -βI, and -ε, are targets for O-GlcNAc modification (90). Additionally, aortas incubated with PUGNac display increased contractile responses to phenylephrine, which are prevented by GF109203X, a PKC inhibitor. The addition of GF109203X (10 μM) in arteries contracted with phenylephrine results in a time-dependent inhibition of the contractile response, which is greater in arteries incubated with PUGNac. Finally, contraction induced by PDBu (10 nM), a PKC activator, is augmented in aortas incubated with PUGNac, compared with control (53). Myristoylated alanine-rich C kinases (MARCKs) represent a specific substrate for PKC, and MARCK phosphorylation is considered a direct measure of PKC activity (45, 48, 57, 121). MARCK is a regulator of the spatial distribution of calmodulin within the cytoplasm of smooth muscle cells (45, 152). In agreement with our functional data, MARCKs phosphorylation is increased in aortas incubated with PUGNac, indicating that increased O-GlcNAc modification contributes to increased PKC activation (90). Together, these results suggest that increased vascular O-GlcNAcylation increases activation of PKC, contributing to augmented vascular contraction. Therefore, it is likely that O-GlcNAc modification of PKC isoforms found in the vasculature, such as PKC-α, PKC-β, PKC-γ, PKC-ε, and PKC-ζ (134) can interfere with vascular processes regulated by these enzymes.

MAPKs. MAPKs are a family of serine/threonine kinases that are classically associated with vascular smooth muscle cell contraction, migration, adhesion, collagen deposition, cell growth, differentiation, and survival (119). Of the major MAPKs, extra-cellular signal-regulated kinases (ERK1/2), p38 MAPK, and stress-activated protein kinase/c-Jun NH2-terminal kinases (SAPK/JNK) are the best characterized. The complex signaling networks that underlie MAPK activation typically require phosphorylation by a MAPK kinase also known as MEK. The ERK1/2 phosphorylation cascade involves MEK1/2 (MAP/ERK kinase), whereas the signaling processes leading to SAPK/JNK and p38 MAPK activation involve MEK4/7 and MEK3/6, respectively (119). Activation of MAPKs has been reported to be primarily dependent on the nonreceptor tyrosine kinase c-Src in different cell types. To date, at least 14 Src-related kinases have been identified, of which the 60-kDa c-Src is the most abundantly expressed isoform in vascular smooth muscle cells and rapidly activated by G protein-coupled receptors. Other proximal regulators of MEK include the Ras-Raf pathway, which may not necessarily involve c-Src (82, 101, 114).

G protein-coupled receptors can also signal by interacting with various small G proteins that are generally classified by structural similarity into five subfamilies: Ras, Rho, Arf, Rab, and Ran family GTPases (9, 99). The biological effects of Ras proteins are exerted through the activation of several downstream effectors, including Raf, Rac, PI3K, and Ras. Raf stimulation of the serine/threonine kinase Raf1 is followed by activation of the downstream kinase MEK1/2, which in turn phosphorylates ERK1/2 (9, 99). These events may bypass c-Src.

MAPKs activation mediates important ET-1-induced vascular effects, such as contraction of vascular smooth muscle cells (16, 20, 87), NADPH oxidase-driven superoxide production, and p47(phox) upregulation (107, 130), cellular hypertrophy, growth, and inflammatory process (12).

Both ERK1/2 and p38 MAPKs are involved in ET-1-induced vascular smooth muscle cell contraction and proliferation. Whereas PKC mediates ERK1/2 activation induced by ET-1 in human vascular smooth muscle cells (20), spleen tyrosine kinase (Syk) mediates p38 activation in rat aortic smooth muscle cells (87). In human endothelial cells, ET-1 induced Nox5 activation, via Ca2+/calmodulin-dependent, Rac-1-independent mechanisms, potentially leads to growth and inflammatory responses (107). ET-1 induces c-Raf phosphorylation in vascular smooth muscle cells, and this event is not affected by c-Src knockdown, suggesting that ET-1-induced phosphorylation of MAPKs might be mediated by c-Src-independent and Ras-Raf-dependent mechanisms (175).

MAPKs AND O-GlcNAc. The MAPKs p38 and ERK1/2 have been reported to be phosphorylated in response to increased O-GlcNAc levels (84). A positive correlation between phosphorylation of the MAPK cascade (ERK1/2 and p38) and nuclear O-GlcNAcylation was observed in fetal human cardiac myocytes exposed to high glucose (51). In isolated rat hearts, perfusion with 5 mM glucosamine increases O-GlcNAc levels and confers cardioprotection against ischemia-reperfusion (181). Interestingly, although glucosamine does not alter the response of either ERK1/2 or Akt (protein kinase B) to ischemia-reperfusion, it significantly attenuates the ischemia-induced increase in p38 phosphorylation, as well as the increased p38 phosphorylation at the end of reperfusion, suggesting that glucosamine-induced cardioprotection may be mediated via the p38 MAPK pathway (71).

Augmented O-GlcNAc levels in mouse hippocampal synapses increases phosphorylation of synapsin I/II at Ser9 (cAMP-dependent protein kinase substrate site), Ser62/67 [ERK1/2 (MAPK 1/2) substrate site], and Ser603 (calmodulin kinase II site). Activation-specific phosphorylation events on ERK1/2 and calmodulin kinase II are also increased in response to elevation of O-GlcNAc levels (126).

Advanced glycation end products induce ROS accumulation, apoptosis, MAPK activation, and nuclear O-GlcNAcylation in human cardiac myocytes (89). In addition, exposure of neutrophils to PUGNac or glucosamine also stimulates the small GTPase Rac, which is an important upstream regulatory element in p38 and ERK1/2 MAPK signaling in neutrophils, and these MAPKs are implicated in chemoattractant signal transduction.

Conversely, alterations in MAPK pathways can also have effects on the enzymes responsible for the regulation of O-GlcNAc (84). In neuro-2a neuroblastoma cells, increased OGT
expression on glucose deprivation occurs in an AMP-activated protein kinase-dependent manner, whereas OGT enzymatic activity is regulated in a p38 MAPK-dependent manner. OGT is not phosphorylated by p38, but rather it interacts directly with p38 through its COOH terminus. The interaction with p38 does not change the catalytic activity of OGT, but p38 regulates OGT activity within the cell by recruiting it to specific targets (21).

Together, these data indicate that O-GlcNAcylation is an important signaling element, and it modulates the activities of several critical signaling kinases (80). Thus, it is possible that signaling kinases, such as proteins from MAPK, PKC, and RhoA/Rho kinase pathways, are also regulated by O-GlcNAc modifications in the vasculature and that this posttranslational modification not only modulates vascular responses to constrictor stimuli, but also may play a role in the abnormal function of kinases involved in hypertension-associated vascular dysfunction.


An inverse functional relationship between NO and RhoA/Rho kinase signaling pathways is well established (10). Accordingly, Rho-kinase activation suppresses eNOS expression, and decreased sensitivity of contractile proteins to Ca\(^{2+}\) is considered a key mechanism in NO-induced relaxation of vascular smooth muscle cells (10).

NO also induces vasodilation through the inhibition of the RhoA/Rho-kinase signaling pathway (10, 17, 24, 135). Accordingly, NO-mediated increases in cGMP and activation of cGMP-dependent protein kinase (cGK) lead to inhibition of RhoA (135, 136). Recombinant RhoA is phosphorylated by cGK at Ser\(^{188}\), resulting in the inhibition of RhoA stress fiber formation. Sodium nitroprusside and constitutively active cGK inhibit PE-induced translocation of RhoA from the cytosolic to membrane fraction in rat aorta (10). Therefore, inhibition of Rho-kinase activity by NO/cGK is physiologically important because it induces vasodilation (10, 24).

RhoA/Rho kinase and O-GlcNAcylation. The small G protein RhoA and its downstream target, Rho-kinase, play a direct role in the regulation of MLC phosphatase activity. In the active state, RhoA engages downstream effectors, such as Rho-kinase, which then phosphorylates the myosin binding subunit of MLC phosphatase (MYPT1 Thr\(^{885}\), inhibiting its activity and thus promoting the phosphorylated state of MLC (25).

So far, recent data strongly suggest that increased O-GlcNAcylation augments vascular reactivity to constrictor stimuli via changes in the RhoA/Rho-kinase pathway. Arteries incubated with PUGNAc (1 \(\mu\)M, for 24 h) display increased vascular content of O-GlcNAc-proteins, as well as increased reactivity to the \(\alpha\)-agonist phenylephrine, which is prevented by Y-27632, a Rho-kinase inhibitor. Additionally, ET-1-incubation of vascular smooth muscle cells, which augments O-GlcNAc levels, increases expression of RhoA and augments activation of CPI-17 (measured by phosphorylation of CPI-17 at Thr\(^{38}\)). Accordingly, MYPT-1 phosphorylation, which favors vascular contraction, is increased after ET-1-incubation. OGT-inhibition, either pharmacologically or by silencing RNA, prevents increased RhoA expression, CPI-17 phosphorylation and MYPT-1 phosphorylation, supporting that increased O-GlcNAcylation augments vascular reactivity to constrictor stimuli via changes in the RhoA/Rho-kinase pathway (94).

Increased vascular O-GlcNAcylation also decreases eNOS/NO signaling (83). It is possible that increased RhoA/Rho-kinase activity, in response to reduce NO bioavailability, contributes to increased reactivity to vasoconstrictor stimuli in the presence of augmented O-GlcNAc levels. Furthermore, the interactions between NO and RhoA/Rho kinase may directly impact the activation of endothelial ET\(_B\) receptors, which activate signaling pathways that promote the release of NO, prostacyclin, and endothelium-derived hyperpolarizing factors (31, 131). ET\(_B\) receptor-mediated release of NO from endothelial cells not only accounts for the transient vasodilator action of ET-1, but also antagonizes the constrictor actions of ET-1 on vascular smooth muscle cells (31, 131). This is important because both the endothelium and the Rho-kinase pathway play a central role in the regulation of vascular tone and also modulate diverse vascular responses, including wall remodeling and inflammation-related processes, all important in hypertension-associated vascular disease.

O-GlcNAc and ET-1-Mediated Vascular Effects

ET-1 production is increased in the vasculature of salt-sensitive forms of hypertension, including DOCA-salt hypertensive rats. In these animals, ET-1 not only induces vasoconstriction, but it also activates transcriptional factors responsible for the coordinated increase in many cytokines and enzymes, thus enhancing inflammation, oxidative stress, and tissue damage (37, 65, 79, 81, 122, 139, 140, 154, 155), which are all important in hypertension-associated vascular dysfunction.

In addition, salt-sensitive hypertension is common in African Americans compared with Caucasians, and this population has been shown to have higher plasma ET-1 concentrations than white hypertensive subjects and to have enhanced ET\(_A\)-dependent vasoconstrictor tone (18, 41, 145). Additional clinical studies in this area would not only provide important information for conditions such as salt-sensitive hypertension but may also provide information on the impact of O-GlcNAcylation in the vasculature of hypertensive subjects.
Considering that O-GlcNAc is increased in the vasculature from mineralocorticoid and angiotensin II plus salt hypertensive rats (90, 127) and the above-mentioned studies showing that ET-1 contributes to vascular dysfunction in arteries from salt-sensitive subjects, we sought to investigate whether O-GlcNAc underlies effects of ET-1 on vascular function. We recently reported that ET-1 augments vascular O-GlcNAcylation (Fig. 4A) and that this posttranslational modification contributes to the vascular changes produced by the peptide (92). The effects of ET-1 on vascular reactivity, as well as on O-GlcNAc levels, are abrogated when vessels are previously transfected with neutralizing antibodies against OGT or are incubated with a selective OGT inhibitor (3-(2-adamantanyl-ethyl)-2-[4-chlorophenyl]azamethylene]-4-oxo-1,3-thiazaper...
hydriune-6-carboxylic acid] (Fig. 4B) (92). In addition, treatment of DOCA-salt rats with an ET, receptor antagonist (atrasentan) prevents augmented O-GlcNAc levels as well as increased contractile responses in arteries of these hypertensive animals. Of importance, chronic ET-1 infusion does not produce consistent increases in blood pressure, glucose levels, or insulin sensitivity, and atrasentan attenuates, but does not normalize, blood pressure in DOCA-salt rats (92). These data and the observation that spontaneously hypertensive rats do not exhibit changes in vascular O-GlcNAc levels indicate that vascular O-GlcNAc protein modification does not result from high blood pressure levels.

In vitro, ET-1 also decreases, in a time-dependent and transient way, the vascular expression of OGT, which may represent a compensatory mechanism for the augmented vascular levels of O-GlcNAc proteins. However, rats chronically infused with low doses of ET-1, which also exhibit increased vascular O-GlcNAc proteins and augmented vascular contractile responses, do not exhibit changes in vascular OGT expression at 14 days, which may be related to the transient changes in OGT expression produced by ET-1 (92).

ET-1 treatment decreases OGA expression (92), similarly to what has been demonstrated in aortas from DOCA-salt rats (93). In addition, ET-1 infusion for 14 days decreases OGA expression in thoracic aortas (92), suggesting that increased vascular O-GlcNAcylation induced by ET-1 treatment may be mediated by decreased OGA expression/activity. Whether this may offer a possible explanation for the augmented O-GlcNAc levels induced by ET-1, it opens a wide array of questions: 1) what are the mechanisms linking ET-1 receptors to OGA and OGT expression/activity; 2) how do changes in O-GlcNAc levels modify vascular reactivity to contractile agents; 3) is ET-1-induced O-GlcNAcylation coupled/associated with changes in phosphorylation levels of proteins important in vascular function; and 4) do other vasoactive agents, such as angiotensin II, also induce their effects partially via O-GlcNAc modification of proteins?

To address some of these questions, we are currently using silencing RNA technique to suppress OGT expression in cultured vascular smooth muscle cells. In addition, it will be very important to examine the use of inhibitors of OGT in vivo. It will be very interesting to test whether inhibition of OGT during the development of cardiovascular disease, particularly salt-sensitive hypertension, will ameliorate vascular dysfunction associated with this pathological condition. The use of OGT inhibitors in vivo will allow very rapid advances in the area.

O-GlcNAcylation and Cardiovascular Disease

Abnormal O-GlcNAcylation seems to contribute to the etiology of important human diseases, particularly diabetes. Elevated O-GlcNAcylation on insulin receptor substrate reduces its interactions with PI3K, thus blocking insulin signaling at an early stage (43, 161). Transgenic mice overexpressing glutamine fructose-6-phosphate transferase in skeletal muscle and adipose tissue develop insulin resistance and hyperleptinemia (29, 59). Glucosamine infusion in vivo also results in skeletal muscle insulin resistance (118, 158, 159). In addition, hyperglycemia, hyperlipidemia, and/or hyperinsulinemia were all shown to produce increased O-GlcNAcylation disturbing signaling, transcription, and other cellular functions (105, 117). Furthermore, a correlation between a polymorphism in the OGA gene and type 2 diabetes has been reported in Mexican-Americans (88).

Increased O-GlcNAcylation has also been implicated in cardiomycocyte dysfunction, generally present in animal models of metabolic disease (55). Alterations in excitation-contraction mechanisms, consistent with decreased SERCA activity, have been described in cardiomyocytes from diabetic animals, as well as in normal cardiomyocytes incubated with glucosamine or hyperglycemic media (36, 40, 124, 125). In addition, streptozotocin-induced diabetic mice display increased cardiac OGT expression and O-GlcNAc levels, which is accompanied by cardiomyocyte dysfunction (125). Increased OGT expression prolongs Ca2+ transient decays, whereas overexpression of OGA improves Ca2+ transients and decreases SERCA mRNA and protein expression in neonatal cardiomyocytes, providing a direct link between increased O-GlcNAc levels and cardiomyocyte dysfunction (64). Increased O-GlcNAc levels, in response to hyperglycemia or glucosamine treatment, have also been associated with activation of the transcriptional regulator p53, decreased cell growth, increased angiotensin II synthesis, and increased apoptosis. These are all events that potentially lead to the rapid onset of heart failure seen in diabetic patients following myocardial infarction (55).

It is interesting to note that augmented O-GlcNAc levels may differentially interfere with a specific response in different tissues/organs. To illustrate this, augmented O-GlcNAc levels increases phenylephrine-induced vascular contraction, whereas it decreases phenylephrine-induced inotropic responses in the intact heart. One may speculate that differential proteins are targeted by O-GlcNAc in these tissues. In neonatal rat cardiomyocytes, hyperglycemia mediated by excessive flux through the HBP (or augmented O-GlcNAc) decreases capacitative Ca2+ entry induced by PLC activation (116). Whereas distinct isoforms of PKC are being O-GlcNAc-modified in cardiomyocytes and vascular smooth muscle cells is unknown. In addition, the effects of augmented O-GlcNAc levels on Ca2+ homeostasis in vascular smooth muscle cells have been poorly investigated. Another possibility is that acute and chronic increases in O-GlcNAc within same tissue/organ levels lead to activation of distinct and unrelated pathways and, consequently, differential temporal responses.

On the other hand, in vivo tissue-targeted deletion of OGT not only showed that cells require a functional Ogt allele, but also demonstrated that O-GlcNAcylation modulates both protein phosphorylation and expression in essential signaling pathways (113). Thus, OGT is required for embryonic stem cell viability (144). In addition, one of the earliest responses to cellular stress is a rapid and global increase in O-GlcNAcylation on many proteins (178). Experimentally decreasing OGT and/or O-GlcNAcylation levels result in cells that are less stress tolerant, whereas increasing O-GlcNAc levels result in cells that are more viable after severe stress. Clearly, O-GlcNAcylation represents an important cell survival mechanism (178).

O-GlcNAc modification is also augmented in arteries from DOCA salt rats, a minelocorticoid model of hypertension (93) and in vessels from angiotensin II plus salt hypertensive rats (127). Interestingly, increased levels of O-GlcNAc (in-
duced by treatment with PUGNAC, an OGA inhibitor) leads to changes in mechanisms that are commonly observed in hypertensive animals, such as decreased production of NO as a consequence of decreased activity of eNOS (90, 91, 93), suggesting that augmented O-GlcNAc may contribute to abnormal vascular reactivity in salt-sensitive hypertension.

An interesting point to be investigated is whether strategies that restore physiological levels of O-GlcNAcylation ameliorate vascular dysfunction in arteries from hypertensive animals. Of importance, we have recently shown that OGT inhibition prevents vascular dysfunction induced by ET-1 (92). This opens the possibility that O-GlcNAcylation may represent a novel therapeutic target for the treatment of pathological conditions associated with augmented vascular ET-1 expression, such as salt-sensitive forms of hypertension and diabetes.

**O-GlcNAcylation and O-Phosphorylation**

O-GlcNAcylation is similar to protein phosphorylation (O-phosphate attachment) in that both modifications occur on serine and threonine residues, both are dynamically added and removed in response to cellular signals, and both alter the functions and associations of the modified protein (162). Dr. Hu et al. (63) have elegantly reviewed evidence showing that although O-GlcNAcylation and phosphorylation have many similar properties, these two posttranslational modifications are regulated very differently. Several specific kinases and phosphatases, encoded by distinct genes, regulate phosphorylation. In contrast, as previously discussed, O-GlcNAcylation is controlled exclusively by two enzymes, OGT and OGA, which are encoded by a single highly conserved gene in animals (56).

One big challenge in this field has been to map the sites where the attachments are simultaneously occurring. In this sense, the development and improvement of some techniques, such as electron capture dissociation and electron transfer dissociation has opened new possibilities to map O-GlcNAcylation and phosphorylation sites. Please refer to the following comprehensive and excellent reviews for further information regarding O-GlcNAc enrichment methods (98, 120, 164, 176).

Augmented O-GlcNAcylation levels, as a result of OGA inhibition, result in lower phosphorylation at 280 sites and increased phosphorylation at 148 sites, which implies that the interaction between these two translational modifications is large. Additionally, the cross-talk between O-GlcNAcylation and phosphorylation may occur by steric competition as well as by regulation mechanisms that are enzymatically dependent (163). For example, increased O-GlcNAc modification of eNOS is associated with decreased phosphorylation of eNOS at Ser1177 (eNOS-Ser1177) and decreased eNOS activity (38, 108). The steric competition between phosphorylation and O-GlcNAcylation occurring in eNOS is an example of competition that is occurring in the same site of occupancy. However, in other proteins, the competition occurs in proximal or distant sites (163). Whereas inhibition of eNOS activity by O-GlcNAc may represent one of the mechanisms leading to impaired vascular reactivity in hypertension, the enzymatic pathways that modulate protein O-GlcNAcylation remain incompletely understood. Therefore, the physiological significance of the crosstalk between O-GlcNAc and phosphorylation warrants further investigation and new techniques allowing the recognition of several O-GlcNAc sites will further clarify how different cellular stimuli interfere with these post-translational modifications (26, 54, 72) (Fig. 2).

Therefore, one potential mechanism by which O-GlcNAcylation may change vascular function includes the complex interplay between O-GlcNAcylation and phosphorylation (63, 165, 179). In addition, the interplay between O-GlcNAc and phosphorylation may represent an additional link between ET-1-activated signal transduction mechanisms and the effects of O-GlcNAc in the vasculature.

Other mechanisms by which O-GlcNAcylation may modulate vascular function include protein targeting to specific substrates, transient complex formation with other proteins, and subcellular compartmentalization upon glycosylation of specific proteins (15). An exciting challenge in the future will be to better understand the cellular dynamics of the modification, as well as the signaling pathways and mechanisms by which O-GlcNAc is regulated on specific proteins in the vasculature, as well as the physiological consequences of this event.

**Perspectives and Significance**

The incidence of hypertension in the U.S. population is very high, and a variety of pharmacological preparations are available for therapy. Despite these options, vascular dysfunction persists in many patients, and end-organ injury remains to be a serious complication. Augmented O-GlcNAcylation in the vascular may be important for four main reasons: 1) the vasculature regulates total peripheral resistance and organ perfusion through constrictor, and dilator mechanisms and abnormalities in these mechanisms may lead to increased blood pressure and abnormal organ perfusion; 2) O-GlcNAcylation modifies the function of numerous proteins, including kinases, phosphatases, transcription factors, and cytoskeleton proteins important in vascular function; 3) the metabolic abnormalities associated with insulin resistance and diabetes such as hyperglycemia, hyperlipidemia, and hyperinsulinemia are all associated with elevated O-GlcNAc levels, and hypertension often coexists with diabetes; and 4) arterial hypertension poses a major public health challenge worldwide and has been fueled mainly by increasing obesity and aging of the population.

Most of the deleterious effects associated with abnormal O-GlcNAcylation have been described in diabetic or hyperglycemic conditions but have not been investigated in hypertensive conditions. Hypertension often coexists with diabetes, and > 80% of patients with type 2 diabetes mellitus develop hypertension, whereas ~20% of patients with hypertension develop diabetes (28). In addition, diabetes mellitus doubles the risk of cardiovascular events in patients with hypertension (1, 75, 149). As is the case for hypertension, the frequency of diabetes mellitus is increasing rapidly worldwide, both in industrialized and in developing countries (78, 180). This combination of risk factors will certainly account for a large proportion of cardiovascular morbidity and mortality. Therefore, the investigation of new potential therapies to treat vascular dysfunction associated with hypertensive disease, and more specifically the impact of modifying O-GlcNAc-modified proteins in the vasculature, should be encouraged.
REFERENCES


