**O-GlcNAcylation of connexin 40: a sweet connection between diabetes and endothelial cell dysfunction? Focus on “O-GlcNAcase overexpression reverses coronary endothelial cell dysfunction in type 1 diabetic mice”**

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CARDIOVASCULAR COMPLICATIONS are the leading cause of morbidity and mortality in patients with diabetes mellitus. The vascular alterations that develop in diabetic patients relate to the pathogenesis of both macrovascular (increased incidence and severity of stroke and myocardial infarction) and microvascular (neuropathy, nephropathy, retinopathy, and erectile dysfunction) systems. Endothelial dysfunction has been implicated in the development of macro- and microvascular diseases. Chronic hyperglycemia is believed to constitute a major contributing factor to the pathogenesis of vascular complications in diabetic patients. Among the various mechanisms that have been involved in the so-called glucotoxicity phenomenon, O-linked N-acetylglucosaminylation (O-GlcNAcylation) of proteins is emerging as an important player (for review see Ref. 5).

O-GlcNAcylation is a posttranslational reversible modification that involves the addition of N-acetylglucosamine (GlcNAc) on serine or threonine residues of cytosolic and nuclear proteins. Only two enzymes, O-linked N-acetylglucosamine (O-GlcNAc) transferase (OGT) and β-N-acetylglucosaminidase (OGA), regulate O-GlcNAc level on protein according to glucose availability (Fig. 1). O-GlcNAcylation has been shown to control the activity, subcellular localization, and stability of numerous proteins, including transcription factors, metabolic enzymes, and signaling molecules. O-GlcNAcylation can also control the phosphorylation of proteins, either by directly competing with the phosphate group for the same residue (yin-yang mechanism) or by regulating the phosphorylation of nearby residues (5).

Nitric oxide (NO), the principal endothelium-dependent relaxing factor, is a key component of vascular homeostasis. In diabetes, endothelial cells fail to produce sufficient amounts of NO and induce vasorelaxation in response to endothelium-dependent vasorelaxants (e.g., acetylcholine, bradykinin, and shear stress) (1). Several studies have implicated O-GlcNAc in the deleterious effects of diabetes on NO production. Endothelial NO synthase (eNOS), the enzyme responsible for NO production in endothelial cells, is activated via its phosphorylation on S1177 by Akt. In bovine and rat aortic endothelial cells, high glucose resulted in increased O-GlcNAc on eNOS and decreased phosphorylation of the protein on the Akt site, suggesting a reciprocal relationship between eNOS phosphorylation and O-GlcNAcylation (5). In addition, Musicki et al. (10), studying erectile function in diabetic rat penis, also observed increased O-GlcNAcylation of eNOS, associated with decreased phosphorylation on S1177 and reduced erectile response to shear stress or VEGF, providing a potential mechanism for diabetes-associated erectile dysfunction. Moreover, Lima et al. (6), using rat artery segments, showed that O-GlcNAcylation induced by O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenylcarbamate (PugNAC), an inhibitor of OGA, blunted vascular reactivity to the vasorelaxing effect of acetylcholine, and this was again associated with decreased S1177 phosphorylation on eNOS. Importantly, these alterations in vascular reactivity were not observed in endothelium-denuded vessels, indicating that the effect of PUGNAC was mediated by endothelial cells (6).

Impaired angiogenesis also participates in cardiovascular complications in diabetes, through impaired wound healing, exacerbated peripheral limb ischemia, and cardiac mortality associated with reduced collateral vessel development. Elevated O-GlcNAc levels also inhibited the potency of endothelial cells to migrate and form capillary-like tubes. This effect was mediated, at least in part, by O-GlcNAcylation of Akt, which decreased its phosphorylation and, thereby, its activity (7).

Therefore, several lines of evidence argue for a contribution of O-GlcNAcylation to hyperglycemia-associated cardiovascular defects. In this issue of American Journal of Physiology-Cell Physiology, Makino et al. (8) add an additional brick to the wall. To determine whether reducing O-GlcNAcylation levels in mouse coronary endothelial cells (MCECs) may improve endothelial dysfunctions in diabetic rats, Makino et al. generated a tetracycline-inducible endothelium-specific OGA transgenic mouse. In the absence of doxycycline, streptozotocin-induced diabetes resulted in an increase in protein O-GlcNAcylation in MCECs, associated with a decrease in OGA expression level. Doxycycline induction restored OGA expression levels and markedly reduced O-GlcNAcylation in MCECs. Overexpression of OGA also restored to normal levels the reduced capillary density in the ventricle myocardium of diabetic rats, an indicator of oxygen transport efficiency and diffusion ability in the muscular tissue. Moreover, using coronary artery rings from these animals, Makino et al. showed that whereas the effect of acetylcholine on endothelium-dependent relaxation and endothelium-derived hyperpolarization-dependent relaxation was markedly impaired in diabetic mice, OGA overexpression fully restored these endothelial cell functions. Neither diabetes nor OGA overexpression affected endothelium-independent relaxation (evaluated using sodium nitroprusside treatment).

In agreement with the studies mentioned previously, Makino et al. (8) found that eNOS O-GlcNAcylation was increased in
diabetic MCECs. However, overexpression of OGA in these mice was not sufficient to restore normal O-GlcNAc level on eNOS.

Endothelium controls vascular tone not only by releasing NO, but also by other pathways, including hyperpolarization of the underlying smooth muscle cells. Gap junctions between endothelial cells and between endothelial cells and myoendothelial cells allow the propagation of electrical signals that participate in vascular relaxation (2). Connexin 40 (Cx40), a protein involved in gap junction communication, participates in endothelium-derived hyperpolarization-dependent relaxation. Gap junction communications are also involved in capillary network formation, playing an important part in the repair and revascularization that are necessary to maintain normal tissue capillary density.

Previously, Makino et al. (9) showed that impaired endothelium-derived hyperpolarization-dependent relaxation in diabetic mice was associated with decreased Cx40. Makino et al. (8) now report that Cx40 expression was decreased in diabetic mice but that OGA induction did not restore its expression. However, Cx40 was also found to be markedly O-GlcNAcylated in diabetic endothelial cells, and this modification was corrected by OGA induction in these animals. Interestingly, in cultured endothelial cells, high-glucose conditions also reduced both Cx40 protein level and gap junction communication (evaluated using dye-transfer experiments). This effect could be corrected by adenoviral overexpression of Cx40 in high-glucose-cultured cells, but pharmacological inhibition of OGA using PUGNAc prevented rescue by Cx40 overexpression. This result suggests that Cx40 O-GlcNAcylation impairs its function.

Therefore, using endothelial-specific OGA-inducible transgenic mice, Makino et al. (8) suggested that, in diabetes, increased O-GlcNAcylation affects endothelial cell-mediated vascular relaxation not only through regulation of eNOS, but also by reduction of gap junction communication involved in transmission of electrical signals.

However, a number of important questions remain to be answered. The mechanism by which diabetes decreased Cx40 is unclear. In their previous work, Makino et al. (9) observed that impaired Cx40 expression was associated with a decreased level of Sp1, a transcription factor that regulates the Cx40 gene. Sp1 was one of the first transcription factors shown to be O-GlcNAcylated, but depending on the sites that are modified, different functional consequences were observed, resulting in an increase or a decrease in its transcriptional activity, as well as changes in its stability (reviewed in Ref. 4).

Other important questions concern the mechanism by which O-GlcNAc regulates Cx40 activity. For instance, it will be important to determine whether O-GlcNAc affects Cx40 export to the plasma membrane or its assembly with other subunits to make the gap junction. Moreover, identification of the residues that are O-GlcNAcylated in Cx40 may help us understand the role of this modification. For instance, given that connexins are known to be regulated by phosphorylation (3), it will be crucial to determine whether O-GlcNAc competes for a phosphorylation site or, rather, regulates (positively or negatively) the phosphorylation of adjacent sites. Clearly, considerable additional work is needed to fully establish the connection between Cx40 O-GlcNAcylation and diabetes-associated endothelial cell dysfunction.

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**DISCLOSURES**

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AUTHOR CONTRIBUTIONS

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