ABSTRACT

Vascular complications from uncontrolled hyperglycemia are the leading cause of death in patients with diabetes mellitus. Previous reports have shown a strong correlation between hyperglycemia and vascular calcification, which increases mortality and morbidity in individuals with diabetes. However, the precise underlying molecular mechanisms of hyperglycemia-induced vascular calcification remain largely unknown. Transdifferentiation of vascular smooth muscle cells (VSMC) into osteoblast-like cells is a known culprit underlying the development of vascular calcification in the diabetic vasculature. Pathological conditions such as high glucose levels and oxidative stress are linked to enhanced osteogenic differentiation of VSMC both in vivo and in vitro.

It has been demonstrated that increased expression of runt-related transcription factor 2 (Runx2), a bone-related transcription factor, in VSMC is necessary and sufficient for the induction of VSMC calcification. Pathological conditions such as high glucose levels and oxidative stress are linked to enhanced osteogenic differentiation of VSMC both in vivo and in vitro. Overall increases in the O-GlcNAcylation of cellular proteins have been closely associated with cardiovascular complications of diabetes. In this review, the authors provide molecular insights into cardiovascular complications, including diabetic vasculopathy, that feature increased O-GlcNAcylation in people with diabetes.

Keywords: O-GlcNAc modification; Hyperglycemia; Vascular calcification; Vascular smooth muscle cells; Runx2

INTRODUCTION

According to the International Diabetes Federation, approximately 463 million adults aged 20–79 years are living with diabetes, and the number of people with diabetes will rise to 700 million by 2045. People with diabetes are at an elevated risk of multiple complications, such as cardiovascular disease (CVD), diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy. In all Westernized countries, CVD is the most common cause of death in people with diabetes. Therefore, the therapeutic and prognostic value of diabetic complications, such as vascular calcification, in patients with diabetes cannot be underestimated.
As epidemiological evidence of the prevalence of vascular calcification in patients with diabetes mellitus and resulting cardiovascular complications, reports have repeatedly described an increased incidence of arterial stiffness and thrombosis, which lead to elevated rates of morbidity and mortality.\textsuperscript{1,2} Vascular calcification, previously considered to be a passive deposition of calcium phosphate crystals in the vasculature, has now been recognized as an active process regulated by multiple relevant cell types, similar to bone mineralization.\textsuperscript{4} A key cell type involved in the development of vascular calcification, vascular smooth muscle cells (VSMC) undergo transdifferentiation into osteoblast-like cells and deposit calcium as hydroxyapatite crystals in the arteries of patients with diabetes.\textsuperscript{5,6}

Hyperglycemia in individuals with diabetes and animal models of diabetes is closely linked to increased vascular calcification.\textsuperscript{6,7} According to previous studies, a high rate of modification of target proteins by the addition of O-linked β-N-acetylglucosamine (O-GlcNAc) was observed in the vasculature of patients with diabetes and in diabetic mouse models.\textsuperscript{8-10} Through the hexosamine biosynthetic pathway, extracellular glucose is metabolized into UDP-β-D-N-acetylglucosamine (UDP-GlcNAc), an active sugar donor for O-GlcNAcylation of cellular proteins.\textsuperscript{11} Hence, hyperglycemia—defined as excessive blood levels of glucose, a substrate for the production of UDP-GlcNAc—may result in enhanced O-GlcNAcylation in patients with diabetes, as well as cardiovascular events.\textsuperscript{6,8,12}

More recently, it was shown that aggravated vascular calcification is accompanied by increased O-GlcNAcylation in a low-dose streptozotocin (STZ)-induced diabetes model via O-GlcNAc-mediated activation of the AKT signaling pathway.\textsuperscript{10} Since vascular calcification is a major contributor to increased morbidity and mortality in patients with diabetes, we review the molecular mechanisms underlying O-GlcNAcylation-mediated vascular calcification and, most importantly, provide crucial molecular insights into the function of O-GlcNAcylation in regulating diabetic vasculopathy.

**ROLE OF O-GLCNACYLATION IN DIABETIC VASCULOPATHY**

1. **O-GlcNAcylation: a double-edged sword in cardiovascular pathologies**

It has been reported that acute increments of O-GlcNAcylation confer protection from oxidative stress—induced calcium overload and structural damage in ischemia-reperfusion models of heart failure.\textsuperscript{13,14} The cardioprotective effects of O-GlcNAcylation have been attributed to improved tolerance of mitochondrial oxidative damage through enhanced O-GlcNAc modifications of voltage-dependent anion channels, thereby attenuating the loss of mitochondrial membrane potential and ultimately leading to the survival of cardiomyocytes.\textsuperscript{15,16}

In an in vitro model of ischemia-reperfusion injury, upregulated protein O-GlcNAcylation and subsequent increases in the expression and translocation of members of the Bcl-2 protein family ameliorated the mitochondrial dysfunction and apoptotic cell death induced by ischemic injuries.\textsuperscript{17} More directly, cardiomyocyte-specific deletion of O-GlcNAc transferase (OGT) aggravated heart failure by reducing the heart’s compensatory capacity in mice with myocardial infarction, clearly demonstrating the necessity of cardiac OGT expression in the failing heart.\textsuperscript{18}

Chronic hyperglycemia exerts devastating impact on vascular function, thereby leading to cardiovascular complications including diabetic cardiomyopathy, nephropathy, retinopathy, neuropathy, and atherosclerosis secondary to diabetes mellitus.\textsuperscript{19} Diabetic vascular dysfunction...
manifests in the early stages of this complex disease and continuously accompanies the progression of pathology in diabetes, ultimately leading to morbidity and mortality from cardiovascular complications. In this process, chronic elevation of O-GlcNAcylation—possibly through chronic hyperglycemia, as occurs in diabetes—exerts adverse effects on the cardiovascular system. Hyperglycemia led to significant increases in both OGT expression and O-GlcNAcylation of cellular proteins, reducing insulin secretion in the pancreas of diabetic Goto-Kakizaki rats and in isolated islets through enhanced O-GlcNAcylation of pancreatic and duodenal homeobox-1 (PDX-1), a critical regulator of β-cell survival and a transcription factor for insulin production in the pancreas. It was also demonstrated that enhanced O-GlcNAcylation of insulin signaling machinery resulted in impaired insulin signal transduction, leading to insulin resistance and dyslipidemia in the hepatic system.

In patients with diabetes, traditionally defined as metabolic vascular syndrome, the increased incidence of cardiovascular complications has been linked to the adverse effect of hyperglycemic milieu on macrovascular and microvascular beds. The cardiovascular complications caused by diabetes mellitus can be largely characterized as endothelial dysfunction, which involves functional impairment of the vascular endothelium via reduced nitric oxide (NO) bioavailability. In the vasculature, NO is mainly synthesized by endothelial NO synthase (eNOS) through a series of redox reactions in the endothelium. As it diffuses into VSMC, NO activates guanylate cyclase, yielding a concomitant increase in cyclic guanosine monophosphate. Through this mechanism, it induces relaxation of VSMC, resulting in reduced NO generation and impaired NO signaling. Therefore, the endothelial layer performs a variety of functions that are closely involved in the maintenance of homeostasis; for instance, it controls immune cell adhesion, smooth muscle cell proliferation, angiogenic migration, vascular integrity, and vessel wall inflammation. Endothelial dysfunction is closely associated with multiple pathological conditions such as diabetes, atherosclerosis, and hypertension. Therefore, in early works demonstrating the role of O-GlcNAcylation in vascular function, many studies focused on the identification of molecular mechanisms underlying hyperglycemia-induced endothelial dysfunction; in one of these mechanisms, hyperglycemia in diabetes leads to the chronic impairment of eNOS activity in bovine aortic endothelial cells through O-GlcNAcylation of the activation domain within the enzyme. Hyperglycemic conditions in diabetes result in the downregulation and inactivation of eNOS, with a concomitant increase in the O-GlcNAcylation of the protein. Hyperglycemia-induced O-GlcNAcylation of eNOS at Ser1177, a primary phosphorylation-dependent activation site, contributes to erectile dysfunction in diabetes patients through reduced NO generation. Moreover, reduced NO production due to enhanced O-GlcNAcylation of eNOS results in endothelial cell dysfunction via the regulatory role of NO in vasodilation and the inhibitory effect of the diffusible gas on platelet aggregation, thereby promoting the development of cardiovascular complications in patients with diabetes. Beleznai and Bagi also reported that hyperglycemia-induced O-GlcNAcylation contributed to the impaired NO-mediated vasodilation of skeletal muscle arterioles isolated...
from male Wistar rats, further confirming the role of O-GlcNAcylation as a contributor to microvascular complications in patients with diabetes. In rats chronically fed a high-sugar diet in an animal model of metabolic syndrome, interference with the vasorelaxant function of perivascular adipose tissue (PVAT) was closely linked to decreased generation of NO, reduced expression of eNOS, and increased O-GlcNAcylation of eNOS, implying that O-GlcNAcylation of eNOS contributes to hyperglycemia-induced PVAT dysfunction and suggesting a therapeutic target for diabetes-associated vascular dysfunction (Fig. 1).

2. Vascular calcification: implications for diabetic vasculopathy

In the patients with diabetes, cardiovascular complications are the leading cause of increased mortality and morbidity. Efforts have been made to reduce the high death rate among adults with diabetes; however, mortality still remains high in people with CVDs caused by the diabetic milieu. Since early detection of CVD is highly valuable from a diagnostic standpoint, strategies for developing reliable and non-invasive medical tests are urgently necessary to reduce the need for intensive therapies and to minimize the economic burden faced by people with diabetes. Since all major risk factors for CVD, including diabetes, have shown close associations with the development of vascular calcification, the medical assessment of arterial calcification in individuals with diabetes might be highly useful as a marker for risk stratification of patients with cardiovascular complications.

Vascular calcification can be classified into 2 distinct but overlapping types, intimal and medial calcification, which correspond to different etiologies. Calcifications of the intimal layer are mainly dependent upon the classic risk factors for CVD such as high blood pressure, high cholesterol, and smoking, and are evoked by oxidative stress or inflammatory responses in atherosclerosis, which could result from lipid accumulation, inflammation, fibrosis, and development of focal plaques. In contrast, medial vascular calcification involves the nucleation and deposition of calcium hydroxyapatite nanocrystals along the elastic lamina and extracellular matrix, in close association with diabetes and chronic kidney disease. However,
both forms of vascular calcification often coincide and frequently overlap with each other.

There is ample evidence that patients with diabetes have a higher propensity for developing vascular calcification than people without diabetes, as highlighted by elevated expression of osteogenic markers such as osteopontin, osteocalcin, and alkaline phosphatase in the medial layer of the vasculature. In an 18-year longitudinal study of 833 individuals with type 2 diabetes and 1,292 individuals without diabetes, arterial calcification was shown to be a strong predictor of cardiovascular and all-cause mortality in patients with diabetes, establishing the prognostic value of aortic calcification in symptomatic subjects.

Aortic stiffness, a hallmark of the aging process, can be described as elastic resistance to deformation, culminating in reduced vascular compliance. Expressed as aortic pulse wave velocity (PWV), arterial wall stiffening represents a strong predictor of cardiovascular and all-cause mortality. In a meta-analysis of 17 longitudinal studies evaluating the aortic PWV with 15,877 participants and a mean follow-up of 7.7 years, it was shown that adjusted rates of cardiovascular events, cardiovascular mortality, and all-cause mortality increased by 14%, 15%, and 15%, respectively, for every 1 m/s increment in the aortic PWV. Arterial wall stiffening occurs in arteries frayed by mechanical stress caused by several disrupting factors, including vascular calcification. A well-established marker of bone metabolism, osteoprotegerin (OPG), was linked to the incidence of aortic stiffness and the extent of coronary artery disease. In a 15-year follow-up study of cardiovascular complications in patients with type 1 diabetes, the authors observed that OPG was closely associated with arterial calcification, leading to the onset of aortic stiffness and accompanying cardiovascular events. Therefore, diabetic medial calcification could be directly linked to the onset of arterial stiffening, which is a prelude to multiple CVDs, such as elevated blood pressure, increased cardiac afterload, and impaired vascular reactivity.

3. O-GlcNAcylation: a strong inducer of vascular calcification

It has been shown that an overall increase in O-GlcNAcylation promoted osteoblastic differentiation of MC3T3-E1 cells with enhanced expression of bone-related markers such as alkaline phosphatase, osteocalcin, and bone sialoprotein via transcriptional activation of runt-related transcription factor 2 (Runx2), an osteogenic transcription factor. Moreover, high glucose levels and PUGNAC, an inhibitor of O-GlcNAcase, induced osteogenic differentiation of human cartilage endplate stem cells and MC3T3-E1 cells via O-GlcNAcylation of Runx2, demonstrating the ability of O-GlcNAcylation to stimulate mineralization of extracellular matrix.

In a diabetic mouse model, STZ treatment combined with an atherogenic diet induced accelerated atherosclerosis in response to hyperglycemia, as shown by exaggerated fatty streaks and atherosclerotic plaques resembling those of human type II lesions. Recently, Heath et al. demonstrated that STZ-induced hyperglycemia was associated with elevated vascular O-GlcNAcylation and aortic calcification in a murine diabetic model involving multiple low-dose STZ injections, suggesting a positive correlation between O-GlcNAcylation and calcification in diabetes. With impaired vascular compliance, as found in mice, increased O-GlcNAcylation in diabetic arteries is directly associated with AKT activation and upregulated expression of Runx2. O-GlcNAcylation of AKT at T430 and T479 promotes AKT phosphorylation and activation, which in turn enhances the expression of Runx2 and calcification of VSMC in vitro. In summary, O-GlcNAcylation, which is enhanced by hyperglycemia in diabetic vasculature, may promote the osteogenic differentiation of VSMC, thereby leading to increased aortic calcification and reduced vascular compliance in patients with diabetes.
4. Mechanistic perspectives on O-GlcNAcylation in VSMC

Dysfunction of VSMC in the vasculature significantly contributes to the development of vascular pathologies such as atherosclerosis, hypertension, and restenosis, which are commonly associated with diabetes, via phenotypic switching of VSMC from the contractile state to the synthetic state.\(^{55-57}\) Diabetes-mediated VSMC dysfunction, broadly defined as a transition into pro-inflammatory phenotype or dedifferentiated status, is accelerated by multiple pathological factors associated with diabetes such as high glucose levels, heightened oxidative stress, and altered lipid metabolism.\(^{58-61}\) Therefore, the pathophysiological manifestations of VSMC dysfunction could involve enhanced inflammatory responses, migration, proliferation, and dedifferentiation via multiple downstream signaling pathways and transcriptional activators.\(^{59,61-63}\)

In rat aortic smooth muscle cells cultured in high-glucose media, increased expression and activity of OGT were mainly observed in the nucleus, and altered patterns of O-GlcNAc-modified nuclear proteins were clearly demonstrated compared to control rats.\(^{64}\) It was also found that the elevated production of matrix hyaluronan (HA) observed in diabetic arteries was triggered by O-GlcNAcylation of HA synthase 2, the main HA synthase in aortic smooth muscle cells, through stabilization of the enzyme.\(^{65}\) This finding provides further confirmation that increased HA synthesis through this process could mediate VSMC dedifferentiation, which is critical for vascular pathologies in diabetes.\(^{66}\)

It was previously shown that O-GlcNAcylation of transcription factor specificity protein 1 (Sp1) in VSMC confers protection against proteasomal degradation, providing a causative link between this versatile transcriptional regulator and a nutritional checkpoint.\(^{67}\) The hyperglycemic milieu often enhances the migration, proliferation, and inflammation of VSMC by modulating the signaling molecules and growth factors involved in each process, thereby providing putative therapeutic targets to protect the vascular system in people with diabetes.\(^{68-70}\)

In rat aortic smooth muscle cells, plasminogen activator inhibitor-1 (PAI-1), a well-known stimulator of VSMC migration (especially in the setting of chronic transforming growth factor [TGF]-β1 activation), is upregulated via Sp1-binding to the PAI-1 promoter region through the release of a transcriptional repressor from Sp1 complexes under high-glucose conditions.\(^{71}\) Barnes et al.\(^{72}\) demonstrated that higher O-GlcNAcylation of Sp1 in pulmonary arterial smooth muscle cells from idiopathic pulmonary arterial hypertension facilitated cell migration via increased expression of vascular endothelial growth factor. It was also shown that the increased expression of TGF-β1 in endothelial cells cultured under high-glucose conditions was mediated by elevated O-GlcNAcylation of Sp1,\(^{73}\) which may be implicated in VSMC proliferation through the synthesis of proteoglycans and extracellular matrix proteins.\(^{74}\)

Chen et al.\(^{75}\) observed that increased expression of PAI-1 promoted apoptotic resistance of VSMC in association with enhanced FLIP activity in the vascular wall through a signaling pathway mediated by nuclear factor (NF)-κB and extracellular signal-regulated kinases (ERK), demonstrating a causative link between PAI-1-induced VSMC proliferation and cardiovascular pathologies, including restenosis after coronary intervention. It has been reported that upregulation of vascular cell adhesion molecule-1 (VCAM-1) in rat glomerulus cells is prompted by enhanced NF-κB binding to the VCAM-1 promoter in hyperglycemia.\(^{76}\) Furthermore, high glucose-mediated augmentation of the O-GlcNAcylation of NF-κB has been found to result in production of tumor necrosis factor alpha and interleukin-6 in rat placenta.\(^{77}\) This data led to the hypothesis that O-GlcNAcylation of NF-κB may participate in inflammatory responses of VSMC. Under hyperglycemic conditions, increased expression of VCAM-1 is induced by the O-GlcNAcylation of NF-κB through the elevated translocation
of NF-κB to the nucleus via the separation of NF-κB and IκB, an inhibitor of NF-κB in rat vascular smooth muscle tissue. This indicates that O-GlcNAcylation of NF-κB may contribute to exaggerated inflammatory responses in VSMC during the progression of diabetes.78 Recently, aldose reductase, a well-known obligatory mediator of the inflammatory changes in diabetic hyperglycemia, was shown to be essential for protein kinase C and NF-κB–induced expression of intercellular adhesion molecule-1 and VCAM-1, further suggesting that under high-glucose conditions, NF-κB could be a critical determinant of the inflammatory response of injured diabetic vasculature.79

As mentioned earlier, Runx2 is a master regulator of vascular calcification in both physiological and pathological conditions. Post-translational modifications of Runx2 by phosphorylation, ubiquitination, and acetylation exert a variety of significant influences on its activity, stability, and interactions with other signaling co-factors downstream of key osteogenic cues.80 In a previous report, O-GlcNAcylation of Runx2 at multiple sites was reported to be closely linked to increased activation of the transcription factor in MC3T3-E1 pre-osteoblasts.81 More interestingly, overall increases in O-GlcNAcylation of cellular proteins, including Runx2, were detected in bone marrow-derived mesenchymal stem cells cultured in an osteogenic medium in the presence of bone morphogenetic protein 2/7 (BMP2/7), further confirming a strong correlation between O-GlcNAc cycling and the Runx2-dependent regulation of osteogenic differentiation (Fig. 2).81

Fig. 2. Pathological effects of O-GlcNAcylation in VSMC under diabetic conditions. The hexosamine biosynthetic pathway in VSMC. After its entry into VSMC, glucose is phosphorylated to glucose-6-P by HK and further metabolized into fructose-6-p. Conversion of fructose-6-p to glucosamine-6-p is carried out by the rate-limiting enzyme, GFAT. The end-product of the hexosamine biosynthetic pathway, UDP-GlcNAc serves as an obligatory substrate for OGT, generating O-GlcNAc-modified proteins, while OGA catalyzes the removal of O-GlcNAc from the targets. O-GlcNAc modification of transcription factors such as Sp1, NFκB, and Runx2 mediates hyperglycemia-induced pathological responses in VSMC. VSMC, vascular smooth muscle cell; O-GlcNAc, O-linked β-N-acetylglucosamine; GLUT, glucose transporter; HK, hexokinase; GFAT, glutamine:fructose 6-phosphate amidotransferase; UDP-GlcNAc, UDP-β-D-N-acetylglucosamine; OGT, O-GlcNAc transferase; OGA, O-GlcNAcase; NFκB, nuclear factor κB; Runx2, runt-related transcription factor 2; glucose-6-P, glucose-6-phosphate; fructose-6-P, fructose-6-phosphate; glucosamine-6-P, glucosamine-6-phosphate.
CONCLUSION

In conclusion, we reviewed the role of O-GlcNAcylation in vascular pathology associated with diabetes mellitus. Although the spatial and temporal regulation of protein O-GlcNAc modification during the development of cardiovascular pathologies should be carefully considered in future studies, we found strong evidences of associations between the O-GlcNAcylation of cellular proteins such as Sp1, NF-κB, and Runx2 and diabetic vasculopathy. We speculate that modulation of O-GlcNAcylation of target proteins could be an attractive therapeutic target to alleviate vascular pathologies in patients with diabetes.

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