PROTEIN O-GLCNAC MODIFICATION IN DIABETIC VASCULAR CALCIFICATION

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ABSTRACT

Vascular calcification is prevalent in patients of diabetes mellitus, which represents an independent risk factor that positively correlated with morbidity and mortality in these patients. Vascular calcification is now recognized as a cell-regulated process of osteogenic differentiation of vascular smooth muscle cells (VSMC) in response to stress, such as hyperglycemia and oxidative stress. Both hyperglycemia and oxidative stress have been shown to induce protein modification by O-linked β-N-acetylglucosamine modification (O-GlcNAcylation), which is also elevated in diabetes. The present studies aimed to determine the effects of protein O-GlcNAcylation on vascular calcification in diabetes, and uncover the underlying molecular mechanisms.

With the use of an array of comprehensive pharmacologic and genetic approaches, including a novel SMC-specific OGT deletion mouse model, that specifically targeting OGT or OGA, we have demonstrated a causative effect of increased O-GlcNAcylation on VSMC calcification in vitro and diabetic vascular calcification in vivo. Mechanistically, increased O-GlcNAcylation promotes VSMC calcification via AKT activation that leads to upregulation of Runx2, the osteogenic transcript factor that we have previously determined to be an essential and sufficient regulator for VSMC calcification. At the molecular level, we have demonstrated direct modifications of AKT and Runx2 by O-GlcNAcylation leading to upregulation of Runx2 and VSMC calcification. The novel and unique O-GlcNAc modifications on AKT at two sites, T430
and T479, are critical for AKT phosphorylation at S473 leading to Runx2 upregulation and VSMC calcification. Site-directed mutagenesis studies suggested that AKT O-GlcNAcylation at T430 and T479 may facilitate its interaction with mTOR complex 2 and subsequent phosphorylation by the kinase at S473. Similarly, direct O-GlcNAc modification on Runx2 at T412/S413 promoted its phosphorylation and binding to the BMP-regulated Smads that are known to be important for the osteogenic function of Runx2.

Our studies have determined a new causative link between chronic increases in vascular O-GlcNAcylation and vascular calcification in diabetes and uncovered a novel mechanism underlying the regulation of AKT activation and Runx2 transactivity by their O-GlcNAcylation. These results have provided molecular insights into targeting O-GlcNAcylation and specifically O-GlcNAcylation of AKT/Runx2 signaling as potential therapy for vascular calcification in diabetes.

Keywords: O-GlcNAc, OGT, OGA, vascular calcification, Runx2, AKT
DEDICATION

To my parents, Bruce and Jean Heath, for their constant guidance and support, and most especially for being my role models for how to be a good human being.
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<td>advanced glycation end-product</td>
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<td>BMP-2</td>
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<td>CA-AKT</td>
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<td>ColIA1</td>
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<td>ESRD</td>
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<td>hexosamine biosynthesis pathway</td>
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<td>TPR</td>
<td>tetratricopeptide repeat</td>
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INTRODUCTION

Vascular calcification has emerged as an important pathological condition associated with major cardiovascular diseases, including diabetes, atherosclerosis, and end-stage renal disease (ESRD). The presence of vascular calcification is positively correlated with morbidity and mortality in these diseases\(^1-3\). Previously considered to be passive calcium deposition in the vessel walls, vascular calcification is now recognized as a cell-regulated process of osteogenic differentiation of vascular cells in response to stress\(^4,\,5\). Among all the cell types, vascular smooth muscle cells (VSMC) contribute most significantly in the pathogenesis of vascular calcification\(^6\). These cells actively regulate the calcification process through trans-differentiation into an osteogenic phenotype, during which the expression of smooth muscle marker proteins is decreased and the expression of osteogenic proteins normally observed in osteoblasts is induced\(^7-9\).

While much progress has been made in elucidating the key mechanisms involved in vascular calcification, a therapeutic method to reverse existing calcification has not yet been developed.

Diabetic patients have an increased prevalence of vascular calcification, independent of atherosclerosis in the vasculature\(^10\). Unlike calcification associated with atherosclerosis, diabetic mineralization is deposited in the media, where the VSMC physiologically reside\(^11,\,12\). Many diabetic mouse models have been shown to exhibit vascular calcification, including the Akita model (expressing mutated insulin receptor)\(^13\), and the streptozotocin and high-fat-diet (STZ, HFD) model\(^14\). In contrast to atherosclerosis models, which may exhibit vascular calcification after 6-9 months of
disease, diabetic mouse models exhibit vascular calcification much earlier in disease progression: 20 weeks in the Akita mutant mice$^{13}$, and over 4 months in the STZ-HFD model$^{14}$. The molecular mechanisms underlying VSMC osteogenic differentiation in diabetes are largely unknown. In the vasculature of diabetic mice, bone signaling is activated to induce calcification. Studies by Bostrom et al. have elegantly shown the increase in bone morphogenic protein-2 (BMP-2) signaling promotes vascular calcification in multiple murine models of diabetes$^{13}$. In these diabetic mice, increased BMP-2 signaling ultimately lead to upregulation of Runx2, the osteogenic transcription factor that we have shown to be essential and sufficient regulator for VSMC calcification$^{7, 15, 16}$. Studies from the Towler group have linked diabetic calcification in low-density lipoprotein (LDL) receptor deficient mice with upregulation of mural Msx2 and Wnt signaling, which is also associated with upregulation of Runx2 that leads to calcification$^{17, 18}$. Therefore, multiple osteogenic signaling pathways converge in the vasculature to induce upregulation of the key osteogenic transcription factor Runx2 that promotes vascular calcification in diabetes.

Impaired glucose homeostasis leads to hyperglycemia, which is the major characteristic of patients with diabetes. Clinical and experimental studies have linked hyperglycemia with vascular calcification. However, the molecular mechanisms are largely unknown. The present research represents the effort of our group to understand the cellular events and molecular regulators underlying hyperglycemia-induced vascular calcification.
Glucose Toxicity in Diabetes

Both type I and type II diabetes exhibit chronic increases in glucose levels, eventually leading to the complications associated with the disease, including retinopathy, kidney disease, nerve damage, and atherosclerosis, among others\textsuperscript{19}. Over long periods of time, diabetes can cause blindness, myocardial infarction, and limb amputation as a result of chronic damage to the tissue and the vasculature\textsuperscript{20}.

Specifically, hyperglycemia has been shown to be one of the major causes of tissue damage observed clinically in diabetic patients\textsuperscript{19}. Many different cell types have been shown to be susceptible to increased glucose levels due to eventual impairment of glucose transport and metabolism pathways\textsuperscript{21, 22}. Mechanistic studies have revealed several different pathways through which glucose may be metabolized to explain the damage to the tissue (Fig 1). These pathways may all work together to cause the ultimate resulting toxicity in the cells of diabetic tissues.

\textbf{Figure 1. Mechanisms of hyperglycemia-induced diabetic complications.} Glucose works through multiple pathways to confer tissue damage in diabetes. Hyperglycemia results in increased production of advanced glycation end-products (AGEs), which may then modify proteins to change their activity and function in the cell. High levels of glucose may also increase the generation of reactive oxygen species (ROS), leading to oxidative modifications and cellular DNA damage. Glucose has been shown to activate the protein kinase C (PKC) pathway as well, which affects multiple signaling pathways, including
those involved in extracellular matrix production and fibrosis. Glucose flux through the hexosamine pathway increases protein O-GlcNAc modification, leading to changes in protein stability, localization, and downstream signaling. All of these pathways may work together to lead to tissue damage and larger organ system complications associated with diabetes.

Increased production of advanced glycation end-products (AGEs) contributes significantly to tissue damage in diabetes. AGEs modify major transcription factors within the cell that affect transcription of proteins involved in apoptosis, ultimately leading to tissue dysfunction. The modification of the extracellular matrix by AGEs can also cause changes in the cellular motility and secretion of extracellular molecules that may cause fibrosis in the matrix. The secretion of AGEs may also affect proteins in the circulation. These modified proteins bind to receptors to cause increased inflammatory signaling and cytokine release in important tissues, thus increasing overall inflammation in many tissues throughout the body. Increased AGEs have been shown to induce vascular calcification, but the specific links to diabetes, including protein targets and pathways, have remained elusive and not well-defined.

Hyperglycemia increases glucose oxidation in the citric acid cycle, leading to increased generation of reactive oxygen species (ROS). The resulting oxidative damage can be prevented by treatment of the cells with a mitochondrial uncoupler, indicating the importance of the ROS in the mitochondria in contributing to diabetic oxidative stress. Many groups have shown the important role of oxidative stress in diabetes, specifically through the generation of superoxide in the mitochondria. On the other hand, aldose reductase, an enzyme that physiologically converts aldehyde into alcohol to prevent damage to tissues, plays a more sinister role via the polyol pathway when glucose levels are increased by converting glucose to sorbitol, using NADPH as a cofactor.
Glutathione, an antioxidant critical for prevention of oxidative stress in the cell, also uses NADPH. The increased utilization of this essential cofactor makes the cell more vulnerable to oxidative stress. Accordingly, aldose reductase inhibitors have been proven to be effective in prevention of tissue damage in diabetes. Our group and others have demonstrated that oxidative stress potently induces Runx2 upregulation and thus promotes VSMC calcification. Therefore, increased oxidative stress via glucose oxidation may contribute to increased vascular calcification in diabetes.

Hyperglycemia can also activate a critical pathway involved in signaling within the cell, the protein kinase C (PKC) pathway. The activation of PKC can affect expression of a multitude of genes. In the vasculature, expression of genes involved in vessel dilation is decreased, and genes involved in constriction of the vasculature are increased. In addition, expression of proteins involved in production of extracellular matrix is increased, leading to fibrosis and decreased cell-cell communication. The inhibition of PKC has been shown to be therapeutic in studies of diabetes focused on diabetic retinopathy and kidney function.

In addition to producing bioenergetic substrates via the tricarboxylic acid cycle, glucose metabolism through the hexosamine biosynthetic pathway (HBP) generates UDP-β-D-N-acetylglucosamine (UDP-GlcNAc), an active sugar donor for O-linked β-N-acetylglucosamine modification (O-GlcNAcylation). Thus, increased glucose in diabetes may promote O-GlcNAcylation by increased UDP-GlcNAc via HBP. Indeed, increased O-GlcNAcylation on a variety of key cellular mediators involved in the insulin/phosphoinositide signaling pathway is associated with glucose toxicity and insulin resistance, the two hallmarks of diabetes. Previous studies have demonstrated elevation
of O-GlcNAcylation in human diabetic carotid plaques\textsuperscript{43} and diabetic mouse vasculature\textsuperscript{44}. Coincidentally, increased calcified plaques have been identified in diabetic patients\textsuperscript{45} and diabetic mouse models\textsuperscript{13}. However, the contribution of O-GlcNAcylation on diabetic vascular calcification is entirely unknown.

**Cellular O-GlcNAc Signaling**

Protein modification by O-GlcNAcylation has recently become recognized as a key post-translational factor in the regulation of protein and cell homeostasis\textsuperscript{46}. O-GlcNAc modification has a range of biological consequences, depending upon the tissue and cellular environment. Importantly, this modification is strongly linked to metabolism. As shown in Figure 2, glucose feeds into the HBP pathway by first being converted to glucose-6-phosphate by phosphorylation, and then modified to produce fructose-6-phosphate; small amounts of this molecule can then enter the HBP\textsuperscript{47}. Glutamine can also feed directly into the HBP, which may have important implications in the connection between protein metabolism at the level of amino acids and the HBP\textsuperscript{47}. The importance of precursors such as glucose and glucosamine, as well as Acetyl-CoA and ATP\textsuperscript{48}, reflects the possibly crucial role of the HBP and O-GlcNAc modification in acting as a sensor of the nutrient state of the cell, and the state of the metabolic pathways within the cell.
Figure 2. Glucose metabolism through the hexosamine biosynthesis pathway. Glucose is phosphorylated to glucose-6-phosphate, which is further metabolized to fructose-6-phosphate. The rate-limiting step of the pathway is the conversion of this molecule to glucosamine-6-phosphate via GFAT. Glucosamine may also feed into the hexosamine pathway here to induce O-GlcNAc modification. UDP-GlcNAc is the substrate for the O-GlcNAc transferase (OGT) to add the O-GlcNAc modification onto serines and threonines of target proteins. The O-GlcNAcase (OGA) hydrolyzes the modification from these residues to produce the naked protein.

O-GlcNAcylation is controlled by two enzymes which have been evolutionarily conserved in eukaryotes: the O-GlcNAc transferase (OGT), and the O-GlcNAcase (OGA)\(^{48}\). OGT is responsible for the addition of one β-N-acetylglucosamine (GlcNAc) through an O-linkage to serine or threonine residues\(^{49,50}\). The OGT enzyme is expressed in every tissue that as yet examined, and it has the highest level of expression within the pancreas and brain, where glucose is highly utilized and glucose homeostasis is crucial for organ function\(^{46}\). The highest abundance of OGT tends to be in the nucleus\(^{49}\), and nuclear pore proteins were some of the first and most closely studied protein modified by the enzyme\(^{50}\). The OGT gene is located on the X chromosome, and it can be alternatively
spliced to result in three distinct OGT isoforms: nucleocytoplasmic OGT (ncOGT), mitochondrial OGT (mOGT), and short OGT (sOGT). Alternative splicing results in a C-terminus that is identical in all three isoforms, but the N-terminus exhibits unique domains leading to differences in localization, as reflected by their names, and protein targeting. The three main functional domains of the OGT protein are the region containing tetratricopeptide repeats (TPR), a middle linker region, and the catalytic domains at the C-terminus. The TPR is a sequence of 34 amino acids that repeat anywhere from 3 to more than 10 times, depending on the OGT isoform. This region is responsible for interaction between OGT subunits, as well as recognition and binding of the substrate to be modified. The catalytic domain near the C-terminus binds to UDP-GlcNAc and is responsible for enzymatic modification of target proteins. Early studies of OGT activity have shown that it can be post-translationally modified by phosphorylation and O-GlcNAcylation; however, the functional consequences are not very clear at this point.

The hydrolysis of O-GlcNAc modification from proteins is carried out by OGA, which is at highest concentrations in the cytoplasm, but may also be found in various organelles as well. One gene encodes the 917-amino-acid OGA protein. The two major domains of OGA are the N-terminal hexosaminidase domain and the C-terminal histone acetyltransferase domain (HAT). The HAT domain has been shown to have acetyltransferase activity; OGA can acetylate free histones and nucleosomal histones. In addition, caspase 3 can target OGA and cleave it into the two separate enzymatic domains without changing the activity of the domains (the cleaved domains may possibly still interact with each other to retain full activity). The significance of the HAT domain
is not fully clear; nor is the cleavage by caspase 3 or the post-translational modification of OGA\textsuperscript{46}. These may yet prove to be key regulators of cellular homeostasis through their regulation of OGA activity and downstream targets. The complexity of both OGT and OGA surely reflect the importance of O-GlcNAc modification within the cellular environment.

The O-GlcNAc modification has many clear differences from other types of cellular protein glycosylation. While N-glycosylation mainly occurs on the cell surface or in specialized organelles responsible for processing (endoplasmic reticulum, Golgi\textsuperscript{42}), O-GlcNAc modification can be observed in the nucleus, cytoplasm, and mitochondria—anywhere within the cell containing the enzymes necessary for the addition of the modification\textsuperscript{54}. In addition, the O-GlcNAc modification is a single sugar moiety, as opposed to the long chains of sugars found at the cell surface and elsewhere\textsuperscript{55}. Another difference is the dynamic nature of the modification. O-GlcNAc can be added and removed on proteins as rapidly as phosphorylation, thus constantly and precisely sensing and signaling based on the nutrient environment. Other sugar modifications do not change quite as dynamically, and often do not change much at all once their long chains are formed\textsuperscript{56}. Lastly, a major difference is the amino acids at which O-GlcNAc modifies proteins. While there is not a clear sequence that the O-GlcNAc transferase recognizes for O-GlcNAcylation, the modification is always an O-linkage on serine or threonine\textsuperscript{54}. This makes the modification more important for the function of the protein, since phosphorylation can also occur at these residues.
O-GlcNAcylation and Phosphorylation

O-GlcNAcylation is distinct from phosphorylation, since the addition/removal of O-GlcNAc is controlled by only two enzymes. The modification of proteins by phosphorylation has been shown to involve at least 600 different kinases and phosphatases, depending on the cell type, protein, and specific residue being phosphorylated. The complex relationship between O-GlcNAcylation and phosphorylation has only just begun to be elucidated. Early studies suggested that O-GlcNAcylation and phosphorylation may counteract each other and even compete for sites of modification. In more recent publications, however, this Yin-Yang model has been disproven in some instances, since O-GlcNAcylation may be required for phosphorylation of proteins, by modifying protein conformation to allow access to phosphorylation sites. Thus, the new paradigm (example in Fig 3) reflects the fact that the total profile on a protein of O-GlcNAcylation and phosphorylation may decide the level of activity, localization, or function of that protein within key pathways within the cell.

Figure 3. O-GlcNAc modification and phosphorylation determine ultimate protein activity and function. As shown in red, sites of protein phosphorylation (P) may be inhibited by O-GlcNAc modification by competing for the same serine or threonine residues, or by steric hindrance. However, in some cases, addition of O-GlcNAc modification may facilitate phosphorylation through a change in protein conformation or kinase binding. Thus, the total O-GlcNAc modification and phosphorylation profile of a protein may change its overall signaling by fine-tuning its activity and function.
O-GlcNAc in the Diabetic Vasculature

The direct evidence that the HBP is involved in diabetic pathology has been shown in mouse models overexpressing GFAT, the rate-limiting enzyme in the HBP, or OGT in various tissues\(^{59, 60}\). Overexpressing these proteins led to a phenotype closely resembling obesity or type 2 diabetes. GFAT overexpression in muscle and fat, which are insulin-sensitive tissues, causes insulin resistance because of impaired ability of the glucose transporter GLUT4 to be translocated to the membrane\(^{60}\). In addition, OGT overexpression in muscle and fat results in a very similar phenotype as seen in the models overexpressing GFAT\(^{59, 61}\).

One particular exciting area within the field of diabetic O-GlcNAcylation is the study of diabetic retinopathy. Diabetic mouse retinas have been shown to exhibit increased O-GlcNAcylation, particularly on proteins within the ganglion cell layer, the inner nuclear cell layer, the retina pigment epithelium layer, and the inner plexiform layer\(^{62}\). O-GlcNAcylation of hypoxia inducible factor 1α (HIF1α) in the retina was associated with decreased expression of occludin, an important regulator of cellular homeostasis, which contributes to the formation of retinal lesions in diabetes\(^{62}\). Since HIF1α is also a key protein induced by hypoxia and redox imbalance, the retina seems to be fertile ground for further discovery of the mechanisms linking diabetes, redox signaling, and O-GlcNAc modification. Studies using the Akita diabetic mouse model and the oxygen-induced ischemic retinopathy model have shown gradual increase of O-GlcNAcylation in the retina during normal development, and O-GlcNAcylation is even higher in diabetic retinas compared to controls\(^{63}\). In the model of oxygen-induced retinopathy, a link was found showing high O-GlcNAc levels during the stage in which
new vessels are being formed (neovascularization). The increase in O-GlcNAc modification may impair the migration of retinal pericytes, which is beneficial for the retina since pericyte migration induces degeneration of the vasculature observed in later stages of diabetes \(^6^4\). The interplay between O-GlcNAcylation and both physiological and pathological angiogenesis is an exciting prospect, and it seems that the total O-GlcNAc modifications in the retina must be fine-tuned to remain within a certain range—too little may impair physiological angiogenesis, and too much may cause pathological vascular degeneration \(^6^5, 6^6\). Moreover, in some pathologies such as the progression of tumors, highly increased O-GlcNAc modification has been shown to promote angiogenesis \(^6^7\). Just as observed in many animal models, there are many conflicting reports about the effects of increased O-GlcNAc in the vasculature. More studies will be required to determine the specific mechanisms and levels of O-GlcNAcylation that are necessary to maintain cellular homeostasis and physiological levels of redox signaling.

**O-GlcNAcylation and Vascular Calcification**

Emerging clinical and experimental studies have demonstrated a critical role for O-GlcNAcylation in vascular homeostasis and pathogenesis of vascular disease. It appears that acute and chronic increases in O-GlcNAcylation may have inverse effects on cardiovascular outcome. Acute increases in O-GlcNAcylation (within 24 hours) protect cardiomyocytes from oxidative stress-induced calcium overload and structural damage in ischemia/reperfusion models of heart failure \(^4^4, 6^8-7^0\); whereas chronically elevated O-GlcNAcylation, as occurs in diabetes, causes adverse complications in the cardiovascular system \(^7^1\). Nonetheless, a causative link between protein O-GlcNAcylation and vascular
calcification has yet been determined. Elevation of O-GlcNAcylation is found in human diabetic carotid plaques\textsuperscript{43} and diabetic mouse vasculature\textsuperscript{44}. Coincidently, increased calcified plaques have been identified in diabetic patients\textsuperscript{45} and diabetic mouse models\textsuperscript{13}. These studies support a positive correlation between protein O-GlcNAcylation and vascular calcification in diabetic vasculature, leading us to explore the regulation of O-GlcNAcylation on vascular calcification and the underlying molecular mechanisms.

As shown in the following three chapters, our studies have determined O-GlcNAc modification is a key regulator of major proteins involved in diabetic vascular calcification. The O-GlcNAc modification of these proteins regulates their activity and is crucial for their function in osteogenic differentiation of VSMC. These exciting findings not only provide important molecular insights into our basic understanding of protein O-GlcNAcylation in regulation VSMC function, but also identify novel potential therapeutic targets for the treatment of vascular calcification in diabetic patients.
ACTIVATION OF AKT BY O-GLCNACYLATION INDUCES VASCULAR CALCIFICATION IN DIABETES

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ABSTRACT

Vascular calcification is a serious cardiovascular complication that contributes to the increased morbidity and mortality of patients with diabetes. Hyperglycemia, a hallmark of diabetes, is associated with increased vascular calcification as well as increased modification of proteins by O-linked N-acetylglucosamine (O-GlcNAcylation). We sought to determine the role of protein O-GlcNAcylation in regulating vascular calcification and the underlying mechanisms. Low-dose streptozotocin-induced diabetic mice exhibited increased aortic O-GlcNAcylation and vascular calcification, which also was associated with impaired aortic compliance in mice. Elevation of O-GlcNAcylation by administration of Thiamet-G, a potent inhibitor for O-GlcNAcase (OGA) that removes O-GlcNAcylation, further accelerated vascular calcification and worsened aortic compliance of diabetic mice in vivo. Increased O-GlcNAcylation, either by Thiamet-G or OGA knockdown, promoted calcification of primary mouse vascular smooth muscle cells (VSMC). Increased O-GlcNAcylation in diabetic arteries or in the OGA knockdown VSMC upregulated expression of the osteogenic transcription factor Runx2 and enhanced activation of AKT. O-GlcNAcylation of AKT at two new O-sites, T430 and T479, promoted AKT phosphorylation, which in turn enhanced VSMC calcification. Site-directed mutation of AKT at T430 and T479 decreased O-GlcNAcylation, inhibited phosphorylation of AKT at S473 and binding of mTOR complex 2 to AKT, and subsequently blocked Runx2 transactivity and VSMC calcification.

In summary, O-GlcNAcylation of AKT at two new sites enhanced AKT phosphorylation and activation, thus promoting vascular calcification. Our studies have identified a novel causative effect of O-GlcNAcylation in regulating vascular
calcification in diabetes and uncovered a key molecular mechanism underlying O-GlcNAcylation-mediated activation of AKT.
NON-STANDARD ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>α-SMA</td>
<td>smooth muscle specific α-actin</td>
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<tr>
<td>CA-AKT</td>
<td>constitutively active AKT</td>
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<tr>
<td>ColIA1</td>
<td>type I collagen A1</td>
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<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>OC</td>
<td>osteocalcin</td>
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<tr>
<td>OGA</td>
<td>β-N-acetylglucosaminidase</td>
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<tr>
<td>OGT</td>
<td>β-N-acetylglucosaminyltransferase</td>
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<tr>
<td>O-GlcNAcylation</td>
<td>O-linked β-N-acetylglucosamine modification</td>
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<td>OPN</td>
<td>osteopontin</td>
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<td>PWV</td>
<td>pulse wave velocity</td>
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<tr>
<td>Runx2</td>
<td>runt-related transcription factor 2</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cells</td>
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<td>wt-AKT</td>
<td>wild type AKT</td>
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NOVELTY AND SIGNIFICANCE

What is known?

- Diabetic patients have increased prevalence of vascular calcification, correlating with higher risk for adverse cardiovascular events.
- Hyperglycemia, a characteristic feature of diabetes, is associated with increased vascular calcification as well as increased overall protein O-GlcNAcylation.
- Protein O-GlcNAcylation is tightly regulated by two enzymes, OGT and OGA.
- Activation of AKT is important for oxidative stress-induced calcification of vascular smooth muscle cells (VSMC).

What new information does this article contribute?

- STZ-induced diabetic mice exhibit increased O-GlcNAcylation and vascular calcification, which also was associated with impaired aortic compliance.
- Inhibition of OGA in VSMC, either by Thiamet-G or the OGA knockdown, increases O-GlcNAcylation in VSMC, which promotes VSMC calcification.
- Administration of Thiamet-G in diabetic mice further enhances vascular O-GlcNAcylation, accelerated vascular calcification and worsened aortic compliance.
- Increased O-GlcNAcylation in diabetic arteries or in the OGA knockdown VSMC enhances activation of AKT that upregulates expression of Runx2.
- AKT activation mediates increased O-GlcNAcylation-induced VSMC calcification.
- Site-directed mutation of AKT at T430 and T479 decreases O-GlcNAcylation inhibits phosphorylation of AKT at S473 and binding of the mTOR complex 2 to AKT, which leads to inhibition of the Runx2 transactivity and subsequent VSMC calcification.
- O-GlcNAcylation of AKT at T430 and T479 promotes phosphorylation of AKT, which represents a novel mechanism underlying AKT activation and vascular calcification in diabetes.

Summary

Vascular calcification is often observed in diabetic arteries, which increases the frequency of cardiovascular events and mortality in diabetes patients. O-GlcNAcylation is increased in response to stressors observed in the diabetic vasculature, such as hyperglycemia and oxidative stress. The present studies have demonstrated a novel and causative link between protein O-GlcNAcylation and vascular calcification in diabetes, and revealed that O-GlcNAcylation of AKT at two new sites enhances AKT phosphorylation and subsequently induces VSMC calcification. These findings have determined O-GlcNAcylation as a novel regulator for development of vascular calcification and uncovered a novel mechanism underlying AKT activation by its O-GlcNAcylation. Our study identified O-GlcNAcylation of AKT as a potential new target for the development of therapies for vascular calcification in diabetes.
INTRODUCTION

Diabetes has been strongly associated with chronic cardiovascular and renal complications, which leads to an increased morbidity and mortality in affected patients\(^1\). \(^2\) Increased vascular calcification is commonly observed in diabetic arteries, in the intimal and medial layers of the vessel walls\(^3\)–\(^6\), which increases arterial stiffness, reduces compliance of the blood vessels\(^7\)–\(^8\), and increases the risk of cardiovascular events and mortality\(^9\). Therefore, understanding of the molecular mechanisms underlying diabetic vascular calcification should provide important insights into overcoming these adverse clinical outcomes.

Hyperglycemia, or elevated blood glucose, is a characteristic feature of diabetes\(^10\). In addition to producing bioenergetic substrates via the tricarboxylic acid cycle, glucose metabolism through the hexosamine biosynthesis pathway generates UDP-GlcNAc, a substrate for protein O-linked \(\beta\)-N-acetylglucosamine modification (O-GlcNAcylation)\(^11\). Hyperglycemia has also been associated with vascular calcification \textit{in vitro}\(^5\),\(^12\); however, the mechanistic function of hyperglycemia and O-GlcNAcylation in regulating diabetic vascular calcification is unknown. O-GlcNAcylation is a dynamic and reversible modification that regulates the activity and function of numerous cytoplasmic and nuclear proteins\(^13\),\(^14\). Like protein phosphorylation, O-GlcNAcylation occurs on serine and threonine residues, and thus these two modifications on proteins crosstalk to regulate cellular signaling and function. Unlike phosphorylation, however, O-GlcNAcylation is tightly regulated by two specific enzymes: \(\beta\)-N-acetylglucosaminyl-transferase (OGT) adds O-GlcNAc onto target proteins, whereas \(\beta\)-N-acetylglucosaminidase (OGA) removes O-GlcNAc modification.
Protein O-GlcNAcylation regulates a variety of cellular functions in different tissues, including the cardiovascular system, related to diabetes and vascular injury. O-GlcNAcylation was found to serve as a cellular nutrient and stress sensor by modulating the function of specific proteins in response to glucose levels\textsuperscript{15}. In cardiomyocytes, O-GlcNAcylation is associated with cell survival in response to oxidative stress, and preserves heart function in models of heart failure\textsuperscript{16-18}. In contrast, increased O-GlcNAcylation has been observed to negatively influence contractility in left ventricular tissue from humans with heart failure\textsuperscript{19}. In human diabetic carotid plaques, the overall O-GlcNAcylation level is increased\textsuperscript{20}. Additionally, diabetic patients have a higher incidence of calcified plaque\textsuperscript{3}. However, the contribution of elevated O-GlcNAcylation in the diabetic vasculature to vascular calcification is unknown.

The present studies investigate the function of O-GlcNAcylation in regulating vascular calcification and the underlying molecular mechanisms. We have demonstrated elevated O-GlcNAcylation and increased vascular calcification in arteries from diabetic mice, which was associated with impaired aortic compliance. Elevation of O-GlcNAcylation by Thiamet-G treatment, a potent inhibitor for OGA, further accelerated vascular calcification and worsened aortic compliance of diabetic mice \textit{in vivo}. Using primary cultured mouse vascular smooth muscle cells (VSMC), we have determined that O-GlcNAcylation of AKT at two new sites increases phosphorylation and activation of AKT, which promoted VSMC calcification. These studies have demonstrated a novel and causative link between protein O-GlcNAcylation and vascular calcification in diabetes. Understanding the molecular mechanisms underlying hyperglycemia in regulating diabetic vascular calcification should provide important insights into
identification of new targets and strategies for prevention and therapy of diabetic calcification.
METHODS

Experimental Animals — To induce hyperglycemia and diabetes, low-dose streptozotocin (STZ) injection was performed as previously described\textsuperscript{21-23}. Briefly, C57BL/6 mice were intraperitoneally injected with STZ (50 mg/kg) for 5 consecutive days, and blood glucose was monitored weekly for 4 months using the AlphaTrak glucose meter and strips (Abbott, Abbott Park, IL). For Thiamet-G treatment, mice were injected intravenously with Thiamet-G\textsuperscript{24} (20 mg/kg) one week after STZ treatment and weekly for 2 months. Both food and fluid intake were given \textit{ad libitum}. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Tissue Harvest and Processing — At the experimental end points, mice were sacrificed, the aortic arch and descending aorta were dissected under a microscope and used for characterization of calcium content, RNA and protein expression and immunostaining as we previously described\textsuperscript{25}.

Aortic Calcium Measurement — Aortic calcium content was measured by Arsenazo III assay as we previously described\textsuperscript{25}. Descending aortas were homogenized and digested by collagenase. Protein amount was determined by BCA assay\textsuperscript{26}, and calcium was extracted with 0.6 mmol/L HCl and quantified colorimetrically by Arsenazo III calcium measurement kit (StanBio)\textsuperscript{25}. The amount of vascular calcium was normalized to the total protein amount in the tissues and expressed as fold change compared to control.

Echocardiography and Measurement of Pulse Wave Velocity — Pulse wave velocity (PWV) was analyzed by echocardiography with the high resolution imaging system.
VEVO 770 (Visual Sonics, Toronto, Canada). Detailed methods are available in online supplemental materials.

**In Vitro Calcification of VSMC** — Primary VSMC were isolated from the aortas of C57BL/6 mice as we described²⁶. VSMC calcification was induced in osteogenic medium containing DMEM, supplemented with 20% fetal bovine serum, L-ascorbic acid (0.25 mM), β-glycerophosphate (10 mM), and dexamethasone (10⁻⁸ M, Sigma-Aldrich) for 3 weeks. Calcification was determined by Alizarin red staining as we described²⁵. In parallel sets of dishes, cells were lysed with 0.5 N HCl and total calcium content was quantified with Arsenazo III calcium measurement kit (StanBio) and normalized to the amount of total proteins²⁵.

**Induction of O-GlcNAcylation in VSMC** — O-GlcNAcylation was induced by inhibition of OGA, using a pharmacological inhibitor, Thiamet-G²⁴, or OGA knockdown by lentivirus-mediated short hairpin RNA specific targeting OGA (GenBank NC_000085.6, shRNA, Thermo Scientific, Waltham, MA) as we have described previously²⁶.

OGA, OGT and O-GlcNAcylation were determined by Western blot analysis using specific antibodies for OGA (Santa Cruz Biotechnology, Santa Cruz, CA), OGT (Sigma Aldrich, St. Louis, MO), and O-GlcNAcylation (RL-2, Abcam)²⁷.

**O-GlcNAcylation of AKT** — To determine AKT O-GlcNAcylation and its impact on phosphorylation, immunoprecipitation was performed with AKT antibody (Cell Signaling). In brief, cell extracts were incubated with AKT antibody or isotope-matched IgG (Santa Cruz, as negative control) at 4°C overnight and then mixed with protein G agarose beads (Sigma Aldrich) for 3 hours. Beads were washed, and proteins pulled
down were analyzed by Western blotting using specific antibodies to detect O-
GlcNAcylation (RL-2) and AKT phosphorylation (Cell Signaling, listed above).

*Dual-Luciferase Reporter Assay* — Runx2 transactivity was determined as we described
by Dual-Luciferase Reporter assay (Promega, Madison, WI) with the use of a luciferase
reporter construct containing six Runx binding elements (p6xRunx-Luc)<sup>28</sup>.

*Generation of AKT Mutants* — Constructs carrying cDNA encoding wild type (wt-AKT)
and constitutively active AKT (CA-AKT) were originally provided by Dr. Hongju Wu
(Tulane University)<sup>29</sup>. Point mutations in the sequence of the lentiviral CA-AKT were
made at serine 122, threonine 430 and threonine 479 to replace the residues with alanine
using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies, Santa
Clara, CA) and confirmed by sequencing analysis. VSMC stably infected with lentivirus
expressing wt-AKT, CA-AKT, and mutant AKT, CA-AKT-S122A, Ca-AKT-T430A and
CA-AKT-T479A, were characterized for AKT O-GlcNAcylation and their effects on
AKT phosphorylation, Runx2 activity and VSMC calcification. The effects of AKT
mutants on the binding of AKT to its kinases and phosphatase were determined by
immunoprecipitation followed by Western blot analysis of Rictor, mTOR, PDK1, PHLPP
with specific antibodies (Cell Signaling).

*Statistical Analysis* — Results are presented as the mean ± SD. Differences between
groups were determined with the use of Student *t* tests or 1-way ANOVA where
appropriate. Significance was defined as *p*<0.05.
RESULTS

*Increased Vascular O-GlcNAcylation and Calcification in Diabetic Mice*

Using low-dose STZ injection-induced diabetic mouse model, we characterized O-GlcNAcylation and vascular calcification in mice. Blood glucose levels were monitored in the STZ-injected mice and compared with those in the control mice (Fig 1A). Elevation of blood glucose levels was observed at one week in the STZ-injected mice (Fig 1A). Severe hyperglycemia was observed after three weeks, which was sustained until the end of the experiments at 16 weeks after administration of STZ. Echocardiography analysis demonstrated a significant increase in pulse wave velocity (PWV), an indicator for aortic stiffness\(^2\), in the diabetic mice 16 weeks after administration of STZ (Fig 1B), suggesting impaired aortic compliance in the diabetic mice. Therefore, STZ-induced hyperglycemia was linked to impaired aortic function in the diabetic mice.

As hyperglycemia has been linked to increased protein O-GlcNAc modification\(^{30,31}\), we determined protein O-GlcNAcylation profile in the diabetic vasculature. Dramatic increases in O-GlcNAcylation were demonstrated in the aortas from STZ-injected mice (Fig 1C, top panel). Increased O-GlcNAcylation in the diabetic arteries was associated with increased expression of the osteogenic transcription factor Runx2 (Fig 1C, middle panel). We have previously demonstrated that increased Runx2 determines vascular calcification\(^{25,26}\). Consistently, increased calcification was also observed in the aortas from STZ-injected mice (Fig 1D). Additionally, increased O-GlcNAcylation was observed in the vasculature of mice 4 weeks after STZ administration, but calcification was not significantly increased at this time point (Suppl.
Fig 1), indicating that increased vascular O-GlcNAcylation may contribute to calcification in diabetes.

Immunofluorescent staining further demonstrated increased vascular O-GlcNAcylation in the media of arteries from STZ-injected diabetic mice compared to those from controls (Fig 1E, O-GlcNAc). Increased O-GlcNAcylation was correlated with decreased smooth muscle specific α-actin (α-SMA) and increased Runx2 expression (Fig 1E). In addition, increased expression of Runx2 and osteogenic marker genes, including osteocalcin (OC), collagen IA1 (ColIA1), and osteopontin (OPN) was demonstrated in aortas from the diabetic mice (Fig 1F), further confirming an association of O-GlcNAcylation with SMC dedifferentiation and calcification.

*Increased O-GlcNAcylation in VSMC Induces Vascular Calcification*

To determine a direct effect of increased O-GlcNAcylation on vascular calcification, we induced O-GlcNAcylation in cultured VSMC using Thiamet-G, a highly potent and selective inhibitor of OGA that has been shown to increase O-GlcNAc modification\(^2\). We found that Thiamet-G dose-dependently increased O-GlcNAcylation, independent of OGT (Fig 2A, and 2B). Thiamet-G at the concentration used did not affect cell viability and proliferation (data not shown). Importantly, increased O-GlcNAcylation by Thiamet-G was found to induce VSMC calcification, as shown by Alizarin red staining (Fig 2C), calcium content quantification by Arsenazo III assay (Fig. 2D), and increase expression of Runx2 (Fig 2E). Similar to the observation with diabetic arteries *in vivo* (Fig 1F), increased O-GlcNAcylation in VSMC by the Thiamet-G
treatment was found to upregulate expression of Runx2 as well as other osteogenic marker genes (Fig 2E).

To confirm that the effects of Thiamet-G were mediated through OGA, we selectively knocked down OGA in VSMC using lentivirus containing shRNA for OGA. Western blot analysis demonstrated effective knockdown of OGA in VSMC, without affecting the expression of OGT (Fig 3A). The viability and proliferation of VSMC was not affected by the OGA knockdown (data not shown). Consistent with the results from Thiamet-G treatment (Fig 2A), the OGA knockdown in VSMC increased O-GlcNAcylation (Fig 3A). Importantly, the OGA knockdown was sufficient to induce expression of Runx2 and other osteogenic marker genes, as well as VSMC calcification (Fig 3B-D). Therefore, these studies have demonstrated a direct effect of increased O-GlcNAcylation on osteogenic differentiation and calcification of VSMC in vitro.

*Increased O-GlcNAcylation Enhances Diabetic Vascular Calcification In Vivo*

The effects of increased O-GlcNAcylation on vascular calcification was further determined in diabetic mice *in vivo*. STZ-induced vascular O-GlcNAcylation was dramatically enhanced by administration of Thiamet-G (Fig 4A). At 8 weeks after the STZ injection, STZ alone induced a significant increase in vascular calcification. Strikingly administration of Thiamet-G further enhanced vascular calcification (Fig 4B). The effect of Thiamet-G on diabetic vascular calcification (Fig. 4B) was well associated with increased expression of Runx2 and the osteogenic marker genes (Fig 4C). Consistently, administration of Thiamet-G further increased aortic stiffness and worsened aortic compliance, indicated by increased pulse wave velocity, in the diabetic mice (Fig
Taken together, these data demonstrated a causative link between increased O-GlcNAcylation and vascular calcification in diabetic mice in vivo.

**Increased O-GlcNAcylation Enhances Activation of AKT**

To determine the molecular mechanisms underlying O-GlcNAcylation on VSMC calcification, we characterized the activation of protein kinase AKT, a critical upstream kinase that we have previously determined to regulate Runx2 activity and VSMC calcification. Glucose-induced phosphorylation/activation of AKT at serine 473 (S473) and threonine 308 (T308) was demonstrated in the control VSMC (Fig 5A, shScr). Increased O-GlcNAcylation in the OGA knockdown VSMC (shOGA) resulted in basal activation of AKT by phosphorylation at S473, but not T308. Furthermore, increased and sustained phosphorylation/activation at both S473 and T308 was demonstrated in OGA knockdown VSMC after stimulation with glucose (Fig 5A), suggesting a direct effect of O-GlcNAc modification on activation of AKT.

Consistently, increased phosphorylation of AKT at both S473 and T308 was observed in the vasculature of diabetic mice injected with STZ (Fig 5Ba). Furthermore, administration of Thiamet-G enhanced AKT phosphorylation at S473 (Fig 5Bb), suggesting the role of increased activation of AKT in mediating the effect of increased O-GlcNAcylation on vascular calcification in diabetes. Using the AKT IV inhibitor, we demonstrated that inhibition of AKT activation blocked increased O-GlcNAcylation-induced VSMC calcification in the OGA knockdown VSMC (Fig 5C, D), supporting a critical role of the AKT activation in mediating O-GlcNAcylation-induced VSMC calcification.
**O-GlcNAcylation of AKT Directly Regulates Activation/Phosphorylation of AKT and Vascular Calcification**

We further characterized whether AKT was directly modified by O-GlcNAcylation, and how the modification alters the AKT activation. O-GlcNAcylation of AKT was detected by Western blot analysis of the AKT immunoprecipitated complex using O-GlcNAc specific antibody (Fig 6A). Four putative O-GlcNAc modification sites were predicted on AKT with the YinOYang 1.2 software ([http://www.cbs.dtu.dk/services/YinOYang/](http://www.cbs.dtu.dk/services/YinOYang/)): serine 122 (S122), threonine 430 (T430), serine 473 (S473), and threonine 479 (T479, Fig 6B). To determine the effect of O-GlcNAcylation on activation of AKT, a lentiviral constitutively-active AKT vector (CA-AKT) was used to mutate the putative glycosylation sites to encode an alanine residue (A). Because S473 is a known site that determines AKT phosphorylation and activation, it was not targeted for mutagenesis. Mutations at both T430 and T479 inhibited O-GlcNAcylation of AKT, whereas mutation at S122 did not affect O-GlcNAcylation of AKT (Fig 6C).

Since phosphorylation of AKT is known to occur at S473 and T308, we characterized the effects of altered AKT O-GlcNAcylation on its activation with each of the AKT mutants. Decreased O-GlcNAcylation of AKT in either T430A or T479A mutant resulted in decreased phosphorylation of AKT at S473, but not at T308 (Figure 7A). These data support a positive correlation of AKT O-GlcNAcylation at T430 or T479 and its phosphorylation at S473.

To explore the mechanisms underlying the regulation of AKT phosphorylation by AKT O-GlcNAcylation, we determined the effects of these AKT mutations on AKT
binding to kinases and phosphatase that are known to regulate AKT phosphorylation. All AKT mutants were found to bind to PDK1 and PHLPP similarly to wt-AKT or CA-AKT (Fig 7B). Decreased O-GlcNAcylation of AKT in either T430A or T479A mutant, but not in S122A, inhibited AKT binding to mTOR and Rictor (Fig 7B), a component of the mTOR complex 2, known to phosphorylate AKT at S473$^{31}$. Inhibition of mTOR signals with rapamycin inhibited vascular calcification induced by increased O-GlcNAcylation in the OGA knockdown VSMC, which was associated with inhibition of AKT phosphorylation (Suppl. Fig II). Therefore, the reduced binding of the mTOR complex 2 to AKT may contribute to inhibited AKT phosphorylation at S473 by impaired O-GlcNAcylation at T430/479, which led to the decrease in vascular calcification.

The contribution of AKT O-GlcNAcylation to VSMC calcification was further demonstrated in VSMC stably expressing different AKT variants. Importantly, expression of CA-AKT in VSMC is sufficient to promote VSMC calcification, as shown by increased calcium content (Fig 7C), suggesting AKT is a key regulator in the VSMC calcification. Mutation at AKT S122A, which did not affect O-GlcNAcylation and phosphorylation of AKT, had no impact on VSMC calcification. In sharp contrast, T430A and T479A, which are the two mutants that markedly inhibited O-GlcNAcylation and phosphorylation of AKT, significantly reduced VSMC calcification (Fig 7C). Consistently, CA-AKT induced Runx2 transactivity, which was inhibited by AKT mutations of T430A and T479A, but not S122A (Fig 7D). Taken together, these data support a critical role of O-GlcNAcylation of AKT at T430/479 in regulating its phosphorylation/activation at S473, thus inducing Runx2 transactivity and promoting VSMC calcification.
DISCUSSION

Vascular calcification is prevalent in diabetes and is correlated with adverse cardiovascular outcome\textsuperscript{32, 33}; however, the molecular mechanisms underlying increased vascular calcification in diabetes are largely unknown. Elevation of O-GlcNAcylation is found in human diabetic carotid plaques\textsuperscript{20} and diabetic mouse vasculature\textsuperscript{34}. Coincidently, increased vascular calcification has been identified in both type I and type II diabetic patients\textsuperscript{35} and diabetic mouse models\textsuperscript{6}. Nevertheless, the role of O-GlcNAcylation in vascular calcification has not been previously determined. The present study has demonstrated a causative effect of O-GlcNAcylation on diabetic vascular calcification. Our studies revealed that activation of AKT by O-GlcNAcylation in vasculature is key to diabetic vascular calcification. Two novel O-GlcNAcylation sites on AKT play a crucial role in enhancing AKT phosphorylation at S473 to increase vascular calcification. Because O-GlcNAcylation is tightly regulated by two specific enzymes, the new findings have exciting implications for prevention and treatment of diabetic vascular calcification through therapies targeting O-GlcNAcylation and signaling.

We found that increased O-GlcNAcylation in response to chronic hyperglycemia induced vascular calcification in the low-dose STZ-induced diabetic mouse model (Fig 1). Previous studies have demonstrated that acute increases in O-GlcNAcylation (less than 24 hours) protect cardiomyocytes from oxidative stress-induced calcium overload and structural damage in ischemia/reperfusion models of heart failure\textsuperscript{18,36,37}. However, few studies have examined the function of chronic increases in O-GlcNAcylation. This study and others\textsuperscript{38} indicate chronic O-GlcNAcylation over an extended period of time, as
observed in the later stages of diabetes, may cause adverse complications in the cardiovascular system. The distinct function of O-GlcNAcylation in chronic and acute disease model may be related to differential activation of unknown signaling cascades. While the STZ model has its limitation due to its toxicity in vitro and its inhibitory effect on OGA also, its diabetogenic mechanism of action has been shown to be independent of these side effects since STZ has a very short half-life. Using Thiamet-G, a potent and selective OGA inhibitor, our studies have provided the first evidence that increased vascular O-GlcNAcylation enhanced vascular calcification in diabetic mice in vivo. Consistent with the clinical observations demonstrating an association between increased vascular calcification and other vascular complications in diabetes, our findings have demonstrated reduced aortic compliance in the STZ-induced diabetic mice, which was further worsened by increased O-GlcNAcylation achieved by the Thiamet-G treatment. Together, our studies have revealed a causative link between increased O-GlcNAcylation and diabetic vascular calcification and impaired vascular compliance in vivo.

Using VSMC in culture, we further demonstrated that increased O-GlcNAcylation by OGA inhibition or knockdown induced VSMC calcification. Previously considered a passive process by deposition of calcium, vascular calcification has now been recognized as a regulated dynamic process involving osteochondrogenic differentiation of vascular cells. Increased O-GlcNAcylation has been associated with osteogenesis and chondrogenesis; however, the underlying mechanisms are unknown. Our studies have revealed a direct effect of O-GlcNAcylation in regulating osteogenic differentiation of VSMC, which may also provide new insights into the function of O-GlcNAcylation in
regulating differentiation of osteoblasts and chondrocytes. We have previously reported that oxidative stress induces AKT activation and VSMC calcification. Both oxidative stress and high glucose induce vascular calcification, and have been found to increase O-GlcNAcylation. Accordingly, it is also likely that hyperglycemia induces oxidative stress that contributes to increased vascular calcification.

We found that increased activation of AKT was associated with increased vascular calcification in the STZ-induced diabetic arteries (Fig 5B). These observations are consistent with previous studies showing that VSMC in diabetic models exhibit sustained activation of AKT after chronic hyperglycemia, although other studies reported blunted AKT activation in diabetic cardiomyocytes and myotubes. Apparently, AKT may be differentially regulated depending upon cell types, cellular environment and disease status. Using Thiamet-G to induce O-GlcNAcylation in vivo, we demonstrated a direct effect of O-GlcNAcylation on AKT activation in the diabetic vasculature. Consistent with these findings, blockade of OGA in cultured VSMC increased O-GlcNAcylation and simultaneously increased and sustained activation of AKT (Fig 5). Furthermore, inhibition of AKT activation attenuated VSMC calcification, demonstrating an essential role of AKT activation in mediating O-GlcNAcylation-induced vascular calcification. Remarkably, constitutively active AKT was sufficient to induce VSMC calcification of VSMC (Fig 7), demonstrating that AKT activation is a key regulator of vascular calcification.

Importantly, the present studies have provided paradigm-shifting novel mechanisms underlying the regulation of AKT phosphorylation and activation by O-GlcNAcylation. Although activation of AKT has been associated with altered O-
GlcNAcylation in different cells, whether the AKT activation is regulated directly by O-GlcNAcylation is not clear\textsuperscript{14, 46}. Our studies have identified that O-GlcNAcylation at T430 and T479 plays an important role in AKT phosphorylation at S473, which promotes vascular calcification. A recent study suggested that O-GlcNAcylation of AKT at T305 and T312 inhibits AKT phosphorylation at T308\textsuperscript{47}, in COS-7 cells, which is in agreement with the prevailing belief that protein O-GlcNAcylation and phosphorylation reciprocally regulate protein activity\textsuperscript{48}. However, mutation at T305 and T312 did not affect AKT phosphorylation at S473 or VSMC calcification (Suppl. Fig III). In addition, increased O-GlcNAcylation by the OGA knockdown in VSMC did not affect basal AKT phosphorylation at T308 (Fig 5). Furthermore, we found that inhibition of O-GlcNAcylation at T430 and T479 did not affect activation of AKT at T308, but only inhibited activation of AKT at S473, suggesting the selective effect of O-GlcNAcylation on the key residues. Therefore, we have identified unique O-GlcNAc modification at two novel sites, T430/479 that are critical for AKT phosphorylation and its function to promote VSMC calcification.

Moreover, mechanistic studies further revealed that AKT O-GlcNAcylation at T430/479 is important for the binding of AKT to Rictor, a component of the mTOR complex 2 (Fig 7B). Because mTOR complex 2 is known to phosphorylate AKT at of S473\textsuperscript{31}, disruption of AKT binding to Rictor by the T430A and T479A mutations may contribute to the inhibited phosphorylation of AKT at S473. Inhibition of O-GlcNAcylation-induced VSMC calcification by rapamycin (Suppl. Fig II) further supports a role of the mTOR signals in mediating O-GlcNAcylation-induced AKT activation and VSMC calcification. The precise mechanism of how O-GlcNAcylation at
T430/479 affects its binding to Rictor remains to be determined. The T430, T479 and S473 residues lie in the hydrophobic motif of AKT, which plays a major role in AKT protein stability. It is possible that O-GlcNAc modifications at T430/479 may change the conformation of the hydrophobic motif so that facilitates its binding to mTOR complex 2 and thus leading to its phosphorylation at S473 site. Although S473 does not reside in the catalytic domain of AKT, phosphorylation of S473 may lead to a conformational change that modulates complete kinase activity and phosphorylation of downstream targets. This study revealed an essential role of AKT phosphorylation at S473 in regulating osteogenic transcription factor Runx2 and VSMC calcification.

The function of O-GlcNAcylation-induced phosphorylation/activation of AKT in promoting VSMC calcification appear to be independent of its regulation of cell proliferation and apoptosis, as we found that increased O-GlcNAcylation, by the OGA knockdown, did not affect VSMC proliferation/viability. Consistently, we have reported that AKT inhibition does not induce apoptosis of VSMC. Further studies are warranted to dissect the precise signaling cascades that are responsible for AKT activation-induced Runx2 upregulation. Nevertheless, the novel regulation of AKT activation by O-GlcNAcylation uncovered in this study may have significant impact not only on the biological function of AKT activation, but also provide novel mechanistic insights into pathogenesis of vascular disease featuring increased activation of AKT.

In summary, the present studies have demonstrated a novel causative link between chronic increases in vascular O-GlcNAcylation and vascular calcification in diabetes, and uncovered a novel mechanism underlying the regulation of AKT activation by its O-GlcNAcylation, which induces Runx2 upregulation and promotes VSMC calcification.
(Suppl. Fig IV). These findings have determined O-GlcNAcylation as a novel contributor to the process of vascular calcification and identified O-GlcNAcylation of AKT as a possible target for the development of therapies for vascular calcification in diabetes.
ACKNOWLEDGMENTS

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expression by increasing sp1 glycosylation. Proc Natl Acad Sci USA. 2000;97:12222-12226


**FIGURE 1**

*Figure 1. Increased O-GlcNAcylation and vascular calcification in diabetic mice.* **A)** STZ injection increased blood glucose. Mice were injected intraperitoneally with sodium citrate buffer (Control) or 50 mg/kg STZ for 5 consecutive days. Blood glucose was measured using the AlphaTrak glucose meter for 16 weeks following the STZ injection (n=6 mice for each group, *p<0.05, **p<0.01 compared to control). **B)** Increased aortic stiffness in STZ-injected diabetic mice. Echocardiography was performed in mice 16 week after the STZ injection, to determine pulse wave velocity, an indicator for aortic stiffness (n=4, *p=0.02). **C)** Increased vascular O-GlcNAcylation and Runx2 expression in diabetic mice. Descending aortas from control and STZ-injected mice were explanted, and protein extracts were analyzed for O-GlcNAcylation and Runx2 by Western blot. **D)** Increased vascular calcification in diabetic mice. Calcium content was determined in descending aortas from control and STZ-injected mice. The calcium content in aortas from control mice was defined as 1 (n=4 mice for each group, *p=0.002). **E)** Increased O-GlcNAcylation and Runx2 in aortic media of diabetic mice. Aortic sections from control and STZ-injected mice were stained with specific antibodies as labeled. White dashed lines delineate aortic media (m) and adventitia (a). Scale bar=100 µm. **F)** Increased expression of Runx2 and osteogenic markers in aortas from diabetic mice. Real-time PCR analysis was performed to determine the expression of Runx2 and osteogenic marker genes, including OC, ColIA1 and OPN, in aortas from control and STZ-injected diabetic mice (n=4, *p<0.001).
**Figure 2.** Inhibition of OGA increases O-GlcNAcylation and calcification in VSMC.  
A) and B) Thiamet-G increased O-GlcNAcylation in VSMC. VSMC were treated with Thiamet-G for 6 hours at 0-10 μM, and Western blot was performed to determine O-GlcNAcylation. Representative blots from 4 independent experiments are shown in A. B) The intensity of the bands in each condition in A was quantified by NIH ImageJ and compared with that in the control (Thiamet-G, 0), defined as 1.  
C) and D) Thiamet-G induced VSMC calcification. VSMC were treated with osteogenic medium alone (Control) or osteogenic medium with 10 μM Thiamet-G for 3 weeks. Calcification was determined by Alizarin red staining (C) or quantified by Arsenazo III assay (D, n=3, *p<0.001).  
E) and F) Thiamet-G increased the expression of Runx2 and osteogenic marker genes, as determined by Western blot (E) and Real-time PCR analysis (F) in parallel experiments as in C and D (n=3, *p<0.01 compared to control).
**Figure 3. Knockdown of OGA increases VSMC calcification.**

**A)** Knockdown of OGA in VSMC by shRNA increased O-GlcNAcylation and Runx2. VSMC were infected with lentivirus containing scrambled shRNA (shScr) or shRNA for OGA (shOGA). Infected cells were selected using puromycin. The amount of OGA, OGT, Runx2 and O-GlcNAc was determined by Western blot analysis. Representative blots from 4 independent experiments are shown. **B)** and **C)** OGA knockdown induced VSMC calcification. Control and OGA knockdown cells were cultured in osteogenic medium for 3 weeks. Calcification was determined by Alizarin red staining (B) or quantified by Arsenazo assay (C) in separate dishes (n=3, *p<0.001). **D)** OGA knockdown increased expression of Runx2 and osteogenic marker genes. Real-time PCR analysis was performed to determine the expression of Runx2 and osteogenic marker genes in parallel experiments as in B and C (n=3, *p<0.005 compared to shScr).
Figure 4. Thiamet-G treatment accelerates vascular calcification in diabetic mice. Mice were treated with control, STZ and STZ plus Thiamet G for 8 weeks as described in material and methods. A) Thiamet-G increased vascular O-GlcNAcylation and Runx2 in diabetic mice. Descending aortas were explanted, and protein extracts were analyzed for aortic O-GlcNAcylation and Runx2 by Western blot. B) Thiamet-G accelerated vascular calcification in diabetic mice. Calcium content was determined in mouse descending aortas and compared with that in the vehicle-treated control group, which is defined as 1 (n=4, *p<0.05 compared with the control, #p<0.05 compared with STZ). C) Thiamet-G increased the expression of Runx2 and osteogenic marker genes in diabetic mice. Real-time PCR analysis was performed to determine the expression of Runx2 and osteogenic marker genes in descending aortas from mice treated with vehicle control, STZ and STZ plus Thiamet-G (n=4, *p<0.05 compared with control, #p<0.05 compared with STZ alone). D) Thiamet-G increased aortic stiffness of diabetic mice. Echocardiography was performed to determine pulse wave velocity (n=4, *p<0.05 compared with the control, #p<0.05 compared with STZ).
Increased O-GlcNAcylation enhances activation of AKT. A) OGA knockdown enhanced AKT activation. Control or OGA knockdown VSMC were exposed to glucose (25 mM) for 0 to 60 minutes. Western blot analysis was performed to determine phosphorylation/activation of AKT at S473 and T308. Representative blots from 3 experiments are shown. B) Increased vascular O-GlcNAcylation is associated with increased AKT activation in diabetic mouse aortas. Western blot analysis was performed to determine AKT phosphorylation at S473 and T308, in a) aortas from control or STZ-injected mice after 16 weeks of STZ injection; and b) aortas from control, STZ and STZ plus Thiamet G-treated mice 8 weeks after STZ injection. Representative blots of 4 mice in each group are shown. C) and D) Inhibition of AKT blocks increased O-GlcNAcylation-induced VSMC calcification. Control and OGA knockdown VSMC were cultured in osteogenic medium with or without AKT inhibitor IV (5 µM) for 3 weeks. Calcification was determined by Alizarin red staining (C) or quantified by Arsenazo III assay (D) in separate dishes (n=3, *p<0.001)
Figure 6. O-GlcNAc modification of AKT at T430 and T479.  

A) AKT was O-GlcNAcylated in VSMC. Immunoprecipitation was performed with antibody for AKT in cell lysate from VSMC cultured in growth media with high glucose (25 mM), and subsequently analyzed by Western blotting. Immunoprecipitation with control IgG antibody was used as a negative control. 

B) Putative glycosylation sites on AKT. YinOYang software was used to predict putative O-GlcNAcylation sites of AKT at serine and threonine residues. 

C) T430 and T479 are required for O-GlcNAcylation of AKT. Site-directed mutagenesis was performed on the constitutively active AKT to replace the putative O-GlcNAcylation sites with alanine. Lentivirus carrying wild-type (wt-AKT), constitutively active (CA-AKT), and the AKT point mutants (CA-AKT-S122A, CA-AKT-T430A, CA-AKT-T479A) were stably transfected into VSMC. Immunoprecipitation was performed with AKT antibody, followed by Western blot analysis to determine O-GlcNAcylation of AKT with RL-2 antibody.
Figure 7. O-GlcNAcylation of AKT at T430 and T479 is required for AKT activation and vascular calcification. A) Effect of O-GlcNAcylation of AKT on its phosphorylation/activation. Western blot analysis was performed with cell lysate from VSMC stably expressing wild type (wt), constitutively active (CA) and mutant AKT to determine activation of AKT by phosphorylation at S473 and T308. B) AKT O-GlcNAc mutants exhibit decreased binding to Rictor. Immunoprecipitation was performed in cell lysates from A with AKT antibody, followed by Western blot analysis to determine the binding of AKT to PDK1, Rictor, mTOR and PHLPP. Representative blots form 3 independent experiments are shown. C) Effect of O-GlcNAcylation of AKT on VSMC calcification. VSMC stably expressing wt-AKT, CA-AKT and AKT mutants were cultured in osteogenic medium for 3 weeks. Calcification was quantified by Arsenazo III assay. D) Effect of O-GlcNAcylation of AKT on Runx2 transactivity. VSMC stably expressing wt-AKT, CA-AKT and AKT mutants were transfected with a luciferase reporter for Runx2 transactivity and a Renilla luciferase reporter plasmid. Luciferase activity was measured and normalized to Renilla activity (n=4 for each experiment; *p<0.05 compared to wt-AKT, #p<0.05 compared to CA-AKT).
SUPPLEMENTAL METHODS

**VSMC Culture** — Primary VSMC were isolated from the aortas of C57BL/6 mice and confirmed by flow cytometry with the use of α-SMA antibody as we described\(^1\). All experiments were performed with VSMC at passages 3–5. Cells were cultured in DMEM (Life Technologies) containing glucose (25 mM), L-glutamine (584 mg/L), and sodium pyruvate (110 mg/L), supplemented with 20% fetal bovine serum (Life Technologies).

**Western Blot Analysis** — Protein extracts were isolated and protein concentration was measured as we previously described\(^1\). Proteins were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. Immunoblotting was performed using specific antibodies and detected with a Western blot chemiluminescence detection kit (Millipore). Antibodies used were as follows: anti-phospho-AKT S473, anti-phospho-AKT T308, anti-AKT, anti-Rictor, anti-mTOR, anti-PHLPP, anti-PDK1 (Cell Signaling), anti-O-GlcNAc (RL-2, Abcam)\(^2\), and anti-OGT (Sigma-Aldrich). The expression of β-tubulin (Cell Signaling) was used as a loading control. Quantification was performed using ImageJ analysis software (NIH).

**Real-time Polymerase Chain Reaction (PCR)** — The expression of osteogenic factors in aortic tissues and VSMC was determined by real-time PCR. Total RNA was isolated using Trizol (Invitrogen) and reverse transcribed into cDNA. SYBR Green-based real-time PCR was performed using specific primers for Runx2, type I collagen (Col Ia1), osteocalcin (OC), and osteopontin (OPN), using iQ SYBR Green Supermix (Bio-Rad) on an iCycler Thermal Cycler (Bio-Rad).
**Dual-Luciferase Reporter Assay** — Runx2 transactivity was determined as we described by Dual-Luciferase Reporter assay (Promega, Madison, WI) with the use of a luciferase reporter construct containing six Runx binding elements (p6xRunx-Luc)\(^3\). VSMC were transiently transfected with p6xRunx-Luc and a Renilla reporter plasmid (control for transfection efficiency) using Lipofectamine 2000 transfection reagent (Invitrogen). Luciferase activities were determined 24 hours later with the Dual-Luciferase assay kit (Promega), and normalized to the activity of Renilla luciferase.

**Immunofluorescent Staining** — For immunostaining, descending aortas were embedded in paraffin (Sakura Tissue-Tek, Torrance, CA) and serial sections (8μm in thickness) were collected. Immunofluorescent staining was performed as we described previously\(^4\). Anti-phospho-AKT S473 (Cell Signaling, Boston, MA), anti-O-GlcNAc (RL-2)\(^2\), anti-Runx2 (MBL, Woburn, MA), and anti-smooth muscle specific α-actin (α-SMA, Sigma-Aldrich, St. Louis, MO) antibodies were used. Slides were washed extensively before the addition of species-specific fluorescently labeled secondary antibody (Alexa Fluor® 488 or 594, Invitrogen). 4′, 6-diamidino-2-phenylindole (DAPI) was used for nuclear localization.

**Echocardiography and Measurement of Pulse Wave Velocity** — Analysis of pulse wave velocity (PWV) by echocardiography was performed with the high resolution imaging system VEVO 770 (Visual Sonics, Toronto, Canada). Animals were anesthetized with inhalation of 1-2% isoflurane, two Millar Mikro-tip 1.0F pressure transducers (0.47 mm; Millar Instruments) were introduced into the aortic arch just before the descending aorta and the bifurcation of the abdominal aorta. The propagation time for the pulse wave moving from the aortic arch to the abdominal aorta was measured as the time interval
between the upstroke (foot) of the pulse wave front recorded at each transducer. Measurements were performed by averaging at least 10 consecutive normal cardiac cycles. After euthanasia and without altering the position of the transducers, the aorta was exposed and the distance between the transducers (ie, the pulse-wave propagation distance) was determined *in situ* using a slide caliper. The PWV was obtained by dividing this distance by the time interval between the 2 pressure wave fronts.
**Supplementary Figure I.** *STZ increases vascular O-GlcNAcylation at 4 weeks.* To determine whether STZ affects vascular O-GlcNAcylation and calcification at an earlier time point, we evaluated the O-GlcNAcylation of aortas from mice treated with STZ after 4 weeks. Increased O-GlcNAc modification was observed in the vasculature of mice treated with STZ for 4 weeks compared with control mice, as demonstrated by Western blot analysis (A, n=4 mice per group). At this time point, there was no significant difference in vascular calcification (n=3/per group, NS, not significant), suggesting that increased vascular O-GlcNAcylation precedes vascular calcification in the STZ treated mice.
Supplementary Figure II. Rapamycin inhibits O-GlcNAcylation-induced VSMC calcification. The effect of inhibition of mTOR signaling on O-GlcNAcylation-induced VSMC calcification was determined with the use of rapamycin, an mTOR signaling inhibition. OGA knockdown VSMC, which exhibited increased O-GlcNAcylation (Fig 3), were grown in osteogenic media without (control) or with rapamycin (Rapamycin, 1 μM) for 3 weeks to induce calcification. Rapamycin treatment markedly inhibited increased O-GlcNAcylation-induced VSMC calcification (A, Alizarin red staining, representative results of two experiments in duplicates), which was associated with inhibition of AKT phosphorylation/activation (B, pAKT) and decreased Runx2 (B, Runx2). The data suggest that mTOR signaling mediates increased O-GlcNAcylation-induced AKT activation and VSMC calcification.
Supplementary Figure III. Mutation of AKT at T305 or T312 does not affect AKT activation and VSMC calcification. VSMC were stably infected with lentiviruses expressing AKT constructs, including wild type (wt-AKT), constitutively active (CA-AKT) or CA-AKT with point mutations at S305A or T312A. A) Effect of AKT T305A or T312A mutation on AKT phosphorylation. Immunoprecipitation was performed with AKT antibody, followed by Western blot analysis of O-GlcNAcylation (O-GlcNAc) of AKT. The expression and phosphorylation of AKT in cell lysates were determined by Western blot with antibodies for total AKT or AKT phosphorylation at S473 and T308. Representation blots from two independent experiments are shown. B) Effect of AKT T305A or T312A mutation on VSMC calcification. VSMC stably expressing wt-AKT, CA-AKT and the AKT mutants were cultured in osteogenic medium for 3 weeks. Calcification was quantified by Arsenazo III assay (n=3, *p<0.05 compared with wt-AKT).
Supplementary Figure IV. Increased AKT O-GlcNAcylation in VSMC promotes AKT phosphorylation and activation, which induces vascular calcification in diabetes. Increased protein O-GlcNAcylation and vascular calcification are demonstrated in diabetic vasculature. Elevation of O-GlcNAcylation in VSMC by OGA inhibition, using Thiamet-G or shRNA, promotes AKT phosphorylation, Runx2 upregulation, expression of osteogenic markers and VSMC calcification. Increased O-GlcNAcylation-induced VSMC calcification is blocked by AKT inhibition (AKT IV). Inhibition of AKT O-GlcNAcylation at T430 and T479 blocks its phosphorylation at S473 but not T308, which leads to inhibited Runx2 transactivity and reduced VSMC calcification.
SUPPLEMENTAL REFERENCES


2. Gao Y, Miyazaki J, Hart GW. The transcription factor PDX-1 is post-translationally modified by O-linked N-acetylglucosamine and this modification is correlated with its DNA binding activity and insulin secretion in min6 beta-cells. *Arch. Biochem. Biophys.* 2003;415:155-163


O-GLCNACYLATION OF RUNX2 NUCLEAR MATRIX TARGETING SIGNAL FACILITATES VASCULAR CALCIFICATION

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ABSTRACT

Vascular calcification increases morbidity and mortality of patients with atherosclerosis, diabetes, and renal disease. Runx2 is a key regulator of this process in vascular smooth muscle cells. O-GlcNAcylation is increased in these diseases as well, and has been shown to have detrimental effects in animal models of these diseases. The goal of this study was to determine the specific regions of Runx2 required for its function in vascular smooth muscle cells, and how O-GlcNAcylation may affect this function. Through overexpression of serial truncations of Runx2 protein, the nuclear matrix targeting signal (NMTS) was determined to be crucial for Runx2 function and VSMC calcification. Pharmacological inhibition, as well as knockdown and deletion strategies, revealed binding of the BMP-regulated Smad family of proteins (Smad1, 5, and 8), along with co-Smad Smad4, to the NMTS to be required for Runx2 function and vascular calcification. Site-directed mutagenesis was utilized to determine sites of O-GlcNAcylation within the Runx2 NMTS. Mutation of threonine 412, serine 413, and tyrosine 425 were found to disrupt protein-protein interaction between BMP-regulated Smads and the Runx2 NMTS, resulting in decreased Runx2 activity and VSMC calcification. Mutations at these sites also decreased serine and threonine phosphorylation on Runx2.

The Runx2 NMTS is a crucial region required for Runx2 activity, BMP-regulated Smad binding, and VSMC calcification. O-GlcNAcylation of the Runx2 NMTS at three sites facilitates Smad binding and enhances Runx2 functional activity. O-GlcNAcylation at these sites is necessary for vascular calcification to occur. Thus, these studies reveal
novel mechanistic insights and therapeutic targets for treatment and prevention of Runx2 function and vascular calcification.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>O-GlcNAcylation</td>
<td>O-linked $\beta$-$N$-acetylglucosamine modification</td>
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<td>VSMC</td>
<td>vascular smooth muscle cells</td>
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<tr>
<td>$\alpha$-SMA</td>
<td>smooth muscle specific $\alpha$-actin</td>
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<tr>
<td>NMTS</td>
<td>nuclear matrix targeting signal</td>
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<td>Runx2</td>
<td>Runt-related transcription factor 2</td>
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INTRODUCTION

Vascular calcification increases morbidity and mortality in patients with diabetes, atherosclerosis, and renal disease\(^1\), \(^2\). The deposition of calcification in medial and/or intimal layers of the vasculature is an active process driven by the osteogenic differentiation of vascular smooth muscle cells (VSMC)\(^3\)–\(^5\), resulting in complications ranging from increased pulse wave velocity to increased risk of thrombosis\(^6\). The importance of vascular calcification has increasingly been recognized, although therapeutic strategies to reduce or prevent the osteogenesis of VSMC have yet to be developed.

As we and others have shown, upregulation of Runx2 in VSMC is a crucial event leading to vascular calcification\(^7\), \(^8\). Commonly known as a regulator of osteogenesis during development, Runx2 expression is necessary and sufficient for VSMC osteogenic differentiation and calcification\(^7\). The specific mechanisms by which Runx2 causes VSMC osteogenic differentiation have not been fully elucidated. In osteoblasts, Runx2 is localized to the nucleus via its nuclear localization signal, and further localizes to subnuclear puncta via the nuclear matrix targeting signal (NMTS)\(^9\). Runx2 may form a regulatory complex by binding to Smads in order to become fully activated within the cell\(^10\).

Post-translational modification of Runx2 has been shown to alter its function\(^11\). However, the effects of protein O-linked β-N-acetylglucosamine modification (O-GlcNAcylation) of Runx2 on its cellular function have not been studied. O-GlcNAcylation is a tightly controlled process that has various ramifications in physiological function of many proteins, and it has been shown to have complex roles in
several disease models, including both type I and type II diabetes\textsuperscript{12, 13}. Importantly, crosstalk between phosphorylation and O-GlcNAcylation may regulate overall protein signaling within the cell. Runx2 phosphorylation at multiple N-terminal sites increases activity of the protein\textsuperscript{14}, whereas phosphorylation at serine 125 and serine 472 leads to ubiquitination and degradation\textsuperscript{11}. Therefore, O-GlcNAcylation of Runx2 may provide a key regulatory target for therapies and treatment of vascular calcification.

These studies explore the specific regions of Runx2 responsible for vascular calcification, as well as the role of Runx2 O-GlcNAcylation in VSMC function and osteogenic differentiation. We have demonstrated that the Runx2 NMTS is required to induce VSMC calcification in primary mouse vascular smooth muscle cells (VSMC). We have found that Runx2 O-GlcNAcylation at three novel sites regulates Runx2 phosphorylation, BMP-regulated Smad binding, and Runx2 activity. O-GlcNAcylation at these three sites is necessary for vascular calcification to occur. The studies presented here emphasize the importance of Runx2 subnuclear localization and reveal a novel post-translational Runx2 regulatory pathway. Addition of these mechanistic pathways to the understanding of the role of Runx2 in VSMC osteogenic differentiation reveals new therapeutic targets for treatment of vascular calcification.
METHODS

VSMC Culture — Primary VSMC were isolated from the aortas of C57BL/6 mice and confirmed by flow cytometry with the use of α-SMA antibody as we described\(^7\). All experiments were performed with VSMC at passages 3–5.

In Vitro Calcification — Calcification of VSMC was induced in osteogenic medium containing 0.25 mM L-ascorbic acid, 10 mM β-glycerophosphate, and 10\(^{-8}\) M dexamethasone (Sigma-Aldrich) for 3 weeks. Mineralization was determined by Alizarin red staining as previously described\(^15\). In separate parallel sets of dishes, cells were lysed with 0.5 N HCl and total calcium in the cell lysates was quantified with Arsenazo III calcium measurement kit (StanBio)\(^15\).

Western Blot Analysis — Protein extracts were isolated and protein concentration was measured as we previously described\(^16\). Proteins were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. Immunoblotting was performed using the following antibodies: Runx2 (MBL, Woburn, MA), O-GlcNAc (RL-2), phospho-serine (Abcam, Cambridge, MA), and phospho-threonine (Abcam). Secondary antibodies labeled with horseradish peroxidase (Santa Cruz) were detected with a Western blot chemiluminescence detection kit (Millipore, Billerica, MA).

Immunoprecipitation — O-GlcNAcylation of Runx2 was detected by immunoprecipitation. Briefly, cell extracts were incubated with IgG (Santa Cruz) or Runx2 antibody at 4°C overnight, and then mixed with protein G agarose beads (Sigma Aldrich) for 3 hours. Beads were washed and proteins pulled down were analyzed by Western blotting.
**Dual-Luciferase Reporter Assay** — Runx2 transactivity was determined as we described by Dual-Luciferase Reporter assay (Promega, Madison, WI) with the use of a luciferase reporter construct containing six Runx binding elements (p6xRunx-Luc)\(^{16}\). VSMC were transiently transfected with p6xRunx-Luc and a Renilla reporter plasmid (control for transfection efficiency) using Lipofectamine 2000 transfection reagent (Invitrogen). Luciferase activities were determined 24 hours later with the Dual-Luciferase assay kit (Promega), and normalized to the activity of Renilla luciferase.

**Generation of Runx2 Mutants** — Constructs carrying cDNA full-length Runx2 (513) and truncated Runx2 mutants (495, 432, 391, 370) were generated from genomic Runx2 DNA. Point mutations in the sequence of the 432 amino acid Runx2 were made at threonine 408, threonine 412, serine 413, and threonine 425 to replace the residues with alanine using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) and confirmed by sequencing analysis. VSMC stably infected with lentivirus expressing the various Runx2 constructs were used for immunoprecipitation and Western blot analysis of O-GlcNAcylation and phosphorylation of Runx2, luciferase reporter assay for Runx2 activity and characterization of VSMC calcification.

**Statistical Analysis** — Results are presented as the mean ± SD. Differences between groups were determined with the use of Student t tests. Significance was defined as \(p<0.05\).
RESULTS

*Nuclear Matrix Targeting Signal is Necessary for Runx2 Function*

To determine the specific regions required for Runx2 function in VSMC calcification, we generated lentiviral constructs encoding a series of truncations of Runx2 protein, as shown in Figure 1A. Each of the truncated Runx2 proteins was successfully expressed in VSMC (Fig 1B). Overexpression of Runx2 at all lengths except the 391 amino acid Runx2 were observed to induce calcification (Fig 1C), indicating the important role of the nuclear matrix targeting signal (NMTS) in Runx2 function. In Runx2 knockout VSMC, overexpression of full-length Runx2, Runx2-495, and Runx2-432 was able to induce vascular calcification (Fig 1D). Overexpression of Runx2-391 and Runx2-370 was unable to induce VSMC calcification. As noted in Figure 1A, the NMTS also serves as the Smad-interacting domain (SMID).

*Runx2 NMTS is Required for BMP-Regulated Smad Binding*

The nuclear matrix targeting sequence contains a Smad-interaction domain, and Runx2 serves as a scaffold for Smad binding and downstream function. Thus, we determined the effect Smad binding to Runx2, by overexpression of either the 432- or 391-amino-acid Runx2 protein. First, chemical inhibition of TGF-β-regulated Smads (Smad 2/3) with inhibitor SB431542 did not affect induction of calcification by 432-amino-acid Runx2. However, inhibition of BMP-regulated Smads (Smad 1/5/8) inhibited the VSMC calcification (Fig 2Aa). This was correlated with a decrease in Runx2 activity upon inhibition of BMP-regulated Smads (Fig 2Ab).
We next investigated the specific Smad proteins required for Runx2 function in VSMC calcification. We used Smad1 deletion (Smad1 Δ/Δ) VSMC to determine the role of this BMP-regulated Smad in Runx2-dependent calcification. Smad1 floxed cells were infected with lentivirus expressing Cre protein and lentivirus encoding the 432 amino acid Runx2 protein. As expected, Smad1 deletion resulted in attenuated Runx2-induced VSMC calcification (Fig 2Ba). The Runx2 activity was also decreased in the Smad1 deletion cells expressing the 432 amino acid Runx2 (Fig 2Bb). To determine the effects of the other Smads in calcification, VSMC were infected with lentivirus containing shRNA for Smad2, 3, 5, and 8, as well as the 432-amino-acid Runx2 protein. The knockdown of Smad5 and 8 but not Smad2 and 3 decreased VSMC calcification induced by Runx2 (Fig 2Ca). Runx2 activity in the Smad5 and 8 knockdown VSMC was also decreased (Fig 2Cb). Taken together with the decrease in bone markers in the Smad1 deletion and Smad 5 and 8 knockdown VSMC (data not shown), these data clearly demonstrate the crucial role of BMP-regulated Smads in Runx2-dependent VSMC calcification.

\textit{O-GlcNAcylation of NMTS Is Essential for VSMC Calcification}

We further determined whether Runx2 is directly O-GlcNAc modified within the NMTS and if that modification affects Smad binding and Runx2 activity. Three putative O-GlcNAc modification sites within the NMTS were predicted using YinOYang 1.2 software (http://www.cbs.dtu.dk/services/YinOYang/): threonine 408, threonine 412, and serine 413 (Fig 3A). An additional potential site of Smad binding near a region key for Smad binding was determined from previous studies\textsuperscript{10}. The 432 amino acid Runx2
construct was mutated at these four sites to determine effects on Runx2 functional activity (Fig 3A). Mutations at threonine 412, serine 413, and tyrosine 425 attenuated total Runx2 O-GlcNAcylation, whereas mutation at threonine 408 did not affect O-GlcNAcylation of the protein (Fig 3B). This effect may be due to the decreased binding of the O-GlcNAc transferase (OGT), but not the O-GlcNAcase (OGA), to Runx2 upon mutation at these sites (Fig 3B). The role of Runx2 O-GlcNAcylation in vascular calcification was next determined in the VSMC overexpressing the Runx2 O-GlcNAc mutants. As expected, the cells overexpressing the T412A, S413A, and Y425A mutants exhibited decreased calcification (Fig 3C). The mutations at these three sites decreased VSMC osteogenic differentiation, as shown by attenuated expression of bone marker proteins in VSMC expressing these constructs (Fig 3D).

_BMP-Activated Smads and Smad4 are Responsible for Runx2-Dependent VSMC Calcification_

We next determined the mechanisms for decreased Runx2 activity upon decreased O-GlcNAcylation at the NMTS. Using immunoprecipitation, we found that the BMP-regulated Smad binding was decreased as a result of mutation at the O-GlcNAcylation sites (Fig 4A). This indicates the essential function of Smads in the BMP signaling pathway for Runx2 function in vascular calcification. In addition, because phosphorylation of Runx2 has been shown to affect its function in osteoblasts\(^\text{17}\), we elucidated how mutation of the Runx2 O-GlcNAcylation sites affected its overall phosphorylation profile. The three mutations resulting in decreased Runx2 O-GlcNacylation also resulted in decreased serine and threonine phosphorylation of Runx2.
(Fig 4B). This led to a net decrease in Runx2 activity measured by luciferase reporter assay (Fig 4C). These data show a complexity in the correlation between Runx2 O-GlcNAcylation and phosphorylation.
DISCUSSION

Vascular calcification is a major cause of morbidity and mortality in patients with atherosclerosis, diabetes, and renal disease. Although Runx2 upregulation has been demonstrated to be critical to induce calcification in VSMC\textsuperscript{15, 18}, the precise protein domains and mechanisms responsible for upregulating vascular Runx2 and its activity have not been fully elucidated. Runx2 post-translational modification is an important regulator of the protein’s stability, activity, and function\textsuperscript{19}. Although elevated O-GlcNAcylation is found in areas of human and mouse vascular calcification\textsuperscript{20, 21}, the role of O-GlcNAcylation in the regulation of vascular Runx2 has not been determined. The preceding studies clearly show the critical role of the nuclear matrix targeting signal (NMTS) in regulating Runx2 function. Specifically, the binding of BMP-activated Smads facilitated through O-GlcNAcylation at three novel sites elucidates a detailed mechanism of Runx2 regulation during VSMC calcification. These studies demonstrate for the first time that O-GlcNAcylation is critical for Runx2 function, and they provide a new target to disrupt Runx2-Smad binding. Because of the important role of O-GlcNAc in diseases which also exhibit vascular calcification, including atherosclerosis, diabetes, and renal disease, these studies may be easily translated to treat vascular calcification in these diseases.

We clearly demonstrated that the Runx2 NMTS is crucial for VSMC calcification induced by Runx2. Although similar observations have been made in osteoblasts\textsuperscript{9}, these studies were not focused on the specific residues modified in the Runx2 NMTS or the O-GlcNAcylation of the Runx2 protein. In the present studies, we showed that the NMTS is required for binding of Runx2 to BMP-regulated Smads and co-Smad Smad4. These
detailed mechanistic studies, using knockdown and deletion of each of the Smads, are the first to clarify the specific Smads necessary for Runx2 function within the vasculature during VSMC calcification. In previous studies, the NMTS has been shown to be crucial for in vivo Runx2 function in osteoblasts\textsuperscript{22, 23}; mice lacking the NMTS lack the ability to develop bone because osteoblasts do not fully mature\textsuperscript{9}. Our immunoprecipitation experiments indicate the formation of a complex including Runx2, BMP-regulated Smads, and Smad4 is necessary for Runx2-dependent vascular calcification. This has implications for targeting the individual components of this complex in order to attenuate and/or prevent calcification of the vasculature.

With the detailed use of protein knockdown and deletion strategies in an in vitro VSMC culture system, we determined the direct effect of O-GlcNAcylation of the Runx2 NMTS in vascular calcification. Other post-translation modification of Runx2 has been extensively studied, primarily in osteoblasts. Importantly, several of these modifications are facilitated by Smad binding to Runx2. Ubiquitination of Runx2 via binding to Smurf1 has been shown to be mediated by binding to Smad6, an inhibitor of the BMP signaling pathway\textsuperscript{24}. On the other hand, acetylation of Runx2 is mediated by Smad1 and Smad5 to protect against ubiquitination and increase Runx2 transactivity\textsuperscript{25}. As we have shown, O-GlcNAcylation of Runx2 precedes binding of Runx2 to BMP-regulated Smad proteins, facilitating the binding of the Smads to the Runx2 NMTS. This indicates the importance of O-GlcNAcylation, since this modification may be upstream of many of the previously studied pathways regulating Runx2 stability and activation.

Using immunoprecipitation of Runx2 O-GlcNAc mutants, we have demonstrated the complex crosstalk between phosphorylation and O-GlcNAcylation on the Runx2
protein. Runx2 phosphorylation has been shown to have various effects on Runx2 function, depending upon the site of phosphorylation. In previous studies, treatment of osteoblast precursors with a chemical inhibitor of MEK/ERK signaling resulted in ablation of Runx2-Smad1 binding\(^\text{25}\), indicating the importance of phosphorylation for binding of BMP-regulated Smads to the Runx2 protein. However, the specific residues affected by the ERK phosphorylation have not yet been determined. In contrast, phosphorylation of a serine residue in the C-terminus of Runx2 is required for Sox9-mediated degradation of Runx2 in HEK293 cells\(^\text{26}\). Thus, the phosphorylation profile of the Runx2 protein, depending on the kinases and phosphatases bound to the protein, determines the expression and activity of Runx2. However, because O-GlcNAcylation is tightly regulated by only two enzymes, the O-GlcNAc transferase (OGT) and the O-GlcNAc hydrolase (OGA), the O-GlcNAc profile of Runx2 may be determined through the protein’s conformation and through the interplay between O-GlcNAc and the other post-translational modifications of Runx2.

The site-directed mutagenesis studies indicated that O-GlcNAcylation of the Runx2 NMTS indicated that O-GlcNAcylation at threonine 412, serine 413 and tyrosine 425 facilitates Smad binding to Runx2 and is required to induce Runx2 functional activity. Phosphorylation of Runx2 at serine 472 has previously been shown to inhibit Runx2 function in osteoblasts\(^\text{27}\), a modification which may be inhibited by the O-GlcNAcylation shown here. The O-GlcNAcylation at tyrosine 425 actually corresponds to a known Smad binding motif, possibly explaining the change in Smad binding caused by mutation at this site. The changes in overall Runx2 O-GlcNAcylation and phosphorylation as a result of these mutations may be attributed to the change in the
conformation of Runx2, possibly deforming the Smad binding sites to facilitate or inhibit protein-protein interaction. Further studies are required to determine how O-GlcNAcylation of the NMTS changes protein conformation and protein binding.

These studies have revealed important insights into the mechanisms of Runx2 functional activity within VSMC. We have found an important new layer of post-translational modification of the Runx2 NMTS which is required for binding of key proteins to Runx2. Three new sites of O-GlcNAcylation have been shown to affect BMP-regulated Smad binding, and mutation of these sites abolished the activity of Runx2 in VSMC, resulting in decreased vascular calcification. These findings have vast implications on therapeutic targets to abolish Runx2 function in VSMC and prevent vascular calcification in atherosclerosis, diabetes, and renal disease.
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Figure 1. Overexpression of Runx2 amino acids 1-513, 1-495, and 1-432, but not 1-391, were sufficient to induce VSMC calcification in WT VSMC. Lentiviruses containing the truncated Runx2 sequences shown in A were produced and infected into primary VSMC. FL: full-length Runx2. B) Western blot was performed to confirm overexpression of Runx2 truncations in VSMC. C) VSMC overexpressing truncated Runx2 were cultured in osteogenic medium for 3 weeks to determine effects on calcification by Alizarin red staining. D) Runx2 deletion VSMC were infected with the truncated Runx2 and calcification was confirmed.
**Figure 2.** BMP2 but not TGFβ activated Smads are responsible for Runx2 dependent VSMC calcification. A) VSMC infected with 391- or 432-amino-acid Runx2 were treated with TGFβ Smad pathway inhibitor SB431542 (20 µM) or BMP Smad pathway inhibitor Noggin (500 ng/mL) to determine effects on a) calcification by Alizarin Red staining and b) Runx2 activity by luciferase assay. B) Smad1 floxed and knockout VSMC were infected with Runx2 truncations; a) calcification was determined by Alizarin red staining and b) luciferase assay was used to determine Runx2 activity. C) Lentiviruses containing shRNA for specific Smads were co-infected with the truncated Runx2 to determine a) calcification by Alizarin red staining and b) Runx2 activity by luciferase assay. (n=6, **p<0.01 compared to 1-391)**
Figure 3. Runx2 O-GlcNAcylation is associated with increased VSMC calcification. A) VSMC infected control scrambled shRNA or shRNA against OGA were immunoprecipitated with Runx2 antibody and blotted with RL-2 O-GlcNAc antibody to determine Runx2 O-GlcNAcylation in VSMC, and B) Runx2 activity was determined in these cells via luciferase assay. C) Lentiviruses containing control shRNA or shRNA for OGT were infected into VSMC, and a) baseline levels of OGT expression and O-GlcNAcylation were determined by Western blot; after 3 weeks in osteogenic medium, b) Runx2 activity was determined by luciferase assay, and a) calcification was determined by Alizarin red staining. (n=6, **p<0.01 compared to 1-391)
Figure 4. O-GlcNAcylation of Runx2 at 412-413 is required for functional activity. A) Schematic representation of Runx2 NMTS and putative O-GlcNAc sites, each of which was mutated to alanine. The tyrosine at 425, also mutated to alanine, is adjacent to the Smad binding site within the NMTS. B) Site-directed mutagenesis was performed on the 432-amino-acid Runx2. Lentivirus carrying mutated Runx2 constructs were stably transfected into VSMC. Immunoprecipitation was performed with Runx2 antibody, followed by Western blot analysis to determine Runx2 O-GlcNAcylation with RL-2 antibody, and OGT and OGA binding with specific antibodies for those enzymes. C) VSMC stably expressing GFP, 432-amino-acid Runx2, and Runx2 O-GlcNAc mutants were cultured in osteogenic medium for 3 weeks. Calcification was quantified by Arsenazo III assay. D) VSMC stably expressing Runx2 mutants from C were collected and mRNA was isolated for real-time PCR to determine osteogenic gene expression.
Figure 5. O-GlcNAcylation of Runx2 in NMTS is required for Smad binding and VSMC calcification. A) Protein lysate from VSMC overexpressing Runx2 O-GlcNAc mutants was collected and incubated with Runx2 antibody and protein G agarose beads to determine effects on Runx2 binding. Antibodies for specific Smads were used as described in methods. B) Specific antibodies for phosphorylation of serine and phosphorylation of threonine were used in separate immunoprecipitation studies to determine Runx2 total phosphorylation in mutant proteins. C) Luciferase assay was performed to determine the effects of Runx2 mutations on transcriptional activity. D) Immunoprecipitation was performed with Runx2 O-GlcNAc mutants to determine effects on binding of previously identified kinases to Runx2 protein.
TARGETING O-GLCNAC TRANSFERASE FOR DIABETIC VASCULAR CALCIFICATION THERAPY

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ABSTRACT

Vascular calcification progresses rapidly in patients with diabetes mellitus and increases risk of cardiovascular events and death. We and others have demonstrated vascular calcification in experimental diabetic models. We found that increased vascular calcification was associated with elevated protein O-linked GlcNAc modification (O-GlcNAcylation) in human diabetic arteries, and in low-dose streptozotocin (STZ)-induced and Akita mutant diabetic mice. The present study determined the effects of inhibition of O-GlcNAc transferase (OGT), the enzyme that catalyzes O-GlcNAcylation, on vascular calcification in diabetic mice. We generated smooth muscle cell (SMC)-specific OGT deletion mice (ΔOGT\textsuperscript{SMC}) by crossing OGT floxed C57BL/6 mice with SMC-specific Cre transgenic mice (SMMHCCre). Cre-mediated OGT deletion was induced by tamoxifen injection in mice at 8 weeks of age. SMC-specific OGT deletion did not affect blood glucose, but significantly inhibited protein O-GlcNAcylation exclusively in SMC. STZ-induced vascular calcification was markedly inhibited in the ΔOGT\textsuperscript{SMC} mice. At the molecular level, OGT deletion decreased expression of Runx2, a key regulator of VSMC osteogenic differentiation, in the diabetic vasculature. Activation of AKT, an important upstream signal for Runx2 upregulation, was significantly inhibited by OGT deletion. Mechanistic studies demonstrated that several inflammatory mediators highly expressed in calcified arteries were decreased in arteries from diabetic ΔOGT\textsuperscript{SMC} mice.

We have demonstrated a crucial role for vascular O-GlcNAcylation and OGT in regulating diabetic vascular calcification. Our studies have uncovered novel mechanisms linking glucose metabolism to vascular dysfunction and revealed therapeutic targets for diabetic vascular calcification.
NON-STANDARD ABBREVIATIONS

$\alpha$-SMA smooth muscle specific $\alpha$-actin

CA-AKT constitutively active AKT

mTOR mammalian target of rapamycin

OGA $\beta$-N-acetylglucosaminidase

OGT $\beta$-N-acetylglucosaminyltransferase

O-GlcNAcylation O-linked $\beta$-N-acetylglucosamine modification

Runx2 runt-related transcription factor 2

VSMC vascular smooth muscle cells
INTRODUCTION

Diabetes has been strongly associated with chronic cardiovascular and renal complications, leading to an increased morbidity and mortality in affected patients\(^1\). Diabetic patients also tend to have a higher burden of calcified plaque\(^3\). Vascular calcification is commonly observed in diabetic arteries, in the intimal and medial layers of the vessel walls\(^3\)-\(^5\).

The presence of calcification in the diabetic vasculature increases arterial stiffness, reduces compliance of the blood vessels\(^6\)-\(^7\), and increases the risk of cardiovascular events and mortality\(^8\). Thus, the causes and underlying mechanisms of calcification in the vasculature represent an important avenue for clinical investigation.

Diabetic hyperglycemia has been shown to directly increase the development and progression of vascular calcification in vitro\(^4\)-\(^9\). However, the mechanistic role of hyperglycemia in the development of vascular calcification is unknown. In addition to producing bioenergetic substrates via the TCA cycle, glucose metabolism through hexosamine biosynthesis generates UDP-GlcNAc\(^10\). O-GlcNAc modification can regulate proteins by changing conformation or localization to induce transcription or downstream signaling in the cell\(^11\)-\(^12\). Two enzymes are responsible for the tightly coupled regulation of O-GlcNAcylation in all cell types. The β-N-acetylglucosaminyltransferase (OGT) binds to and adds O-GlcNAc modification onto serines and threonines of proteins, and the β-N-acetylglucosaminidase (OGA) removes O-GlcNAc modification.

O-GlcNAc modification may be utilized by the cell to sense the nutrient state of the cellular environment; the levels of O-GlcNAc substrate are affected by glucose and
glucosamine concentrations among other signals\textsuperscript{13}. We have previously shown that increased O-GlcNAc modification enhances vascular calcification in a diabetic mouse model\textsuperscript{14}. The studies which are shown here sought to investigate the direct role of decreased O-GlcNAcylation in vascular calcification. We generated smooth muscle cell-specific OGT knockout mice and induced diabetes in the mice via STZ injection. We observed decreased medial expression of OGT and O-GlcNAc modification in the knockout mice, both in control and diabetic models. This decrease was associated with attenuated vascular calcification in arteries from the diabetic mice. Using primarily cultured mouse VSMC, we determined that activation of AKT by phosphorylation at S473 was blunted in the aortas of OGT knockout mice. Expression of inflammatory markers was also decreased in these vessels. We have shown a clear and causative link between O-GlcNAcylation and diabetic vascular calcification. Future studies building on this work may lead to novel therapeutic targets for alleviating vascular calcification, including proteins modified by O-GlcNAcylation.
METHODS

Experimental Animals — OGT floxed mice were bred with mice overexpressing inducible SMMHC Cre to obtain OGT f/Y SMMHC Cre/+ mice, and OGT +/- SMMHC Cre/+ littermate controls. At 2 months of age, mice were injected intraperitoneally for 5 consecutive days with vehicle or 40 mg/kg tamoxifen. n=6 for each group shown.

Tissue Harvest and Processing — At the experimental end points, mice were sacrificed, the aortic arch and descending aorta were dissected under a microscope and used for characterization of calcium content, RNA and protein expression and immunostaining as we previously described.15

Immunohistochemistry — Consecutive sections were processed for immunohistochemistry as we previously described.16 Anti-OGT (Sigma Aldrich), anti-smooth muscle specific α-actin (α-SMA), and anti-O-GlcNAc (RL-2) antibodies were used. Sections exposed only to the secondary antibody served as negative controls. Sections were counterstained with hematoxylin. H&E staining was performed as previously described.16

In Vitro Calcification of VSMC — Primary VSMC were isolated from the aortas of C57BL/6 mice as we described.17 VSMC calcification was induced in osteogenic medium containing DMEM, supplemented with 20% fetal bovine serum, L-ascorbic acid (0.25 mM), β-glycerophosphate (10 mM), and dexamethasone (10^-8 M, Sigma-Aldrich) for 3 weeks, with or without 0.3 mM hydrogen peroxide to induce calcification. Calcification was determined by Alizarin red staining as we described.15 In parallel sets of dishes, cells were lysed with 0.5 N HCl and total calcium content was quantified with
Arsenazo III calcium measurement kit (StanBio) and normalized to the amount of total protein\textsuperscript{15}.

\textit{Dual-Luciferase Reporter Assay} — Runx2 transactivity was determined as we described by Dual-Luciferase Reporter assay (Promega, Madison, WI) with the use of a luciferase reporter construct containing six Runx binding elements (p6xRunx-Luc)\textsuperscript{16}.

\textit{Statistical Analysis} — Results are presented as the mean ± SD. Differences between groups were determined with the use of Student \textit{t} tests or 1-way ANOVA where appropriate. Significance was defined as \( p < 0.05 \).
RESULTS

*Smooth Muscle-Specific OGT Deletion Attenuates Diabetic Vascular Calcification*

In order to delete OGT specifically in VSMC, we bred the OGT floxed mice with mice overexpressing an inducible SMMHC Cre. The OGT gene resides on the X chromosome, and thus males are hemizygous for the mutation or deletion of OGT. Also, the SMMHC Cre is expressed on the Y chromosome, such that only males are able to overexpress this Cre. Therefore, the genotypes used for the study were OGT +/Y SMMHC Cre controls and OGT f/Y SMMHC Cre experimental animals. Because OGT has been shown to be essential during development of many tissues\(^{18}\), we waited until 8 weeks after birth to induce the OGT deletion, in order to confer a better chance for survival of the animal. The animals were injected with tamoxifen for 5 consecutive days to induce OGT deletion, and then they were monitored for 2 months to ensure the overall physical health of the animals before pursuing further treatments. We used the low-dose STZ injection-induced diabetic calcification background, as we have previously shown\(^{14}\), to then induce diabetes and calcification after 4 months of STZ treatment.

The deletion of OGT in VSMC achieved a dramatic decrease in the expression of OGT in the vascular media, resulting in a similar decrease in medial O-GlcNAcylation (Fig 1). This decreased expression was specific to the media, as the brown staining was still present in the endothelium and the adventitia. This decrease in expression was observed in control and STZ-treated diabetic mice, showing that the normal phenomenon of STZ-induced increase in O-GlcNAcylation was attenuated by the deletion of OGT.

The decreased O-GlcNAcylation was concomitant with a decrease in diabetic calcification of the vasculature, as measured by calcium content in the aortic lysate of the
control and STZ diabetic mice (Fig 2A). This was due to the decreased expression of bone marker proteins in the vessels, namely Runx2 and its transcriptional target osteocalcin (OC) (Fig 2B). This is an indication that the OGT deletion and resultant decrease in vascular O-GlcNAcylation somehow attenuates the osteogenic trans-differentiation of the VSMC which is responsible for vascular calcification.

**OGT Deletion Attenuates Hydrogen Peroxide-Induced VSMC Calcification and Blunts AKT Activation**

In order to determine the mechanism linking decreased O-GlcNAcylation to attenuation of vascular calcification, we used primary VSMC from the OGT deletion mice to recapitulate the calcification model in vitro. Hydrogen peroxide has been shown by our group to strongly induce VSMC calcification in vitro\(^ {17}\), and so we used this stimulus to study the changes in VSMC signaling as a result of OGT deletion. Hydrogen peroxide induced calcification in VSMC from control mice, as shown by Alizarin red staining, but this effect was dramatically attenuated in VSMC from OGT deletion mice (Fig 3A). This was confirmed using Arsenazo III assay to measure total cellular calcium content (Fig 3B).

Our lab has recently shown that AKT protein O-GlcNAc modification results in increased activation at S473\(^ {14}\). Therefore, we determined the AKT activation in the OGT deletion VSMC to explore the effects of decreased O-GlcNAcylation on AKT activation. The activation of AKT by glucose was blunted specifically at S473 in the OGT deletion VSMC, indicating a possible cause of decreased calcification in the cells (Fig 3C).
**Chronically Decreased O-GlcNAcylation Protects VSMC from Inflammation**

O-GlcNAcylation has been shown in previous studies to reduce inflammatory signaling in the vasculature and attenuate stress responses\(^9\). Thus, we determined the effects of the decreased O-GlcNAcylation in the vasculature of the OGT deletion mice on the inflammation that normally accompanies vascular calcification, as we have shown\(^{16}\). A microarray study of the vascular calcification in several different models has shown caspase 1 and interleukin-10 receptor b (IL-10Rb) to be highly expressed in vascular calcification accompanying atherosclerosis and diabetes (data not shown). Therefore, these and other markers of calcification inflammation were the focus of the expression studies in the OGT mice.

As shown in Figure 4, real-time PCR studies revealed that caspase 1 and other inflammatory markers were all decreased in OGT deletion VSMC. This change in inflammatory signaling may be another key link between OGT and the vascular system under chronic stress.
DISCUSSION

Vascular calcification is a major risk factor for negative cardiovascular outcomes in diabetes\textsuperscript{20, 21}. The work shown here gives a clear picture of how O-GlcNAcylation may play a role in that calcification and potential targets for future therapies. O-GlcNAcylation is increased in many disease models and human diseased tissues\textsuperscript{22, 23}. Specifically, diabetic patients and mouse models have been shown to have increased O-GlcNAcylation and vascular calcification\textsuperscript{6, 14, 24}. However, no studies have been performed to determine the effects of reducing vascular O-GlcNAcylation in vitro to target its effects on vascular calcification. This study has clearly shown that decreased O-GlcNAcylation may have an ameliorative effect on diabetic vascular calcification. Our studies revealed that OGT deletion resulted in decreased O-GlcNAcylation in vasculature and decreased vascular calcification in the STZ diabetic mouse model. We showed that this may be due to the blunted activation of AKT in the OGT deletion mice, and that inflammation in the vasculature which is normally upregulated during vascular calcification is attenuated by the decrease in O-GlcNAcylation. These discoveries may lead to new therapies in vascular calcification, since the OGT enzyme is the only enzyme controlling O-GlcNAcylation of proteins in the cell.

Work performed by many groups in previous work has suggested that O-GlcNAcylation may protect against vascular stressors to preserve cellular function and phenotype\textsuperscript{25-27}. However, few studies have examined the function of chronic increases in O-GlcNAcylation. With the data shown here in addition to our recent work linking increased O-GlcNAcylation to calcification, it is clear that chronically high levels of O-GlcNAc modification in the vasculature result in detrimental effects on cellular and tissue
integrity\textsuperscript{28}. Diabetic models consistently show increased O-GlcNAcylation and other vascular complications, such as diminished aortic compliance and hypertension\textsuperscript{21, 23, 24}, and with this study the evidence shows that decreasing O-GlcNAcylation in long-term models of disease is beneficial and may be a therapy in the future for the treatment of those with chronic illness.

In vitro, we found that the OGT deletion and decreased O-GlcNAcylation blunted the signaling through AKT which we have shown to be vital for the induction of vascular calcification\textsuperscript{14}. This further strengthens the notion that the vasculature is being actively changed by cellular differentiation via modifications such as O-GlcNAcylation which ultimately affect the expression of proteins to change the cellular phenotype\textsuperscript{17, 29}. In osteoblast precursors, O-GlcNAcylation has been shown to induce a physiological osteogenesis to produce osteoblastic/chondrocytic changes\textsuperscript{28, 30}, but the targets of O-GlcNAcylation in regulating differentiation of osteoblasts and chondrocytes have not been shown by any group up to this point. Our previous reports added with the data here give an indication that oxidative stress-induced vascular calcification in diabetes may be due to O-GlcNAcylation of specific proteins such as AKT\textsuperscript{14, 17}. This may be due to the increased glucose in the diabetic system, which increases O-GlcNAcylation through oxidative stress.

The cytokines activated in the VSMC from STZ diabetic mice have important roles in vascular inflammation, as has been shown previously. However, no role of these inflammatory markers has been studied in vascular calcification thus far. Caspase 1 and the inflammasome were recently proven to have a critical role in the development of atherosclerosis in mice fed a Western diet. Plaque area, number of macrophages, and
number of vascular smooth muscle cells migrating into the plaque area were all decreased in ApoE -/- caspase 1 -/- mice fed a high fat diet\textsuperscript{31}. In support of the role of caspase 1 in calcification, the same study showed that calcium phosphate activates caspase 1 through activation of the inflammasome in macrophages\textsuperscript{31}. Other studies have shown that caspase 1 must be present in the microvesicles of monocytes to maintain vesicle integrity\textsuperscript{32}. This has important implications for the progression of calcification through delivery of calcium phosphate in microvesicles. The expression of inflammasomes is upregulated in human atherosclerotic aortas\textsuperscript{33}. Specifically, the NLRP3 inflammasome is required for progression of atherosclerosis, due to the fact that early deposition of cholesterol crystals activates the NLRP3 inflammasome and caspase 1\textsuperscript{34}.

AKT activation, which is critical for vascular calcification, plays an important role in inflammation in many different settings. The expression of constitutively-active AKT in THP-1 cells is sufficient to increase caspase 1 activation and IL-1\(\beta\), indicating the important connection between AKT signaling and activation of the inflammasome\textsuperscript{35}. Additionally, in macrophages, the PI3K/AKT pathway is required for caspase 1 activation and IL-1\(\beta\) release\textsuperscript{35}. While these studies have shown the critical role of AKT in caspase-1 activation, none has specifically shown that AKT directly binds to the components of the inflammasome or caspase 1. This indicates a novel avenue for mechanistic insights into inflammatory signaling and the role of AKT.

The downstream activation of mTOR complex 1 by AKT has been shown to decrease the activation of caspase 1 in several studies\textsuperscript{36, 37}, indicating the complex relationship between metabolic stress signaling and inflammation. However, upstream of AKT, the mTOR complex 2 has not been studied as it pertains to caspase 1 activation and
inflammatory signaling. In the mTOR complex 2, mTOR interacts with the helper protein rictor to phosphorylate AKT at serine 473. The attenuation of rictor has been shown to increase inflammation in mast cells, due to an impaired AKT activation. However, the specific effects of downregulation of mTOR complex 2 in vascular smooth muscle cells have not been reported. Under hypoxic conditions, both mTOR complex 1 and complex 2 are necessary for VSMC proliferation in pulmonary arterial hypertension, indicating the important role of mTOR in the modulation of VSMC phenotype.

Inflammatory signaling has been associated with an increase in AKT/mTOR signaling in murine smooth muscle cells with knockdown in PTEN expression. This was also associated with increased neointima formation and depression of smooth muscle marker proteins in PTEN +/- mice with carotid ligation. In addition, because neutrophil accumulation is critical for atherosclerotic lesion formation and progression, CXCL1, a neutrophil chemoattractant, is a key marker enhanced in atherosclerosis. Although less studied in atherosclerosis models, CXCL5 has been associated with phenotypic changes in airway smooth muscle cells.

The role of O–GlcNAc in vascular calcification is an important one, mediating oxidative and inflammatory signaling to induce the osteogenic trans-differentiation of VSMC. These studies open new doors for the exploration of O-GlcNAcylation of target proteins in the vasculature and in any model of chronic disease to identify therapeutic applications for alleviation of disease burden.
ACKNOWLEDGMENTS

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Figure 1. Smooth muscle-specific OGT deletion. Two-month-old OGT +/- SMMHC Cre/+ (Control) or OGT f/Y SMMHC Cre/+ (ΔOGT^{SMC}) mice were injected intraperitoneally with tamoxifen. At 16 weeks of age, the mice were injected with saline or STZ to induce diabetes. Four months later, immunohistochemical staining was performed to determine expression of OGT and O-GlcNAcylation. H&E=hematoxylin and eosin staining; specific antibodies for OGT and O-GlcNAc were used as described in methods.
FIGURE 2

**Figure 2.** Smooth muscle-specific OGT deletion inhibits STZ-induced vascular calcification. At 40 weeks of age, control and OGT deletion mice were sacrificed. Aortas were isolated from the mice, and calcification was evaluated by A) measurement of vascular calcium content, and B) real-time PCR to analyze bone marker expression. (n=6, *p<0.05, **p<0.01 compared to control, #p=NS compared to control)
Figure 3. Smooth muscle-specific OGT deletion attenuates VSMC calcification in vitro and blunts AKT activation. Primary vascular smooth muscle cells were explanted from control and smooth muscle-specific OGT deletion mice. A) VSMC were treated for 3 weeks in osteogenic medium alone (Control) or osteogenic medium plus 0.3 mM hydrogen peroxide (H$_2$O$_2$). The plates were stained with Alizarin red to determine calcification. B) VSMC were treated at indicated time points with 25 mM glucose, and Western blot was performed to determine AKT activation at S473 and T308.
Figure 4. Smooth muscle-specific deletion of OGT attenuates diabetic vascular inflammatory marker expression. At 40 weeks of age, 4 months after STZ diabetic induction, aortas were collected from control or OGT mice. Real-time PCR was performed with mRNA isolated from the vessels, with specific primers for inflammatory mediators previously shown to be upregulated in vascular calcification.
DISCUSSION

Vascular calcification is a major risk factor for adverse cardiovascular outcomes in diabetes\textsuperscript{72, 73}. However, the specific causative mechanisms linking diabetes to vascular calcification have not been determined. The studies shown in this body of work have demonstrated a causative effect of O-GlcNAcylation on diabetic vascular calcification, and further shown that activation of AKT and Runx2 by O-GlcNAcylation in the vasculature is key to diabetic vascular calcification. Our finding has uncovered a novel and clinically important pathway in diabetic vascular calcification, and supported that O-GlcNAcylation may represent a new target for therapeutic intervention in this disease, specifically at the level of targeting O-GlcNAc modification of AKT and Runx2.

With the low-dose STZ-induced diabetic mouse model, we have demonstrated that a dramatic increase in O-GlcNAcylation is associated with calcification in diabetic vasculature\textsuperscript{74}. These studies have provided the first evidence linking O-GlcNAcylation and vascular calcification in diabetes \textit{in vivo}. Previous studies have shown varying results of O-GlcNAc modification in diabetes, which may be attributed to the different effects of hyperglycemia in distinct organ and tissue environments as well as the particular stage of diabetes studied in each case\textsuperscript{48, 75}. Although acute increases in O-GlcNAcylation have been found to be protective in the cardiovascular system\textsuperscript{68-70}, our studies and others\textsuperscript{71} have demonstrated that chronic O-GlcNAcylation over an extended period of time, as observed in the later stages of diabetes, causes adverse complications in the cardiovascular system. It is likely that activation of differential signaling cascades in chronic and acute disease models contribute to the effects of O-GlcNAcylation in the pathogenesis of disease. The protective effect of increased O-GlcNAc modification in
the heart and vasculature\textsuperscript{76} in response to short-term stressors such as high glucose may protect against inflammation and decrease damage to the organ caused by the stress\textsuperscript{68-70}. Such an effect reflects the physiological function of O-GlcNAcylation: an acute protective mechanism to maintain cell homeostasis. Chronic increases in O-GlcNAcylation, as shown in our studies\textsuperscript{74} and others\textsuperscript{71}, however, become detrimental to the cardiovascular system when the stresses are maintained at high levels, as we observed in late stage of diabetes. The distinct pathways activated by short-term versus long-term increases in O-GlcNAcylation have yet to be determined. It is possible that some of the same proteins, such as AKT, are protective by acute activation but may become detrimental due to chronically sustained high levels of activity\textsuperscript{77}.

With the use of comprehensive pharmacologic and genetic approaches, we have demonstrated a direct effect of increased O-GlcNAcylation and diabetic vascular calcification \textit{in vivo}. Using Thiamet-G, a potent and selective OGA inhibitor, we have shown that increased O-GlcNAcylation precedes vascular calcification in diabetic mice\textsuperscript{74}. As a functional consequence, reduced aortic compliance was determined in the STZ-induced diabetic mice, which was further worsened by increased O-GlcNAcylation via Thiamet-G treatment. These observations are consistent with the clinical observations demonstrating an association between increased vascular calcification\textsuperscript{78} and other vascular complications\textsuperscript{44, 73} in diabetes. By generating a novel SMC-specific OGT deletion mouse model, we have further determined a definitive function of O-GlcNAcylation in regulating VSMC calcification \textit{in vitro} and diabetic vascular calcification \textit{in vivo}. Consistently, SMC-specific OGT deletion prevented O-GlcNAcylation-induced decreases in aortic compliance in diabetic mice. Although many
studies have postulated the role of O-GlcNAc in pathogenesis of diseases\textsuperscript{13, 74, 78}, to our knowledge, the present study is the first to show the direct effects of tissue-specific OGT deletion and decreased O-GlcNAcylation on vascular function in a chronic disease model in vivo. Altogether, these findings have strongly supported a causative link between increased O-GlcNAcylation and diabetic vascular calcification and impaired vascular compliance in vivo (Fig 1).

Mechanistically, O-GlcNAcylation may act as a sensor of the metabolic state of the cell: globally increased O-GlcNAc signals a high metabolic stress, such as hyperglycemia, to activate survival signaling and other metabolic pathways\textsuperscript{79}. Chronic

\textit{Figure 1. O-GlcNAcylation regulates vascular calcification.} Inhibition of the O-GlcNAcase (OGA), which hydrolyzes O-GlcNAc modification from target proteins, by Thiamet-G or shRNA targeting OGA (shOGA) results in increased protein O-GlcNAcylation that promotes vascular calcification. On the other hand, the O-GlcNAc transferase (OGT) adds O-GlcNAc modification onto serines and threonines of target proteins. Inhibition of OGT by shRNA (shOGT) or SMC-sepcific OGT deletion (ΔOGT\textsuperscript{SMC}) decreases protein O-GlcNAc modification and inhibites vascular calcification.
exposure of many different types of cells to high glucose causes increased O-GlcNAcylation leading directly to changes in insulin signaling, mirroring the signaling observed in diabetic patients. Furthermore, O-GlcNAcylation may even exacerbate hyperglycemia further through its effects in the diabetic liver—high hepatic levels of O-GlcNAc result in excess glucose production in this organ. In addition, chronically high levels of O-GlcNAc in the glucose-sensitive pancreas cause breakdown in the function of the beta cells and degradation of organ function. Our studies have demonstrated a direct regulation of VSMC calcification by intracellular O-GlcNAcylation levels in the diabetic vasculature. Increased O-GlcNAcylation, by OGA inhibition or knockdown, promotes VSMC calcification. In contrast, decreased O-GlcNAcylation by OGT inhibition blocks high glucose and oxidative stress-induced VSMC calcification. Although previous studies have shown that increased O-GlcNAcylation is associated with osteogenesis and chondrogenesis, a causative effect of O-GlcNAcylation on osteogenic differentiation and the molecular mechanisms underlying O-GlcNAcylation in osteogenesis and chondrogenesis are unknown. Our studies have determined a direct effect of O-GlcNAcylation in regulating osteogenic differentiation of VSMC via upregulation of Runx2, the osteogenic transcription factor that we have demonstrated to be essential and sufficient for vascular calcification.

We have determined that activation of AKT mediates the effects of O-GlcNAcylation on Runx2 upregulation and VSMC calcification. We reported previously that AKT activation by oxidative stress is important for VSMC calcification. Both oxidative stress and high glucose have been found to increase O-GlcNAcylation. Consistently, we have demonstrated that increased activation of AKT is associated with
increased O-GlcNAcylation and vascular calcification in the STZ-induced diabetic arteries in vivo and OGA-inhibited VSMC in vitro\textsuperscript{74}. On the other hand, inhibition of O-GlcNAcylation by OGT inhibition blocks AKT activation in diabetic arteries and in VSMC with OGT deletion. Importantly, we found that increased O-GlcNAcylation does not affect VSMC proliferation/viability, suggesting that the function of O-GlcNAcylation-induced AKT activation in promoting VSMC calcification is independent of its regulation of cell proliferation and apoptosis. This finding is consistent with our previous report that AKT inhibition does not induce apoptosis of VSMC\textsuperscript{7}. Our studies revealing AKT as a key target of O-GlcNAcylation in diabetes have exciting implications for future studies. AKT has been shown to be chronically activated in vascular cells exposed to hyperglycemia in diabetic models\textsuperscript{86}, although other studies reported blunted AKT activation in diabetic cardiomyocytes and myotubes\textsuperscript{87, 88}. Therefore, AKT activation may be differentially regulated in diabetes depending upon cell type, cellular environment, and disease status. In VSMC, we have found that inhibition of AKT activation attenuates VSMC calcification whereas constitutively active AKT is sufficient to induce VSMC calcification. These results have demonstrated an essential role of AKT activation in mediating O-GlcNAcylation-induced VSMC calcification, and that activation of AKT is sufficient to promote VSMC calcification.

We have further identified direct O-GlcNAc modifications on AKT that affect AKT activation\textsuperscript{74}. High levels of O-GlcNAc modification have been shown to directly modify several important proteins involved in vascular homeostasis in diabetic models\textsuperscript{79}. For instance, endothelial nitric oxide synthase is modified by O-GlcNAc, which results in attenuation of its phosphorylation and activity\textsuperscript{89}. This causes a decrease in nitric oxide
production and ultimately to impairment in vascular function\textsuperscript{90}. In addition, increased O-GlcNAcylation has been linked to increased expression of pro-fibrotic proteins, leading to higher levels of extracellular matrix production and reduced vascular tone\textsuperscript{91}. This can also lead to dysfunction in the kidney, further exacerbating the renal problems associated with diabetes\textsuperscript{91}. Furthermore, O-GlcNAc modification of PDX1 and NeuroD1, two transcription factors that are known to be involved in metabolism, enhances binding of these proteins to the promoter of insulin gene\textsuperscript{92, 93}, which links protein O-GlcNAcylation with metabolic regulation. Similarly, we identified direct O-GlcNAc modification sites on the Runx2 transcription factor which promote osteogenic differentiation of VSMC. Our finding of direct modification of AKT and Runx2 by O-GlcNAcylation leading to upregulation of Runx2 and VSMC calcification has shed new light on negative functional outcomes resulting from chronically high levels of O-GlcNAcylation in diabetes.

O-GlcNAcylation of AKT and Runx2 was found to regulate their phosphorylation. Protein O-GlcNAcylation has been linked to phosphorylation; the prevailing belief is that protein O-GlcNAcylation and phosphorylation reciprocally regulate protein activity\textsuperscript{48}. For example, increased O-GlcNAcylation of insulin receptor substrate 1 decreases its phosphorylation, leading to decreased glucose transport via glucose transporter type 4\textsuperscript{94}. A recent study also found that O-GlcNAcylation of AKT at T305/T312 inhibits phosphorylation of AKT at T308 in COS-7 cells\textsuperscript{77}. In contrast, we have observed a positive correlation between O-GlcNAcylation of AKT and Runx2 and their phosphorylation, supporting the novel mechanisms underlying the regulation of AKT/Runx2 phosphorylation and activation by their O-GlcNAcylation.
Activation of AKT has been associated with altered O-GlcNAcylation in different cells; however, a direct role of O-GlcNAc modification on AKT activation to regulate cellular function has not been determined\textsuperscript{95}. We have identified unique O-GlcNAc modification at two sites, T430 and T479, which are critical for AKT phosphorylation at S473 leading to VSMC calcification. O-GlcNAcylation of AKT at these sites does not affect T308, a finding which is in contrast to previous studies in COS7 cells showing AKT O-GlcNAcylation at T305 and T312 inhibited its phosphorylation at T308\textsuperscript{77}. Of note, increased O-GlcNAcylation induces AKT phosphorylation at S473 but not T308, suggesting a unique and selective role of O-GlcNAcylation-promoted AKT activation at S473. The mechanism underlying AKT O-GlcNAcylation and phosphorylation has not been fully elucidated. Inhibition of O-GlcNAcylation of AKT by T430 and T479 mutation blocked its binding to Rictor, a component of the mTOR complex 2, the major kinase known to phosphorylate AKT at S473\textsuperscript{96}. Therefore, AKT O-GlcNAcylation at T430 and T479 may facilitate its interaction with mTOR complex 2 and subsequent phosphorylation by the kinase at S473. Although further investigation is warranted to determine the precise molecular details of how this interaction leads to AKT activation, our studies have demonstrated that O-GlcNAc modification of AKT at T430 and T479 is required for its full activation leading to Runx2 upregulation and osteogenic differentiation of VSMC.

Sites of direct O-GlcNAc modification on Runx2 were also determined. Post-translational modifications of Runx2 have been shown to play an important role in Runx2 osteogenic function in osteoblasts\textsuperscript{97,98}. The two novel O-GlcNAcylation sites at T412 and S413 are located in the Runx2 nuclear matrix targeting signal domain (NMTS). This
Runx2 NMTS domain is known to be responsible for the subnuclear localization of the protein, and it contains the binding sites for Smad proteins\textsuperscript{99}. Although the NMTS domain is important for Runx2 function in osteoblasts\textsuperscript{100}, little is known about the post-translational modifications of Runx2 in the NMTS and how they may affect Runx2 osteogenic function. One study has shown that Runx2 binding to ubiquitin ligases requires the binding of Smad6 to the Smad-interacting domain of the NMTS\textsuperscript{101}, suggesting the involvement of NMTS in regulating Runx2 ubiquitination, an important regulator of osteogenic differentiation in osteoblasts and mesenchymal stem cells\textsuperscript{102, 103}. In addition, Runx2 may also be acetylated at many lysine residues, which may act to stabilize the protein and protect against ubiquitin-mediated degradation\textsuperscript{102}. Our studies have provided the first evidence of a direct post-translational modification by O-GlcNAcylation on Runx2 in the NMTS domain. We have further determined that O-GlcNAcylation of Runx2 at T412 and S413 is important for the Runx2 binding to the BMP-regulated Smads as well as the osteogenic function of Runx2 in promoting VSMC calcification. This exciting new finding adds an entirely new aspect to our understanding of Runx2 post-translational modification and its effects on Runx2 osteogenic function.

We found that O-GlcNAcylation of Runx2 affects its binding to BMP-regulated Smad 1/5/8 but not the TGF-β-regulated Smad 2/3, which may contribute to the unique role of BMP but not TGF-β in Runx2-promoted VSMC calcification. This observation is different from previous studies demonstrating that NMTS binding to Smads that are regulated by both BMP and TGF-β signaling mediates the osteogenic function of Runx2 in osteoblasts\textsuperscript{99}, suggesting a unique role of Runx2 NMTS domain in regulating osteogenesis of VSMC. The precise mechanisms that are responsible for Runx2 O-
GlcNAcylation in regulating its osteogenic function have not been determined. We found that decreased Runx2 O-GlcNAcylation by the T412 and S413 mutations inhibited Runx2 phosphorylation on serine and threonine as well as Runx2 binding to Smad 1/5/8. Inhibition of ERK-mediated phosphorylation of Runx2 has been found to prevent its binding to Smad1 and impair osteogenesis of osteoblast precursors, but the exact sites of ERK-regulated Runx2 phosphorylation have not been determined\textsuperscript{102}. Our observation of a positive regulation of Runx2 O-GlcNAcylation on its phosphorylation suggests that O-GlcNAc modification of Runx2 at the T412/S413 may result in a conformation change that facilitates Runx2 phosphorylation in the NMTS. Further exploration into the mechanisms underlying our novel finding of Runx2 O-GlcNAcylation in regulating its phosphorylation and Smad binding will provide important molecular insights into the regulation and function of this master regulator of vascular calcification.

**SUMMARY AND CONCLUSION**

O-GlcNAc modification is important in controlling cellular homeostasis to determine the fate of the cell in acute and chronic conditions. Although acute elevations in O-GlcNAcylation are believed to be protective, chronically sustained upregulation of O-GlcNAcylation is detrimental to the cell and tissue, leading to pathogenesis of disease.

Our studies have demonstrated a novel causative link between chronic increases in vascular O-GlcNAcylation and vascular calcification in diabetes. We have uncovered a novel mechanism underlying the regulation of AKT activation and Runx2 transactivity by their O-GlcNAcylation, which induces Runx2 upregulation and promotes VSMC
Increased O-GlcNAcylation promotes diabetic vascular calcification via direct modification of AKT and Runx2. The diabetic vasculature exhibits enhanced protein O-GlcNAcylation and vascular calcification. Induction of O-GlcNAc modification in VSMC promotes activation of AKT and increased binding of Runx2 to BMP Smads (Smad1/5/8), leading to increased Runx2 expression and transcriptional activity and resulting in vascular calcification. Inhibition of Runx2 O-GlcNAcylation at T412 and S413 inhibits Runx2-Smad binding and attenuates vascular calcification. Mutation of AKT O-GlcNAcylation sites T430 and T479 blocks its phosphorylation at S473 but not T308, leading to reduced Runx2 transactivity and attenuated VSMC calcification.

Using a novel inducible smooth muscle-specific OGT deletion mouse model, we have further demonstrated that OGT deletion in VSMC reduces vascular O-GlcNAcylation and blocks diabetic vascular calcification. SMC-specific OGT deletion inhibits AKT activation and Runx2 upregulation in the diabetic vasculature. The novel regulation of AKT activation by O-GlcNAcylation uncovered in this study may have a significant impact not only on the biological function of AKT activation, but also on novel insights into the pathogenesis of vascular disease featuring increased activation calcification (Fig 2).
of AKT. Furthermore, O-GlcNAcylation of Runx2 was found to regulate its phosphorylation and binding to Smad1/5/8, ultimately leading to VSMC calcification. Taken together, our studies have determined O-GlcNAcylation as a novel contributor to the process of vascular calcification and identified O-GlcNAcylation of AKT/Runx2 signaling as a possible target for the development of therapies for vascular calcification in diabetes.
REFERENCES


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101. Shen R, Chen M, Wang YJ et al. Smad6 interacts with Runx2 and mediates Smad ubiquitin regulatory factor 1-induced Runx2 degradation. *J Biol Chem* 2006 February 10;281(6):3569-76.


APPENDIX A: ANIMAL USE APPROVAL FORM
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: May 1, 2012

TO: YABING CHEN, PhD
SHEL-710 2182
FAX: (205) 975-9927

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: O-GlcNAc Modification in Diabetic Vascular Calcification (Jack Heath)
Sponsor: American Heart Association
Animal Project Number: 120409598

As of April 18, 2012, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

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Animal use must be renewed by April 17, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 120409598 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.