



Impaired intracellular calcium homeostasis enhances protein O-GlcNAcylation and promotes vascular calcification and stiffness in diabetes

Weiping Zhang^{a,b}, Yong Sun^{a,c}, Youfeng Yang^a, Yabing Chen^{a,c,*}

^a Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA

^b Department of Cardiology, First Affiliated Hospital of Xi'An JiaoTong University, Xi'An, PR China

^c Research Department, Birmingham Veterans Affairs Medical Center, Birmingham, AL, USA

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ABSTRACT

Vascular calcification is accelerated in patients with diabetes mellitus and increases risk of cardiovascular events and mortality. Vascular smooth muscle cells (VSMC) play a key role in regulating vascular tone and contribute significantly to the development of diabetic vasculopathy. In this study, the function of stromal interaction molecule 1 (STIM1), an important regulator for intracellular calcium homeostasis, in diabetic vascular calcification was investigated, and the underlying molecular mechanisms were uncovered.

A SMC-specific STIM1 deletion mouse model (STIM1^{Δ/Δ}) was generated by breeding the STIM1 floxed mice (STIM1^{f/f}) with SM22α-Cre transgenic mice. Using aortic arteries from the STIM1^{Δ/Δ} mice and their STIM1^{f/f} littermates, we found that SMC-specific STIM1 deletion induced calcification of aortic arteries cultured in osteogenic media *ex vivo*. Furthermore, STIM1 deficiency promoted osteogenic differentiation and calcification of VSMC from the STIM1^{Δ/Δ} mice. In the low-dose streptozotocin (STZ)-induced mouse model of diabetes, SMC-specific STIM1 deletion markedly enhanced STZ-induced vascular calcification and stiffness in the STIM1^{Δ/Δ} mice. The diabetic mice with SMC-specific STIM1 ablation also exhibited increased aortic expression of the key osteogenic transcription factor, Runx2, and protein O-GlcNAcylation, an important post-translational modulation that we have reported to promote vascular calcification and stiffness in diabetes. Consistently, elevation of O-GlcNAcylation was demonstrated in aortic arteries and VSMC from the STIM1^{Δ/Δ} mice. Inhibition of O-GlcNAcylation with a pharmacological inhibitor abolished STIM1 deficiency-induced VSMC calcification, supporting a critical role of O-GlcNAcylation in mediating STIM1 deficiency-induced VSMC calcification. Mechanistically, we identified that STIM1 deficiency resulted in impaired calcium homeostasis, which activated calcium signaling and increased endoplasmic reticulum (ER) stress in VSMC, while inhibition of ER stress attenuated STIM1-induced elevation of protein O-GlcNAcylation.

In conclusion, the study has demonstrated a causative role of SMC-expressed STIM1 in regulating vascular calcification and stiffness in diabetes. We have further identified a novel mechanisms underlying STIM1 deficiency-induced impairment of calcium homeostasis and ER stress in upregulation of protein O-GlcNAcylation in VSMC, which promotes VSMC osteogenic differentiation and calcification in diabetes.

1. Introduction

Despite of progression in the diagnosis and treatment of diabetes mellitus, developing cardiovascular disease remains a leading cause of morbidity and mortality among patients with diabetes mellitus [1,2]. Diabetic vasculopathy, including microvascular and macrovascular

complications, is one of the major diabetic complications in the cardiovascular system [3]. Vascular calcification is prevalent in diabetes and leads to increased risk of major cardiovascular events and mortality [4,5]. Complex metabolic disorders, including hyperglycemia, insulin insufficiency and excessive oxidative stress, have been linked to increased vascular calcification in diabetes mellitus. Both

* Corresponding author. Dept. of Pathology, University of Alabama at Birmingham, 614 Shelby Biomedical Research Bldg, 1825 University Blvd, Birmingham, AL, 35294, USA.

E-mail address: yabingchen@uabmc.edu (Y. Chen).

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atherosclerosis-independent medial calcification and atherosclerosis-related intimal calcification occur in diabetic vasculature, which accelerate vascular stiffness and worsens diabetic vasculopathy. Calcification of vascular smooth muscle cells (VSMC) is the common pathology in the medial and intimal lesions, contributing predominantly to the development of diabetic vasculopathy [5–7]. Under pathological conditions, such as hyperglycemia and oxidative stress, VSMC can undergo phenotypic switching and osteogenic differentiation, leading to the deposition of hydroxyapatite mineral and calcification in the extracellular matrix. Increased oxidative stress is a significant cause of vascular calcification [8–13]. Hyperglycemia induces oxidative stress, generation of the nonenzymatic advanced glycation end products and activation of inflammation signaling in the vasculature [3,14,15], all of which promote vascular calcification *in vitro* and *in vivo* [8,9,15]. Additionally, increased glucose metabolism via the hexosamine biosynthesis pathway promotes posttranslational protein modification via O-linked β -N-acetylglucosamine modification (O-GlcNAcylation), which is associated with increased vascular calcification in human and mouse diabetic vascular lesions [5,16–19]. Recent studies have demonstrated a causative link between increased O-GlcNAcylation and VSMC osteogenic differentiation and vascular calcification and stiffness in diabetic mice [19].

Increased oxidative stress and O-GlcNAcylation have been linked to the regulation of calcium flux and calcium-dependent signaling [20,21]. Calcium signaling plays a critical role in the development of vascular calcification, as demonstrated in both humans and animal models [22,23]. However, the role of stromal interaction molecule 1 (STIM1), a key intracellular calcium regulator located on endoplasmic reticulum membrane, in VSMC calcification remains largely unknown. STIM1 is highly conserved in eukaryotic cells and regulates intracellular calcium flux through the store-operated calcium entry [24,25]. STIM1 deficiency causes abnormal calcium influx and cellular calcium concentration, which may subsequently affect several cellular events, including cell proliferation and differentiation [25–27]. In humans, patients carrying a homozygous point mutation for the STIM1 gene suffer from immunodeficiency, autoimmune disease, congenital myopathy, ectodermal dysplasia, and partial iris hypoplasia [28,29]. Mice with global STIM1 deletion exhibit sudden and perinatal mortality, immunodeficiency, autoimmune and inflammatory diseases, and muscle weakness [30]. In the vascular system, STIM1 is ubiquitously expressed in all vascular cells but more abundant STIM1 mRNA levels are determined in VSMC [31]. STIM1-dependent signaling is important for VSMC contractility, proliferation and migration [26,32,33]. Nonetheless, it is unknown how this major calcium regulators may modulate VSMC osteogenic differentiation and calcification. Using a SMC-specific STIM1 deletion animal model, the present study uncovered an important role of VSMC-expressed STIM1 in diabetic vascular calcification and elucidated how STIM1 deficiency affects calcium signals and ER stress, leading to augmented O-GlcNAcylation that mediates STIM1 deficiency-promoted VSMC osteogenic differentiation calcification.

2. Methods

2.1. Generation of SMC-specific STIM1 deletion mice

STIM1 exon2 floxed mice (STIM1^{f/f}) mice were bred with the SM22 α -Cre transgenic mice, both were originally from The Jackson Laboratory and backcrossed to C57BL/6 genetic background, to generate SMC-specific STIM1 ablation mice (STIM1 ^{Δ/Δ}). Primer sets for genotyping include: Cre: F-5'-GCGGTCTGGCAGTAAAACTATC-3' and R-5'-GTGAAACAGCATTGCTGTCACTT-3'; STIM1: 5'-CGATGGTCTCACGGTCTCTAGTTTC-3'; 5'-GGCTCTGCTGACCTGGAACATATAGT G-3'; and 5'-AACGCTTGCAGTTGCTGTAGGC-3'.

2.2. Experimental animals

Eight-week old STIM1 ^{Δ/Δ} mice and the STIM1^{f/f} control littermates were intraperitoneally injected with low-dose streptozotocin (STZ, 50 mg/kg) for 5 consecutive days to induce hyperglycemia and diabetes as we previously reported [19]. Blood glucose was monitored bi-weekly for 14 weeks using the AlphaTrak glucose meter and strips (Abbott, Abbott Park, IL). Both food and fluid intake were given *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

2.3. Measurement of pulse wave velocity

Aortic stiffness, indicated by pulse wave velocity (PWV), was analyzed by echocardiography using high resolution imaging system VEVO 770 (Visual Sonics, Toronto, Canada) as we previously described [19,34]. PWV was calculated as the distance divided by the time interval between the pulse wave at the aortic arch and the abdominal aorta (m/s).

2.4. Tissue harvest, processing and analysis

At the end of the experiments, mice were euthanized and aortic tissues were collected for characterization of calcium content and protein expression as we reported [9,34–37].

Total aortic calcium was quantified by Arsenazo III calcium measurement kit (StanBio Laboratory). The amount of aortic calcium was normalized to the total protein amount in the tissues, determined by the bicinchoninic acid assay (Pierce™, PI-23225) and expressed as fold change compared to control. Aortic protein expression was determined by Western blot analysis, or immunostaining on consecutive 7- μ m aortic sections using specific antibodies, including antibodies for O-GlcNAcylation (RL-2; Abcam), Runx2 (MBL, D130-3). Hematoxylin and eosin (H&E) staining was used for histology. Stained specimens were examined microscopically (leica M165 FC), and quantified using ImageJ software (NIH Bethesda, MD) as we described previously [9,34–37].

2.5. Ex vivo aortic calcification

Calcification of aortic rings was performed as previously reported [34,36], using 3-mm mouse aortic rings cultured in osteogenic media for up to 3 weeks. Total calcium content in the aortic rings was quantified by Arsenazo III method. In parallel experiments, aortic rings were fixed in 4% paraformaldehyde, paraffin embedded and consecutive 7- μ m sections were used for Alizarin Red staining and STIM1 expression was determined using specific antibody (Cell signaling) [34,36].

2.6. In vitro VSMC calcification

In vitro calcification was performed with primary VSMC at passages 3–5, as we previously reported [8,9,34–37], in osteogenic media containing 0.25 mM L-ascorbic acid and 10 mM β -glycerophosphate and 10⁻⁸ M dexamethasone (Sigma Aldrich) for 3 weeks with media change every 3 days. Calcification was determined by Alizarin Red staining; or quantified by measuring total calcium in the cell lysates by Arsenazo III method in parallel experiments.

2.7. Inhibition of O-GlcNAcylation in VSMC

O-GlcNAcylation was inhibited in VSMC using ST49. (Sigma-Aldrich, ST045849), a pharmacological inhibitor of β -N-acetylglucosaminyltransferase. Inhibition O-GlcNAcylation was confirmed by Western blot analysis using specific antibodies for O-GlcNAcylation (RL-2, Abcam).

2.8. Intracellular calcium measurement

Intracellular calcium was measured using Fluo4 NW calcium assay kit (Molecular Probes) as we reported [34]. VSMC pre-loaded with Fluo4 NW fluorescent dye (1 μ M) for 30 min were exposed to osteogenic media, and fluorescence signals were recorded for 30 min using the fluorescence microplate reader with excitation at 485 nm and emission at 525 nm (Synergy2, BioTek). Intracellular calcium was calculated by subtracting the basal fluorescence intensity from the total fluorescence intensity.

2.9. Western blot analysis

Western blot analyses were performed with the use of specific antibodies for STIM1 (Cell signaling, 5668S), α -SMA (Sigma, A5228), Col1a (Sigma, SAB2100463), Runx2 (MBL, D130-3), O-GlcNAcylation (RL-2; Abcam), p-eIF2a (Cell Signaling, 9721S), and p-CaMKII (Cell Signaling, 12716S, Phospho-CaMKII-Thr286) and detected with a Western blot chemiluminescence detection kit (Millipore). 4-Phenylbutyric Acid (4PBA, Sigma, Cat# P21005) was used to inhibit ER stress as we described [37].

2.10. Real-time polymerase chain reaction (PCR)

As we reported [8], 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, osteocalcin (OC), α -SMA, alkaline phosphatase (ALP) and collagen Ia (Colla) with SsoFast EvaGreen Supermix (Bio-Rad) on a C1000 Thermal Cycler (Bio-Rad).

2.11. Statistical analysis

Results are presented as the mean \pm SD. Differences between two groups were determined by 2-tailed Student *t*-tests. For multiple group comparison, one-way analysis of variance followed by a Student-Newman-Keuls test was performed. Significance was defined as $p < 0.05$.

3. Results

3.1. STIM1 deficiency induces VSMC osteogenic differentiation and calcification

Using VSMC from the SMC-specific STIM1 deletion mice (STIM1 Δ/Δ) and their control littermates (STIM1 $^{f/f}$), we determined the function of STIM1 in regulating VSMC osteogenic differentiation and calcification. STIM1 deletion induced calcification of VSMC culture in osteogenic media, as highlighted by both Alizarin Red staining (Fig. 1Aa) and total calcium measurements (Fig. 1Ab). Western blot analysis revealed increased protein level of the key osteogenic transcription factor, Runx2 and bone marker protein Col1 α concurrently with decreased expression of the smooth muscle cell marker, α -SMA, in the STIM1 Δ/Δ VSMC (Fig. 1B). Consistently, expression of genes encoding Runx2, Col1 α and bone marker genes, ALP and OC, was upregulated while the α -SMA gene was downregulated in the STIM1 Δ/Δ VSMC. Altogether, these results demonstrated that STIM1 deficiency induces osteogenic differentiation and calcification of STIM1 Δ/Δ VSMC, supporting an important role of STIM1 in regulating VSMC function.

3.2. SMC-specific STIM1 deficiency promotes ex vivo aortic calcification

To further determine the effects of SMC-specific STIM1 deletion on calcification of VSMC in their natural milieu, we employed an *ex vivo* aortic ring culture system as we previously reported [36], using descending aortas from the STIM1 Δ/Δ mice and their control littermates. STIM1 deficiency was evident in the aortic media of the STIM1 Δ/Δ mice, as determined by immunohistochemical staining (Fig. 2A, STIM1). Consistent with the observation from the study using isolated VSMC in Fig. 1, aortas from STIM1 Δ/Δ mice exhibited enhanced calcification after cultured in osteogenic media for 3 weeks, compared with that in aortas from the control STIM1 $^{f/f}$ littermates (Fig. 2A, Alizarin Red). Quantification analysis revealed a markedly increase in vascular calcification in aortas from STIM1 Δ/Δ mice (Fig. 2B), further supporting that SMC-specific STIM1 deletion promoted VSMC calcification.

3.3. SMC-specific STIM1 deletion promotes vascular calcification and stiffness in diabetic mice

Using a low-dose STZ injection-induced diabetic mouse model, the effects of SMC-specific STIM1 deletion on vascular calcification *in vivo*

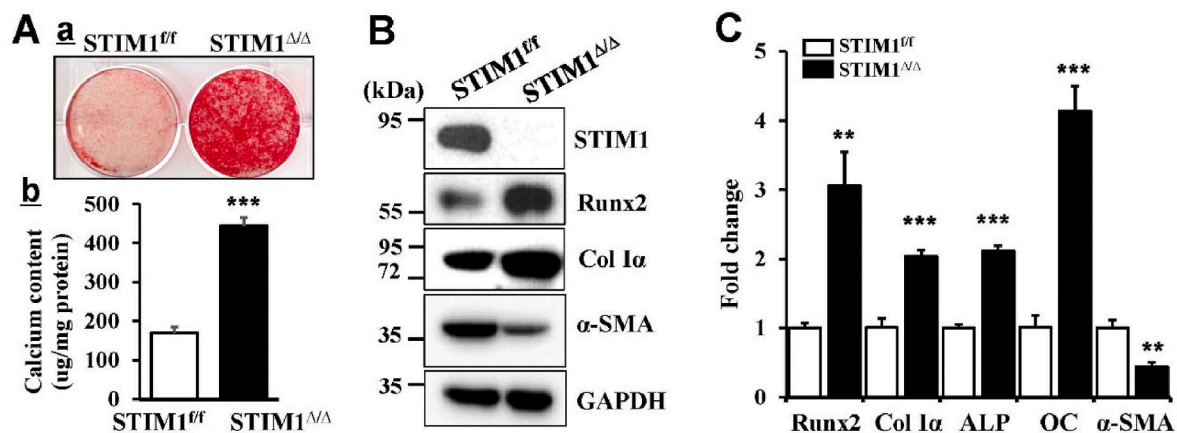


Fig. 1. STIM1 deficiency induces VSMC osteogenic differentiation and calcification. Primary VSMC isolated from STIM1 $^{f/f}$ and STIM1 Δ/Δ mice were cultured in osteogenic media for 3 weeks. **A**) Calcification was determined by **a**. Alizarin Red staining. Representative images of stained dishes from 3 independent experiments are shown; and **b**. Total calcium content quantification by Arsenazo III assay, in parallel sets of experiments. Results shown are normalized by total protein amount. Bar values are means \pm SD ($n = 6$, *** $p < 0.001$). **B**) Western blot analysis of the expression of STIM1, Runx2, type I collagen (Col 1 α) and smooth muscle α -actin (α -SMA), using specific antibodies. The expression of GAPDH was used as a loading control. **C**) Real-time PCR analysis of Runx2, osteogenic markers, including Col 1 α , alkaline phosphatase (ALP), osteocalcin (OC) and SMA. The expression level of each gene in the STIM1 $^{f/f}$ VSMC is normalized as 1 ($n = 3$, *** $p < 0.001$, ** $p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

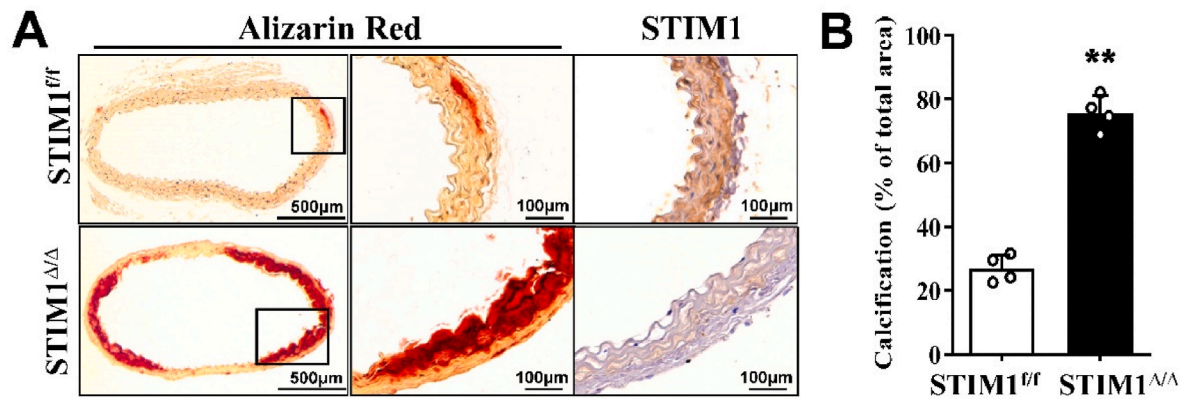


Fig. 2. SMC-specific *STIM1* deletion promotes ex vivo aortic calcification. Aorta rings from *STIM1*^{f/f} and *STIM1*^{Δ/Δ} mice were cultured in osteogenic medium for 3 weeks. **A)** Consecutive sections were stained by Alizarin Red (calcification) and specific antibody for *STIM1*. Representative images from 4 pairs of littermates are shown. **B)** Quantification of vascular calcification in **A** with by NIH Image J software. Results presented are the percentage of positively alizarin red-stained areas in the total area (n = 4, ***p < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were determined. Elevation of blood glucose levels was observed in the STZ-injected *STIM1*^{Δ/Δ} mice similarly to those of the control mice (*STIM1*^{f/f}/STZ) during the 14 weeks after administration of STZ (Fig. 3A). Increased calcification was determined in aortic arteries from the STZ-injected *STIM1*^{Δ/Δ} mice, compared with the STZ-injected control *STIM1*^{f/f} littermates (Fig. 3B). Furthermore, a significant increase in pulse wave velocity (PWV), an indicator for aortic stiffness [38], was demonstrated in the STZ-injected *STIM1*^{Δ/Δ} mice (Fig. 3C). Therefore, SMC-specific *STIM1* deletion in mice promoted vascular calcification and aortic stiffness, without affecting low dose STZ-induced hyperglycemia, in the diabetic mice.

3.4. Increased O-GlcNAcylation and Runx2 are associated with diabetic vascular calcification in the SMC-specific *STIM1* deletion mice

We have previously reported that increased protein O-GlcNAcylation promotes VSMC calcification *in vitro* and vascular calcification in diabetic mice [19]. To evaluate whether SMC-specific *STIM1* deletion-increased vascular calcification in the diabetic mice was associated with O-GlcNAcylation, we determined protein O-GlcNAcylation in arteries from the STZ-injected *STIM1*^{Δ/Δ} mice and the control *STIM1*^{f/f} littermates. Immunohistochemical staining revealed markedly increased vascular O-GlcNAcylation in the media of arteries from STZ-injected *STIM1*^{Δ/Δ} mice compared with those from the *STIM1*^{f/f} mice (Fig. 4Aa, O-GlcNAc) and quantified in Fig. 4Ab. Western blotting analysis further confirmed significant increases in protein

O-GlcNAcylation in aortic arteries from the *STIM1*^{Δ/Δ} mice (Fig. 4B). Increased aortic O-GlcNAcylation in the *STIM1*^{Δ/Δ} mice was accompanied by increased Runx2 (Fig. 3Aa & Ba, Runx2), the key osteogenic transcription factor that determines vascular calcification^{35,86}. Accordingly, increased vascular O-GlcNAcylation in the *STIM1*^{Δ/Δ} mice may be responsible for *STIM1* deletion-promoted vascular calcification in the diabetic mice.

3.5. Increased O-GlcNAcylation mediates *STIM1* deletion-promoted VSMC calcification

The effect of *STIM1* deficiency on O-GlcNAcylation was further characterized using primary VSMC from the *STIM1*^{Δ/Δ} mice and the control *STIM1*^{f/f} mice. *STIM1* deficiency led to significant increases in O-GlcNAcylation in VSMC grown in culture media (Fig. 5A), suggesting a direct contribution of *STIM1* deficiency to increased O-GlcNAcylation in VSMC. The role of increased O-GlcNAcylation in mediating *STIM1* deficiency-promoted VSMC calcification was further determined using ST49, a selective pharmacological inhibitor for O-GlcNAc transferase (OGT), which is the essential enzyme for protein O-GlcNAcylation. Inhibition of O-GlcNAcylation by ST49 not only abolished calcification of the control *STIM1*^{f/f} VSMC cultured in osteogenic media, but also attenuated *STIM1* deficiency-promoted calcification of the *STIM1*^{Δ/Δ} VSMC (Fig. 5Ba). Quantitative measurements of calcium in parallel sets of experiments further confirmed that ST49 potentially inhibited *STIM1* deficiency-promoted VSMC (Fig. 5Bb). Consistently, ST49-inhibited O-

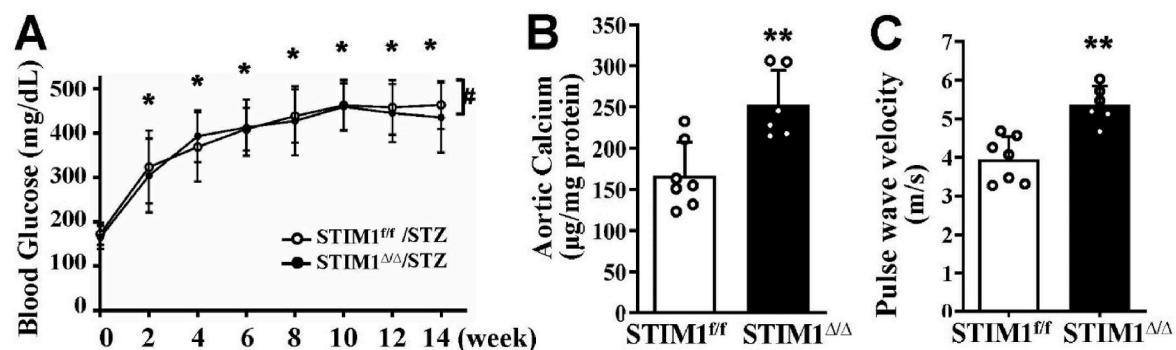


Fig. 3. SMC-specific *STIM1* deletion promotes vascular calcification and stiffness in diabetic mice. *STIM1*^{f/f} and *STIM1*^{Δ/Δ} mice were injected with streptozotocin (STZ) for 5 consecutive days. **A)** Blood glucose levels were monitored using the AlphaTrak glucose meter up to 14 weeks after the STZ injection. (*p < 0.05 compared with week 0; # indicating not significant between two groups). **B)** Calcium contents were determined in descending aortas, the calcium content in each sample was normalized to its protein level (n = 7 and 6 respectively, ***p < 0.001). **C)** Echocardiography was performed in mice 14 week after STZ injection, to determine pulse wave velocity, an indicator for aortic stiffness (n = 7 and 6 respectively, ***p < 0.001).

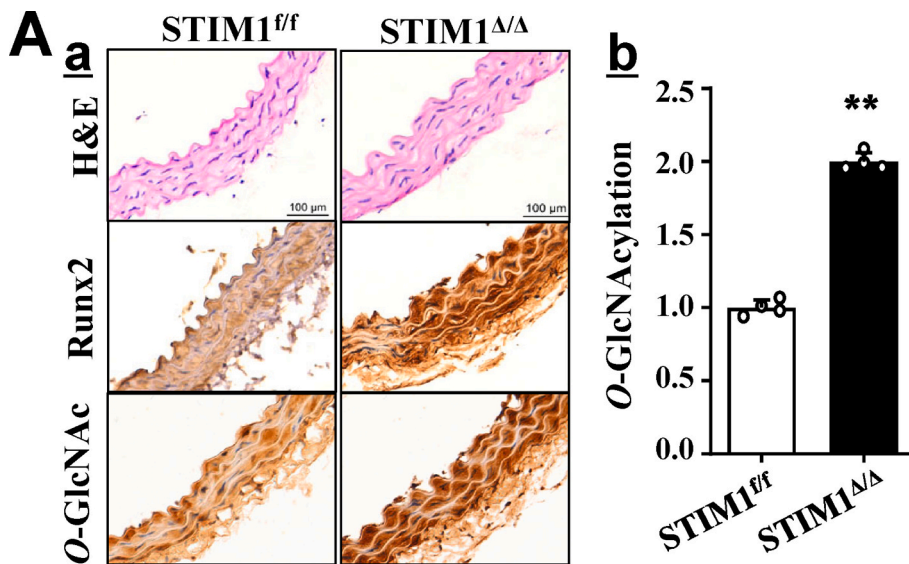


Fig. 4. Increased O-GlcNAcylation and Runx2 are associated with diabetic vascular calcification in the SMC-specific STIM1 deletion mice. The expression of Runx2 and protein O-GlcNAcylation in aortas from the STZ-injected STIM1^{f/f} and STIM1^{Δ/Δ} mice as described in Fig. 3 were determined by A) Immunostaining in aortic sections, using specific antibodies for Runx2 and O-GlcNAc. H&E staining was used for histology. **b.** Vascular O-GlcNAcylation in **a** was quantified by NIH ImageJ and compared with that in STZ-injected STIM1^{f/f}, which was defined as 1, (n = 4/group, **p < 0.001). **B)** Protein extracts from descending aortas (n = 3/group) were used for Western blot with specific antibodies. The expression of GAPDH was used as a loading control. **b.** Total protein O-GlcNAcylation was quantified by the intensity of the bands in **a** (O-GlcNAc), normalized to the intensity of GAPDH. Results are shown as fold changes compared to GlcNAcylation in protein extracts from STIM1^{f/f} mice which was defined as 1 (n = 3, **p = 0.002).

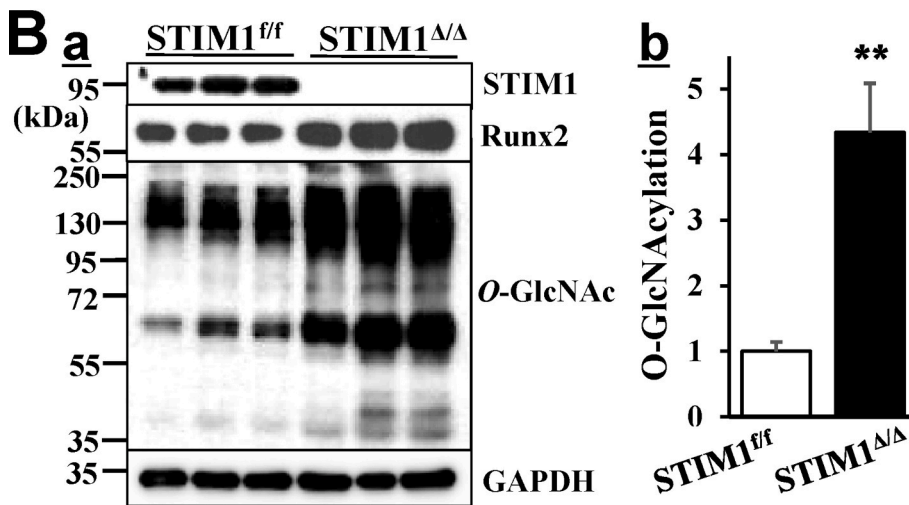


Fig. 5. STIM1 deletion promotes VSMC calcification via increased O-GlcNAcylation. **A)** Western blot analysis of protein O-GlcNAcylation in VSMC isolated from STIM1^{f/f} and STIM1^{Δ/Δ} mice. The expression of GAPDH was used as a loading control. (n = 3/group). **B)** STIM1^{f/f} and STIM1^{Δ/Δ} VSMC were cultured in osteogenic medium with or without inhibitor for O-GlcNAcylation, ST49 (10 μM) for 3 weeks. Calcification was determined by **a.** Alizarin red staining; or **b.** quantified by Arsenazo III assay in separate dishes (n = 3, ***p < 0.001). **C)** Western blot analysis of Runx2 expression and O-GlcNAcylation in protein lysates isolated from **B** with specific antibodies. Representative blots from 3 independent experiments are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

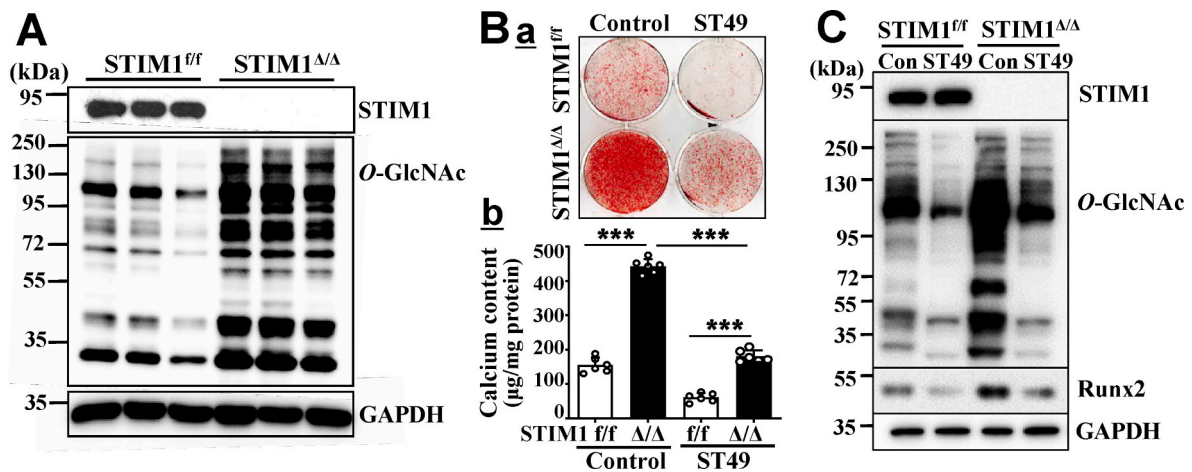


Fig. 5. STIM1 deletion promotes VSMC calcification via increased O-GlcNAcylation. **A)** Western blot analysis of protein O-GlcNAcylation in VSMC isolated from STIM1^{f/f} and STIM1^{Δ/Δ} mice. The expression of GAPDH was used as a loading control. (n = 3/group). **B)** STIM1^{f/f} and STIM1^{Δ/Δ} VSMC were cultured in osteogenic medium with or without inhibitor for O-GlcNAcylation, ST49 (10 μM) for 3 weeks. Calcification was determined by **a.** Alizarin red staining; or **b.** quantified by Arsenazo III assay in separate dishes (n = 3, ***p < 0.001). **C)** Western blot analysis of Runx2 expression and O-GlcNAcylation in protein lysates isolated from **B** with specific antibodies. Representative blots from 3 independent experiments are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

GlcNAcylation in VSMC was associated with inhibition of STIM1 deficiency-induced Runx2 in the STIM1^{Δ/Δ} VSMC. Collectively, these results support a critical role of increased O-GlcNAcylation in mediating STIM1 deficiency-induced Runx2 upregulation and calcification of VSMC.

3.6. STIM1 deficiency-induced calcium signaling and endoplasmic reticulum (ER) stress increase O-GlcNAcylation in VSMC

STIM1 is known to regulate intracellular calcium flux, therefore, we initially compared calcium flux in the STIM1^{f/f} and STIM1^{Δ/Δ} VSMC. Elevation of intracellular calcium was observed in VSMC within minutes after exposure to osteogenic medium, followed by slow decreases after 16 min. STIM1 deficiency in VSMC led to a significant increase in intracellular calcium flux in the STIM1^{Δ/Δ} VSMC. Such signal was sustained at a higher level during the experimental period (Fig. 6A). Consistently, increased phosphorylation and activation of calcium/calmodulin-dependent kinase II (CaMKII), a calcium signal mediator, was observed in the STIM1^{Δ/Δ} VSMC (Fig. 6B, p-CaMKII). As increased intracellular calcium flux has been linked to ER stress, we further evaluated ER stress, as highlighted by increased phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) determined in the STIM1^{Δ/Δ} VSMC. Accordingly, increased calcium flux, activation of calcium signaling and elevated ER stress in the STIM1^{Δ/Δ} VSMC may contribute to STIM1 deficiency-induced O-GlcNAcylation and VSMC calcification.

Using 4PBA, an ER stress inhibitor that inhibits VSMC calcification [37], we further determined whether increased ER stress might mediate STIM1 deficiency-induced O-GlcNAcylation. 4PBA inhibited p-eIF2α, but did not affect activation of pCaMKII in the STIM1^{Δ/Δ} VSMC (Fig. 6C), suggesting that activation of calcium signaling may precede STIM1 deficiency-induced ER stress. Importantly, inhibition of ER stress also decreased O-GlcNAcylation (Fig. 6C, O-GlcNAc), supporting that increased ER stress mediates STIM1 deficiency-induced O-GlcNAcylation in VSMC. Taken together, these results suggest that STIM1 deficiency in VSMC leads to increased intracellular calcium flux, activation of CaMKII, and ER stress, which mediates upregulation of O-GlcNAcylation that promotes VSMC calcification.

4. Discussion

Vascular calcification is prevalent in diabetes mellitus and is

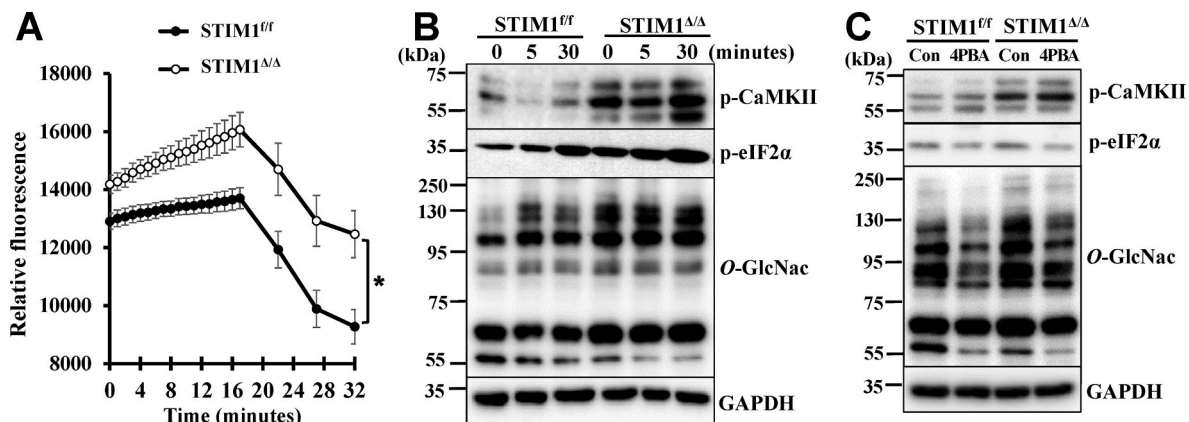


Fig. 6. STIM1 deficiency-induced calcium signaling and ER stress increase O-GlcNAcylation in VSMC. **A)** VSMC from STIM1^{f/f} and STIM1^{Δ/Δ} were exposed to osteogenic media and calcium flux was monitored by Fluo-4 for up to 32 min. Results from 8 independent experiments are shown. (**p* < 0.05). **B)** VSMC from STIM1^{f/f} and STIM1^{Δ/Δ} were serum starved and exposed to osteogenic media for 0, 5 and 30 min. Western blots were performed to determine phosphorylation of CaMKII (p-CaMKII, Ca²⁺/calmodulin-dependent protein kinase), phosphorylation of eIF2α (p-eIF2α, eukaryotic translation initiation factor 2A, an ER stress signal), and protein O-GlcNAcylation (O-GlcNAc). Representative blots from 3 independent experiments are shown. **C)** VSMC were serum starved and then exposed to osteogenic media with or without ER stress inhibitor, 4PBA (10 mM), for 24 h. Western blots were performed to determine p-CaMKII, p-eIF2α and O-GlcNAc using specific antibodies. Representative blots from 3 independent experiments are shown.

correlated with adverse cardiovascular outcome and mortality in diabetic subjects. Emerging studies have improved our understanding of the active cell-driven process of vascular calcification and highlighted the major contributions of the osteogenic differentiation of VSMC in the development of vascular calcification in atherosclerosis, diabetes and chronic renal failure. In diabetic vascular calcification, we and others have demonstrated the major role of increased oxidative stress and upregulation of protein O-GlcNAcylation in promoting VSMC calcification. The present studies further elucidated a novel function of STIM1, a calcium sensor and key regulator of the intracellular calcium signals, in the regulation of VSMC calcification via ER stress-mediated upregulation of O-GlcNAcylation (Fig. 7).

STIM1 mutations have been linked to human diseases, including immunodeficiency and autoimmunity and defects in platelets, fibroblasts, skeletal muscle [28,29]. However, the role of STIM1 in diabetic vascular calcification in human has not been reported. STIM1 expression was reduced in islets from human donor with type II diabetes and STZ-induced diabetic mice [39]. By generating SMC-specific STIM1 deletion mouse model, we have provided the first evidence linking a causative regulation of STIM1 in VSMC calcification *in vitro* and diabetic vascular calcification *in vivo*. Similar to our previous reports, minimal calcification was determined in the control VSMC cultured in the osteogenic media without additional stimuli *in vitro* or *ex vivo*, while STIM1 deficiency was sufficient to increase calcification of the STIM1^{Δ/Δ} VSMC cultured in osteogenic media. Furthermore, decreased SMC marker gene

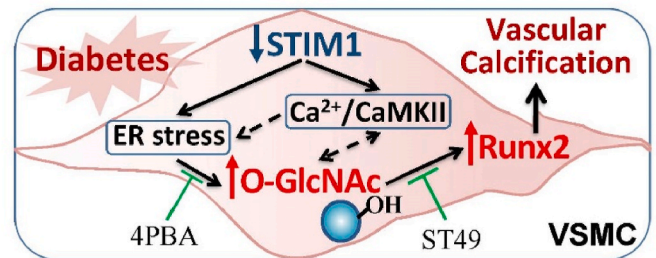


Fig. 7. STIM1 deficiency promotes VSMC calcification in diabetes via ER stress-induced O-GlcNAcylation. Diabetic conditions enhance STIM1 deficiency-induced intracellular calcium/CaMKII activation and ER stress in VSMC, leading to increased protein O-GlcNAcylation that upregulates Runx2 and promotes VSMC calcification.

and increased osteogenic transcription factor Runx2 and bone markers were determined in the STIM1^{Δ/Δ} VSMC, supporting the notion that STIM1 deficiency in VSMC led to dedifferentiation and osteogenic differentiation of VSMC. Consistently, SMC-specific STIM1 deletion markedly increased vascular calcification of the STIM1^{Δ/Δ} mice *in vivo* in the low dose STZ-induced diabetes model, which we have previously reported to induce vascular calcification [19]. Similar to our previous observations in diabetic mice [19], we found that increased vascular calcification in the diabetic STIM1^{Δ/Δ} mice was associated with increased aortic stiffness.

Of note, SMC-specific STIM1 deletion did not affect STZ-induced development of hyperglycemia *per se*, suggesting that intrinsic intracellular signaling in the STIM1^{Δ/Δ} VSMC promotes VSMC osteogenic differentiation and calcification in diabetic conditions. Hyperglycemia has been linked to increased oxidative stress, upregulation of O-GlcNAcylation and activation of calcium signaling [19,20,40], all of which promote VSMC calcification. The observation of an increase in vascular O-GlcNAcylation in the STIM1^{Δ/Δ} mice prompted us to further determine the role of O-GlcNAcylation in VSMC from the STIM1^{Δ/Δ} mice. Intriguingly, deletion of STIM1 in VSMC resulted in elevation of O-GlcNAcylation, a key inducing factor that promotes VSMC calcification *in vitro* and *in vivo* [19]. Importantly, inhibition of O-GlcNAcylation attenuated STIM1-deficiency induced VSMC calcification in the STIM1^{Δ/Δ} VSMC, supporting that STIM1 deficiency induces VSMC calcification via a mechanism mediated by increased O-GlcNAcylation.

Consistent with the known function of STIM1 in regulating intracellular calcium flux, we found that STIM1 deficiency led to increased calcium flux in the STIM1^{Δ/Δ} VSMC. The findings of a sustained elevation of calcium flux in the STIM1^{Δ/Δ} VSMC under basal condition that was further induced by the osteogenic media suggests that STIM1 deficiency may prime VSMC to a status that is prone to osteogenic differentiation. Consistent with this notion, STIM1 deficiency was sufficient to promote VSMC calcification in osteogenic media without additional stimuli.

Differences in the duration of intracellular calcium flux may lead to diverse downstream molecular signals that differentially regulate VSMC physiology and pathology. Unlike physiologic intracellular calcium influx that is associated with excitation-contraction of SMC, sustained increase in intracellular calcium may lead to VSMC pathology, such as calcification [41]. We found the prolonged increases in intracellular calcium in the STIM1^{Δ/Δ} VSMC were associated with the activation of the calcium signaling mediator CaMKII, a multifunctional serine/threonine protein kinase that is expressed abundantly in vascular tissue [42,43]. CaMKII activation has been linked to pathophysiological changes in Ca²⁺ handling and regulating gene expression in cardiomyocytes [44], however, the role of CaMKII signaling in regulating vascular calcification is not clear. Autophosphorylation of CaMKII on Threonine (Thr) 286/287 during sustained calcium transients is associated with a constitutively Ca²⁺/CaM-independent activity even at lower intracellular calcium concentration [45]. Accordingly, sustained increased intracellular calcium led to autophosphorylation and activation of CaMKII in the STIM1 deficiency VSMC. Given that ER stress inhibitor did not affect activation of CaMKII in the control or STIM1 deletion VSMC in the present study, it is possible that increased phosphorylation of CaMKII may precede STIM1 deficiency-induced ER stress. Furthermore, hyperglycemia can promote direct O-GlcNAc modification of CaMKII in diabetes mellitus, which activates CaMKII autonomously [44]. In addition, hyperglycemia-induced O-GlcNAcylation and CaMKII activation increases cytosolic reactive oxygen species (ROS) in mouse myocytes [20]; while ROS can induce CaMKII activation via oxidation of methionine 281/282 in the regulatory domain of CaMKII, similar to it Thr287 autophosphorylation [46]. Therefore, elevated O-GlcNAcylation in the STIM1^{Δ/Δ} VSMC may further promote CaMKII activation. Our observation of increased CaMKII phosphorylation and activation in the STIM1^{Δ/Δ} VSMC has provided new insights into the activation of CaMKII in VSMC.

Our studies revealed that increased ER stress contributed to the upregulation of O-GlcNAcylation in the STIM1^{Δ/Δ} VSMC. STIM1-deficiency in VSMC increased phosphorylation of eIF2α, an ER stress signal transducer that regulates protein translation. In hearts from the SMC-specific STIM1 deletion mice with myocardial infarct, ER stress markers GRP78 and CHOP was reduced while ATF6 was increased [47]. Accordingly, STIM1-deficiency in different cells, VSMC or cardiomyocytes, may differently affect ER stress responsive signals, such as eIF2α for protein translation, CHOP for apoptosis, or ATF6 for protein degradation. In cardiomyocytes, increased intracellular calcium induces ER stress markers, including GRP78 and CHOP, which is associated with O-GlcNAcylation elevation [48]. Increased O-GlcNAcylation impairs calcium cycling and cardiomyocyte function, while inhibition of O-GlcNAcylation improves calcium handling and contractile function of the diabetic heart [49,50], supporting the role of O-GlcNAcylation in regulating calcium flux and cardiomyocyte function in the development of diabetic cardiomyopathy. Furthermore, O-GlcNAcylation of eIF2α was found to regulate eIF2α-mediated ER stress response in the HepG2 cells [51]. Together with our finding of the role of ER stress in mediating STIM1 deficiency-induced O-GlcNAcylation, these studies suggest a dynamic interplay of intracellular calcium, ER stress and O-GlcNAcylation, which contribute to STIM1-regulated VSMC pathophysiology.

As STIM1 mutations have been linked to human diseases but the function of STIM1 in vascular disease are not fully understood, our funding of a causative effect of STIM1 deficiency on VSMC calcification has shed lights on the novel function of STIM1 in the pathogenesis of vascular disease. Of note, reduction of STIM1 expression has been demonstrated in islets from human donor with type II diabetes [39]. In addition, decreased STIM1 expression has been determined in aged arteries, such as rat mesenteric and mouse cerebral arteries [52,53], which may contribute to increased vascular calcification and stiffness with aging. Further investigations are warrant to uncover the regulation and function of STIM1 in different vascular beds in aging and age-related vascular diseases. In addition, the novel mechanisms uncovered in our studies have highlighted STIM1 deficiency-increased upregulation of O-GlcNAcylation in promoting Runx2 upregulation and VSMC calcification. O-GlcNAcylation is emerging recognized as an “integrated sensor” for many diseases, including cardiovascular disease [54], uncovering the STIM1-dependent and independent O-GlcNAcylation signaling network in VSMC may provide important molecular insights into the development of new strategies or targets for detection, prevention and therapy of vascular diseases.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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