High glucose increases the expression of Cbfa1 and BMP-2 and enhances the calcification of vascular smooth muscle cells

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Abstract

Background. Vascular calcification is common in diabetes but the pathogenesis is poorly understood.

Methods. To investigate the pathogenesis, we first examined the histology of inferior epigastric arteries from diabetic and non-diabetic patients undergoing a renal transplant. To examine the role of hyperglycemia, bovine vascular smooth muscle cells (BVSMCs) were incubated with normal (5 mM) or high glucose (25 mM) for 48 or 72 h.

Results. The results demonstrated that diabetic patients, compared with non-diabetic patients, had significantly greater calcification and increased expression of the bone matrix proteins osteopontin, type I collagen, bone sialoprotein and alkaline phosphatase (ALP). The in vitro studies demonstrated that high glucose increased the expression of the osteoblast transcription factor core binding factor alpha subunit 1 (Cbfa1) and its downstream protein osteocalcin by 1.9-fold and 1.8-fold, respectively, and ALP activity by 1.5-fold. These findings were blunted in the presence of an inhibitor to protein kinase C. High glucose also significantly enhanced calcification in BVSMC in a time-dependent manner (2.20 ± 0.50 vs 1.35 ± 0.55 μmol/mg, day 7; 5.04 ± 1.35 vs 3.12 ± 0.92 μmol/mg, day 14; P < 0.05). High glucose also induced the secretion of bone morphogenetic protein-2, a known osteoinductive factor, and further increased the secretion normally seen during calcification by 43% at day 7 and 57% at day 14.

Conclusions. These results demonstrate that vascular calcification in patients with diabetes is a cell-mediated process characterized by a phenotypic change of VSMCs to osteoblast-like cells with increased bone matrix protein expression, and that hyperglycaemia may directly induce these changes.

Keywords: bone matrix proteins; bone morphogenetic protein-2 (BMP-2); core binding factor alpha-I (Cbfa1); high glucose; vascular smooth muscle cells

Introduction

Diabetes is associated with increased cardiovascular mortality compared with the general population [1]. The aetiology of the increased mortality is multifactorial, but recent data now supports that vascular calcification may also be contributory. Vascular calcification is more common in patients with diabetes compared with the general population and is associated with increased mortality, stroke and amputations [2,3]. In patients with kidney disease, diabetes is a prominent risk factor for vascular calcification [4,5]. In addition, glycaemic control appears to be a risk factor in some studies [6].

Pathologically, there are two patterns of vascular calcification. Typical atherogenesis is found in large vessel disease and coronary arteries, and is associated with lipid laden macrophages and intimal hyperplasia. In contrast, medial calcification (also called Mönckeberg’s calcification or medial calcinosi), occurs independently of intimal calcification and atherosclerosis. Medial calcification occurs initially in the medial layer and is not associated with lipid laden macrophages or intimal hyperplasia. As Mönckeberg’s calcification progresses, it forms a dense circumferential sheet of calcium crystals in the centre of the media, bounded on both sides by vascular smooth muscle cells (VSMCs) and can contain bone trabeculae and osteocytes. Mönckeberg’s calcification is most commonly described in distal vessels of patients with diabetes, advanced aging and renal failure. Thus, the elevated glucose concentrations observed in patients with diabetes may directly affect the calcification process, as patients with diabetes are at an increased risk of both atherogenic and medial calcification.

Recent studies have demonstrated that vascular calcification is an active and tightly regulated process that resembles mineralization in bone, with the
production of ‘bone’ proteins by VSMCs. We have demonstrated a similar ‘active’ process in vascular calcification in patients with end stage renal disease (ESRD) [7], finding that there was increased calcification and expression of bone proteins in inferior epigastric arteries. Our data also demonstrates that the osteoblast differentiation factor, core binding factor alpha subunit 1 (Cbfa1) is present in association with calcification in both the intimal and medial layers of arteries from dialysis patients. This localization correlated with the expression of the downstream products of Cbfa1, the ‘bone’ matrix proteins osteopontin and type I collagen [8]. Engelse et al. [9] also demonstrated the expression of Cbfa1 in human atherosclerotic plaques from non-dialysis patients. The in vitro studies demonstrated that uraemic serum [8] induces Cbfa1 expression and enhanced calcification in bovine VSMCs (BVSMCs). Thus, up-regulation of Cbfa1 in VSMCs may lead to differentiation to an osteoblast-like phenotype. These cells can then produce ‘bone’ matrix proteins providing necessary matrix for subsequent calcification. The purpose of this study is to determine how hyperglycaemia may directly lead to increased vascular calcification in patients with diabetes.

**Materials and methods**

**Ex vivo studies**

**Histological analyses.** The inferior epigastric artery was obtained from diabetic and non-diabetic patients undergoing renal transplantation as previously described in detail [4]. The study was approved by the local Institutional Review Board and all patients gave written informed consent. Medical records were reviewed for demographic information, and the presence of diabetes. The inferior epigastric artery was dissected free of fat and subcutaneous tissues. Sections were incubated for 10 min in hydrogen peroxide, rinsed, and placed in sodium carbonate-formaldehyde, followed by Farmer’s diminisher (gifts of Larry Fisher, PhD; National Institutes of Health; LF-123, 1:100 dilution LF-67, 1:50; LF-100, 1:100), or alkaline phosphatase (1:500, Chemicon, Temecula, CA, USA). The sections were developed with DAB and counterstained with haematoxylin. Negative controls were obtained by substituting the primary antibody with 1× blocking solution. Images were recorded on a Digital camera (Nikon Coolpix 950) using a Nikon Eclipse E400 microscope.

**In vitro studies**

**Cell cultures.** BVSMCs were obtained by a modification of the explant method. Small pieces of bovine ascending aorta (1 mm) were placed in a six-well culture dish and cultured for two weeks in DMEM with 10% fetal bovine serum (FBS) in a 95%/5% air/CO2 humidified environment at 37°C. Only cells between passages two to eight were used for experiments. BVSMCs were incubated for various times with DMEM plus 10% FBS in the presence of normal (5 mM) or high glucose (25 mM). In some experiments, 25 mM d-mannitol was used as an osmolarity control. To determine the cell-signalling pathway involved, BVSMCs were incubated with or without a protein kinase (CPKC) inhibitor, GF109203X (10 μM, Calbiochem), or the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor, LY294002 (10 μM, Sigma) for the indicated time. To induce calcification, BVSMCs were treated with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid in the presence or absence of high glucose for 7 and 14 days, as previously described [10]. To determine the role of bone morphogenetic protein-2 (BMP-2) in calcification of BVSMCs, cells were treated with 10 mmol/l β-glycerophosphate, 10−7 mol/l insulin and 50 μg/ml ascorbic acid in the presence or absence of 500 ng/ml recombinant human BMP-2 (R&D Systems).

**Alkaline phosphatase (ALP) assay.** Cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged. Supernatants were assayed for ALP activity (Pointe Scientific, Inc.). One unit was defined as the activity producing 1 nmol of P-nitropheno1 for 30 min. Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad) and ALP activity normalized to cellular protein content.

**Western blot analysis.** Western blotting was performed as previously described [10]. The blots were incubated with rabbit antibodies against Cbfa1 (1:1500, a gift from Dr Andrew Gieser, Eli Lilly and Company) or osteocalcin (LF-32, 1:1000; gift of Dr Larry Fisher, NIH) overnight at 4°C followed by incubating with peroxidase-conjugated secondary antibody (1:5000 dilution). Immunodetection was with the Enhanced Chemiluminescence Kit (Amersham). The band intensity was analysed by scanning densitometry (Quantity One, Bio-Rad).

**Calcium deposition.** BVSMCs were incubated in DMEM with 10% FBS until confluent (= day 0 of calcification assay). At each time point thereafter, BVSMCs were decalcified with 0.6N HCl for 24 h. The calcium content of HCl supernatants was determined colorimetrically by the o-cresolphthalein complex one method (Calcium kit; Pointe Scientific) as previously described [10] and normalized to protein content.
ELISA. Before (pre) and after (post) cell culture incubation, conditioned media from BVSMCs cultures were collected and centrifuged, and 50 μl of conditioned media was used to measure BMP-2 concentrations using Quantikine BMP-2 Immunoassay kit (R & D Systems).

**Statistical analysis**

Semi-quantitative analysis was done for all tissue sections by a single reader, who was blinded to patient characteristics. Scores for two to six sections per stain per subject were determined and averaged. The calcification (MacNeal and Alizarin red stain) and immunohistochemistry was read in a similar blinded, semi-quantitative manner with grading of 0 to 4+. The difference of histological calcification score, the expression of osteopontin, type I collagen, bone sialoprotein and alkaline phosphatase between diabetic and non-diabetic patients were compared by analysis of variance (ANOVA) with Fisher’s *post-hoc* analysis. Demographic and laboratory data were compared by *t*-test, chi-square/Mann-Whitney rank sum test. ALP activity, Cbfa1 and osteocalcin expression and BMP-2 concentrations in response to various treatments were also compared by ANOVA with Fisher’s *post-hoc* analysis. Differences in calcification over time and between groups were analysed with two-way ANOVA with Fisher’s *post-hoc* analysis. The results are expressed as mean ± SD, with *P* < 0.05 considered significant (StatView, SAS Institute, Inc., Cary, NC, USA).

**Results**

**Increased expression of bone matrix proteins and calcification in arteries from patients with diabetes**

We have previously evaluated the histology of the inferior epigastric artery from dialysis patients undergoing renal transplant and demonstrated that the deposition of bone matrix proteins is associated with vascular calcification in ESRD patients [4]. To directly determine the impact of diabetes on vascular calcification, we re-analysed these specimens to directly compare the histology of sections of the inferior epigastric artery from diabetic (*n* = 11; DM) and non-diabetic (*n* = 27; NDM) dialysis patients undergoing renal transplant. There was no significant difference in the age (DM = 48.3 ± 10.6 vs NDM = 45.2 ± 13.3), dialysis vintage (DM = 21 ± 7.5, median 20 months, vs NDM = 28 ± 23, median 23.5 months), percentage female (DM = 37 vs NDM = 27%), percentage Caucasian (DM = 81 vs NDM = 89%), Calcium binder intake (DM = 2.3 ± 2.1 vs NDM = 2.1 ± 2.1 g/day), serum calcium level (DM = 2.37 ± 0.20 vs NDM = 2.40 ± 0.22 mmol/l), serum phosphorus level (DM = 1.78 ± 0.52 vs NDM = 1.74 ± 0.48 mmol/l) or intact PTH (DM = 21.9 ± 23 vs NDM = 62.8 ± 110.8 pmol/l). The vessels were assessed for the presence of calcification by staining with MacNeal’s stain and Alizarin red stain, and histological calcification content determined by semiquantitative analysis as described in the ‘Materials and methods’ section. As shown in Figure 1, compared with non-diabetic patients, diabetic patients had significantly greater calcification scores by MacNeal’s stain (2.1 ± 0.6 vs 0.5 ± 0.2; *P* < 0.004) or Alizarin red stain (2.3 ± 0.5 vs 0.6 ± 0.2; *P* < 0.004). All calcification was in the medial layer, with the exception of one artery from a diabetic patient where there was calcification in both the intimal and medial layers. The expression of bone matrix proteins was also significantly increased in arteries from diabetic patients (DM) compared with that from non-diabetic patients (NDM, Figure 1). The immunostaining scores were 2.8 ± 0.4 (DM) vs 0.8 ± 0.2 (NDM) for osteopontin, *P* < 0.0001; 2.6 ± 0.5 (DM) vs 1.2 ± 0.3 (NDM) for type I collagen, *P* < 0.017; 1.9 ± 0.4 (DM) vs 1.0 ± 0.2 (NDM) for bone sialoprotein, *P* = 0.054; 2.4 ± 0.3 (DM) vs 1.0 ± 0.2 (NDM) for alkaline phosphatase, *P* < 0.001. These results indicate that there is increased expression of bone matrix proteins and enhanced vascular calcification in diabetes.

![Image](hyperglycaemia_and_vascular_calcification.png)

**Fig. 1.** Semi-quantitative analysis of calcification and the expression of bone matrix proteins in inferior epigastric arteries from diabetic and non-diabetic patients. Sections from arteries of diabetic patients with ESRD (DM, *n* = 11, solid bars) and non-diabetic patients with ESRD (NDM, *n* = 27, open bars) were examined for calcification by MacNeal’s and Alizarin staining, and the expression of bone matrix proteins by immunohistochemistry. Sections were scored semiquantitatively from 0 to 4 as described in ‘Materials and methods’ section, and the mean staining score for calcification and bone matrix proteins in the arteries shown (mean ± SD). The results demonstrated that in patients with diabetes there is enhanced vascular calcification and increased bone matrix protein expression. *P* < 0.05, DM vs NDM. OPN, osteopontin; Collagen I, type I collagen; BSP, bone sialoprotein; ALP, alkaline phosphatase.

**High glucose increases the expression of Cbfa1 and bone matrix proteins and enhances the calcification of BVSMCs**

To determine the role of hyperglycaemia in the expression of Cbfa1 and bone matrix protein expression and calcification in BVSMCs *in vitro*, we first examined the expression of Cbfa1 and the bone matrix protein, osteocalcin in BVSMCs treated with normal (5 mM) or high glucose (25 mM) for 48 or 72 h by western blot. The results demonstrated that high
glucose significantly increased the expression of Cbfa1 (Figure 2A, *P < 0.05) and osteocalcin (Figure 2B, *P < 0.05) in BVSMCs. Similarly, high glucose also significantly increased alkaline phosphatase activity in BVSMCs by 72 h (Figure 2C, 135 ± 14 vs 93 ± 13 U/g protein, *P < 0.05). To confirm that these results were not due to the high osmolarity, additional experiments were performed in BVSMCs to compare the effect of treatment with 25 mM D-mannitol, providing an equivalent osmolarity as 25 mM glucose.

This osmolarity control did not significantly change the alkaline phosphatase activity (112 ± 11 vs 109 ± 7 U/g protein), Cbfa1 expression (23 ± 6 vs 22 ± 3 Odu) or osteocalcin expression (76 ± 9 vs 73 ± 8 Odu) in BVSMCs (data not shown), suggesting that the observed enhanced effect on bone matrix protein is specific for glucose.

To further determine the role of hyperglycaemia in calcification of BVSMCs, we examined the mineralization of BVSMCs treated with normal or high glucose for 7 and 14 days by measuring the total calcium deposition by HCl extraction. As shown in Figure 3, compared with normal glucose, high glucose significantly increased calcification in BVSMCs in a time-dependent manner (Figure 3, 2.20 ± 0.50 vs 1.35 ± 0.55 μmol/mg, *P < 0.05, day 7; 5.04 ± 1.35 vs 3.12 ± 0.92 μmol/mg, *P < 0.05, day 14). These results indicate that hyperglycaemia plays an important role in vascular calcification, perhaps via inducing expression of the osteoblast transcription factor Cbfa1 and bone matrix protein expression with subsequent calcification.

Protein kinase C (PKC) is involved in high glucose-induced expression of Cbfa1 and bone matrix proteins in BVSMCs

To determine the signalling mechanism involved in high glucose-induced response in BVSMCs, cells were treated with normal (5 mM) or high glucose (25 mM) in the presence or absence of the PKC inhibitor, GF109203X, for 48 or 72 h. Western blot was performed to determine the expression of Cbfa1 and osteocalcin in BVSMCs, and alkaline phosphatase activity analysed in cell lysate. As shown in Figure 4A, a high concentration of glucose increased Cbfa1 expression in BVSMCs. Treatment with the PKC inhibitor, GF109203X, significantly decreased high glucose-induced Cbfa1 expression in BVSMCs. However, inhibition of PKC had no effect on Cbfa1
expression in BVSMCs treated with normal concentration of glucose (Figure 4A). Similarly, inhibition of PKC significantly decreased high glucose-induced osteocalcin expression but had no effect in VSMC treated with normal glucose (Figure 4B). Furthermore, the increased alkaline phosphatase activity in response to high glucose was prevented in BVSMCs treated with PKC inhibitor (Figure 4C). These data suggest that high glucose enhances the expression of Cbfα1 and osteocalcin and increases alkaline phosphatase activity, at least partially through the activation of protein kinase C in cultured BVSMCs.

Bone morphogenetic protein-2 (BMP-2) is involved in high glucose-induced calcification in BVSMCs

BMP-2 is a potent osteoinductive factor in multipotent mesenchymal cells [11] and BMP-2 is known to upregulate Cbfα1 expression in osteoblasts [12]. We have also demonstrated that BMP-2 significantly increased calcification in BVSMCs (Chen et al., Kidney International, in press). To determine the potential role of BMP-2 in high glucose-induced calcification in BVSMCs, we first examined the effect of high glucose on BMP-2 secretion from BVSMCs by incubating cells with normal or high glucose for 48, 72 and 96 h. Conditioned media were collected and BMP-2 concentration measured by ELISA. The pre-incubation levels of BMP-2 were subtracted from post-incubation levels. Data are shown as mean ± SD from three experiments. *P < 0.05, high glucose vs normal glucose, same time; **P < 0.05, different from 48 h, same condition; †P < 0.05, different from 72 h, same condition.

To determine if PI3-kinase signalling pathway is also involved in high glucose-induced Cbfα1 and bone matrix protein expression in BVSMCs, cells were treated with normal (5 mM) or high glucose (25 mM) in the presence or absence of PI3-kinase inhibitor, LY294002, and the expression of Cbfα1 and bone matrix proteins examined. The results demonstrated that blocking PI3-kinase had no effect on high glucose-induced Cbfα1 and osteocalcin expression and alkaline phosphatase activity in BVSMCs (data not shown).
ELISA on day 7 and 14. The results demonstrated that BMP-2 secretion is progressively increased during calcification in BVSMCs and high glucose further enhanced its secretion by 43% at day 7 and 57% by day 14 (Figure 6, 118.0 ± 10.2 vs 82.5 ± 7.5 pg/ml, P < 0.05, day 7; 195.3 ± 20.5 vs 125.5 ± 11.5 pg/ml, P < 0.05, day 14). These results indicate an important role of BMP-2 in the regulation of calcification of BVSMCs.

Discussion

In the present study, we have demonstrated that diabetic patients with ESRD have a greater degree of medial calcification in the inferior epigastric artery compared with that in non-diabetic patients with ESRD. In addition, the increased calcification in diabetic patients is associated with increased expression of bone matrix proteins in the arteries. Compared with non-diabetic patients, the expression of osteopontin, type I collagen and alkaline phosphatase were all significantly increased in arteries from diabetic patients. Thus, our findings suggest that vascular calcification in diabetes is associated with the expression of bone matrix proteins. In our in vitro studies, we have demonstrated that high glucose increased the expression of the osteoblast transcription factor Cbfa1 and osteocalcin and enhanced calcification in BVSMCs. Furthermore, PKC signalling pathway was involved in high glucose-induced expression of Cbfa1 and bone matrix proteins. We have also demonstrated that BMP-2 secretion from BVSMCs is increased during calcification and high glucose further increased its secretion. These results suggest that the increased vascular calcification in diabetes is at least partially due to the direct effects of hyperglycaemia on VSMC via multiple mechanisms.

Vascular calcification is very common in diabetes, kidney disease and aging [2]. There are several studies that associate medial arterial calcification with morbidity and mortality in diabetes. Lehto et al. [3] in a study of 1059 patients with type II diabetes demonstrated that medial arterial calcification of peripheral arteries was a strong independent predictor of total cardiovascular mortality, and also a significant predictor of future coronary heart disease events, stroke and amputation. These findings have been confirmed in another cohort of patients with diabetes mellitus, where the age-adjusted odds ratio for cardiovascular mortality was 4.2 (95% CI: 1.5–11.3) for medial calcification and 1.6 (0.3–4.3) for intimal calcification using plain thigh radiographs [13]. This relationship was observed regardless of glycaemic control and known duration of diabetes. In contrast, Everhart showed that risk factors for arterial calcification were impaired vibration, duration of diabetes and high plasma glucose [14]. Shanahan et al. [15] found that in distal peripheral arteries with calcification from patients with diabetes, VSMCs expressed a number of osteocytic/chondrocytic markers. Patients with diabetes have increased coronary artery calcification compared with non-diabetic controls, and the presence of even early nephropathy accelerates this [5]. In the present study, we have demonstrated that expression of osteopontin, bone sialoprotein, alkaline phosphatase and type I collagen and calcification in arteries was increased in diabetic compared with non-diabetic patients. These data strongly indicate that diabetes itself contributes to the pathogenesis of vascular calcification, at least partially through a direct action of glucose on VSMCs.

The primary cell responsible for bone mineralization is the osteoblast, which differentiates from a pluripotent mesenchymal stem cell. These same stem cells can differentiate to VSMCs. Primary VSMCs from explants of medial tissue of both normal and diseased arteries transform into phenotypically distinct cells capable of calcification in vitro [16]. These VSMCs facilitate mineralization in vitro by forming ‘nodules’ similar to those produced by osteoblasts, producing bone-associated proteins and forming matrix vesicles. A variety of stimuli have been shown to induce or modulate phenotypic transformation of VSMCs to osteoblast-like cells with subsequent mineralization in vitro, including phosphorus, oxidized LDL, calcitriol, PTH, PTH-related peptide [7], and in the present study, high glucose. These mesenchymal stem cells are known to become osteoblasts with up-regulation of Cbfa1. Cbfa1 knock-out mice fail to form mineralized bone, proving that Cbfa1 is ‘the switch’ that turns a pluripotent stem cell into an osteoblast [17]. We have previously demonstrated that Cbfa1 expression was present in calcified vessels from dialysis patients and serum from dialysis patients up-regulates Cbfa1 expression and increased calcification in BVSMC [8,10]. Cbfa1 expression in human
calcified vessels was also demonstrated in non-CKD patients [9].

The role of hyperglycaemia in this transformation of VSMCs to osteoblast-like cells is demonstrated in the current study. High-glucose concentrations increased the expression of Cbfa1 and osteocalcin and alkaline phosphatase activity in BVSMCs. High glucose also significantly enhanced calcification of BVSMCs. In the in vitro studies, high glucose also induced cell proliferation and expression of osteopontin in cultured VSMCs [18] and in medial layers of the carotid arteries of streptozotocin-induced diabetic rats [19]. Although the changes observed in the short-term (<72h) experiments do not equate to calcification, they are reflective of a phenotypic transformation. We and others believe this is a critical first step in the pathogenesis of vascular calcification as these osteoblast-like cells can then regulate the production of the matrix and subsequent calcification [7,20]. These data strongly suggest that hyperglycaemia may contribute to the pathogenesis of vascular calcification in diabetes.

Recent work has shown that exposure of VSMC to high glucose activates several signal transduction networks responsible for mediating the proliferative and growth-promoting response. PKC has been implicated in a wide variety of cellular responses, including growth, differentiation, gene expression, angiogenesis, contractility and vesicle trafficking [21]. Increase in PKC activity has been demonstrated in several tissues from diabetic animals [22]. In aortic and vascular cells, several PKC isoforms have been shown to be activated in response to hyperglycaemia [22]. Our data also demonstrated that PKC signalling pathway is involved in high glucose-induced expression of Cbfa1, osteocalcin and alkaline phosphatase activity in BVSMCs. Although PI-3 kinase has also been implicated in high glucose signal transduction [21], we did not find an effect of PI-3 kinase in the present study.

Another finding of our current study is that hyperglycaemia augments the secretion of BMP-2 in BVSMCs. BMP-2 is a potent osteo-inductive factor in mesenchymal stem cells and marrow stromal cells [11], and the expression of BMP-2 has been detected in human calcified arteries [23]. Bostrom et al.[16] have demonstrated that BMP-2 increased calcification and osteogenic differentiation in calcifying VSMCs, and we have also confirmed that exogenous BMP-2 significantly increases calcification in BVSMCs (Chen et al., Kidney International, in press). BMP-2 is known to up-regulate Cbfa1 in both osteoblasts and VSMCs [12]. BMP-2 also activates another transcription factor, Msx2, which in turn, up-regulates osterix (Osx), a global transcriptional regulator of mineralization and osteoblast differentiation via a Cbfa1-independent signalling mechanism [24]. Msx2 expression has been observed in human atherosclerotic calcification [25]. The synergistic relationship of Cbfa1 and BMP-2 is not surprising, as multiple studies have demonstrated the two osteo-inductive factors work in concert. Cells derived from calvariae of Cbfa1 −/− mice still synthesize osteocalcin in response to BMP-2, but at levels less than in cells from wild-type embryos [17]. Yang et al. [26] found that overexpression of BMP-2 or Cbfa1 alone in C3H10T1/2 mesenchymal cells modestly induced ALP activity, osteocalcin expression and mineralization, whereas overexpression of both Cbfa1 and BMP-2 in these cells significantly increased osteoblast markers and mineralization by 10-fold. It should be noted that while we focused on BMP-2 which we believe is one of the mediators of high glucose-induced calcification in BVSMCs, inflammatory cytokines may also be involved in vascular calcification in diabetes. A study by Wen et al. [27] demonstrated that hyperglycaemia increased multiple inflammatory cytokines [interleukin (IL)-12, tumour necrosis factor (TNF)-α] in diabetic mice. TNF-α can also promote in vitro calcification in VSMCs [28]. In addition, in response to TNF-α, endothelial cells produce BMP-2 [29] which in turn, can enhance calcification in VSMCs. Nevertheless, our finding that hyperglycaemia potentiates both BMP-2 and Cbfa1 expression in VSMCs may be why vascular calcification is so common in diabetes.

In conclusion, we have demonstrated that vascular calcification in patients with diabetes is associated with increased bone matrix protein expression in arteries. Our study also provides evidence of direct effects of hyperglycaemia on vascular calcification. High glucose concentration induced the expression of BMP-2, Cbfa1 and bone matrix proteins, at least partially mediated by activation of PKC pathway. However, the pathophysiology of vascular calcification in patients with diabetes is almost certainly multi factorial and not solely dependent on hyperglycaemia and PKC pathway. Therefore, additional studies are warranted to further define the pathogenesis of vascular calcification in diabetes.

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Conflict of interest statement. None declared.

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