

Phosphate and vascular calcification: Emerging role of the sodium-dependent phosphate co-transporter PiT-1

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Summary

Elevated serum phosphate is a risk factor for vascular calcification and cardiovascular events in kidney disease as well as in the general population. Elevated phosphate levels drive vascular calcification, in part, by regulating vascular smooth muscle cell (VSMC) gene expression, function, and fate. The type III sodium-dependent phosphate co-transporter,

PiT-1, is necessary for phosphate-induced VSMC osteochondrogenic phenotype change and calcification, and has recently been shown to have unexpected functions in cell proliferation and embryonic development.

Keywords

Phosphate, vascular calcification, PiT-1, kidney disease

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Introduction

Vascular calcification, the inappropriate deposition of calcium phosphate salts in valvular and vascular tissue, occurs at high prevalence in aging, diabetes, and renal disease. In blood vessels, intimal calcification is associated with atherosclerotic plaques and coronary artery occlusion, whereas arterial medial calcification (AMC) is a non-occlusive process that leads to stiffening of vessels (1).

There is a heavy burden of cardiovascular disease in the chronic kidney disease (CKD) population. The prevalence is twice that of the general population, and advances at twice the rate (2). In the US, the one-year mortality following an acute myocardial infarction is 46% in CKD patients vs 26% in those without CKD (3). Using electron beam CT imaging, Braun et al. documented a two- to five-fold increase in coronary artery calcification in dialysis patients, compared with age-matched non-CKD patients with known coronary artery disease (4). Both intimal and medial calcification occur in the elastic arteries of CKD patients to a greater extent than in age-matched controls (5).

AMC in particular is an important cause of decreased vascular compliance in aging, diabetes, and CKD (6). In AMC, hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$] is deposited in the arterial media (7). Paediatric patients present a unique opportunity to examine vascular calcification in CKD in the absence of atherosclerotic risk factors (smoking, hypertension, diabetes, dyslipidaemia). Vessels from paediatric dialysis patients show entirely medial calcification,

without evidence of inflammation or atherosclerosis (8). Increased vascular stiffness translates into increased pulse wave velocity and pulse pressures, both of which are highly associated with increased mortality in the dialysis population (9). Clinically, increased vascular stiffness leads to hypertension, left ventricular hypertrophy, heart failure and compromised coronary perfusion (10). At the arteriole level, AMC causes the ischaemic skin lesions characteristic of calcific uraemic arteriopathy, a condition observed in dialysis patients that carries an extremely high mortality rate (11).

Phosphate balance, vascular calcification and cardiovascular disease

Inorganic phosphorus, in the form of phosphate, is a critical mineral found in bone, phospholipids and nucleic acids; and is required for high-energy phosphate bonds, intracellular signalling and pH buffering. In humans, 85% of total body phosphorus is in bone, 14% is intracellular and only 1% is in extracellular fluid (12). The kidney is the major regulator of phosphate homeostasis, whereby 70% of phosphate reabsorption in the proximal convoluted tubule occurs via the sodium-dependent phosphate co-transporter (NaPi)-IIa (6). Two other phosphate transporters, NaPi-IIc and phosphate transporter (PiT)-2, also contribute to renal phosphate reabsorption (13).

The phosphatonins, or hormonal regulators of phosphate balance, include vitamin D3, fibroblast growth factor 23 (FGF-23) with its cofactor klotho, and parathyroid hormone (PTH) (6). Active vitamin D3 (1,25[OH]₂ D3 or calcitriol) is a steroid hormone primarily synthesised in the kidneys, and is decreased in CKD (14). NaPi-IIb of the duodenal enterocyte is a transcriptional target of the vitamin D receptor (15), but calcitriol deficiency in CKD does not result in decreased intestinal phosphate uptake due to passive phosphate transport through enterocyte paracellular pathways (6). FGF-23/klotho decrease phosphate reabsorption by the NaPi-IIa transporter, and inhibit vitamin D3 production via suppression of 1 α -hydroxylase (16). Uraemic mice with early CKD demonstrate elevated circulating FGF-23 and are able to maintain normal serum phosphate levels (17). It is suggested that klotho acts in a paracrine fashion, where the extracellular domain is shed from the distal convoluted tubule and secreted in the adjacent proximal convoluted tubule where FGF-23 is expressed. Secondary hyperparathyroidism in CKD is a consequence of decline in calcitriol production, hypocalcaemia and hyperphosphataemia. There is parathyroid hyperplasia and increased PTH synthesis, with resultant dysregulated bone remodelling (6). FGF-23 and PTH increase urinary phosphate excretion; however, these defense mechanisms are ineffective as renal function continues to fall in advanced CKD. Hence, hyperphosphataemia in CKD is a consequence of decreased phosphate excretion as well as disordered bone remodelling (renal osteodystrophies) (6).

Elevated serum phosphate has emerged as a non-traditional risk factor for cardiovascular events in CKD (18, 19), as well as in the general community (20). In a study of veterans with CKD, a statistically significant increase in mortality risk was noted with serum phosphate levels >3.5 mg/dl (21). The high risk of mortality is likely mediated, in part, by increased vascular calcification in CKD patients. This was demonstrated in prospective, randomised clinical trials where treatment with a dietary phosphate-binder attenuated vascular calcification (22, 23) and mortality in dialysis patients (23). However, other randomised trials have not confirmed mortality and quality-of-life benefits with lowering of serum phosphate in dialysis patients (24, 25), emphasising the complexity of mineral metabolism of which there is still much to learn. The role of phosphate in vascular calcification has come under intense investigation in the past decade. The vascular smooth muscle cell (VSMC) is now known to play a prominent role in phosphate-induced calcification, and recent data has implicated the involvement of elastin degradation.

Mechanisms of phosphate-induced vascular calcification

Regulation of VSMC fate: Osteochondrogenic phenotype change vs. apoptosis

When the clinical significance of hyperphosphataemia surfaced in the late 1990s, it spurred bench research that reinforced phosphate's role in AMC.

In vitro, no matrix calcification occurs in human VSMCs incubated with 1.4 mM phosphate (physiological level), but dose-dependent calcification occurs when phosphate levels are increased from 1.6 mM to 3 mM (26). Subsequently, Giachelli's group studied phosphate loading using a uraemic mouse whereby a two-step surgery involving partial electrocautery of the right kidney and total nephrectomy of the left kidney results in moderate to severe uraemia (17). Uraemic mice fed a high (0.9%) phosphate diet developed robust AMC without atherosclerosis or inflammation. Treatment with a dietary phosphate-binder (sevelamer) attenuated phosphate-driven vascular calcification in this model (unpublished findings), consistent with findings in uremic rats (27) and in CKD patients (28).

There are two major hypotheses regarding fate of VSMCs in phosphate-induced vascular calcification. The first invokes a profound transition to a bone-forming phenotype. *In vitro* studies have shown that elevated phosphate results in loss of smooth muscle markers (SM alpha actin, SM22alpha) and expression of osteochondrogenic markers (Runx2/Cbfa1, osterix, alkaline phosphatase, osteopontin) (26, 29). Runx2/Cbfa1 is a transcription factor that induces the expression of major components of the bone matrix, including type I collagen, osteocalcin, and osteopontin. In addition, ultrastructural analysis of the surface of VSMCs shows matrix vesicles containing apatite which act as nucleation sites for calcification (similar to the vesicles that bud from osteoblasts and hypertrophic chondrocytes), as well as calcifying collagen fibrils (30). Evidence for *in vivo* VSMC lineage reprogramming to the bone-forming phenotype has been found in calcified vascular lesions from animals (17, 31, 32) and humans (8). The complexity of VSMC phenotype change was recently underscored using whole-genome expression arrays of uremic rats fed on a high phosphate diet; the transition from "muscle-related" to "bone-related" gene expression involved dysregulation of 53 genes (33). Activation of extracellular signal-regulated kinases (Erks) has been implicated; mouse aortic medial cells treated with high phosphate demonstrate increase of phosphorylated Erk1/2 levels along with increase of Runx2/Cbfa1, prior to loss of VSMC lineage markers (32). The canonical Wnt pathway, known to be important in bone formation, is also likely to be involved in phosphate-induced vascular calcification. Secreted frizzled related proteins (SFRPs) inhibit the Wnt pathway, and SFRPs-1, 2 and 4 are upregulated in uraemic rats fed on a high phosphate diet presumably as a defensive response against further ossification (33).

The second hypothesis involves apoptosis-dependent matrix mineralisation. Reynolds et al. cultured human VSMCs in elevated phosphate and calcium conditions, and this led to calcification of matrix vesicles and apoptotic bodies (34). Arteries from paediatric dialysis patients show VSMC loss due to apoptosis with release of matrix vesicles from damaged/dead VSMCs (8). These findings were replicated in a recent study where vessel rings from paediatric subjects were cultured under various calcium/phosphate concentrations (35). Vessels from healthy controls were resistant to calcification whereas dialysis vessels developed dense medial calcification in association with apoptosis, cell loss and deposition of apatite-containing vesicles. Growth arrest-specific gene 6 (Gas6) is

believed to exert anti-apoptotic effects via the Bcl2-mediated PI3K-AKT pathway (36). Gas6 is markedly downregulated along with its receptor Axl in phosphate-induced VSMC calcification (37); phosphorylation inactivates Bcl2 and activates Bad, resulting in caspase-3 activation and apoptosis (36). Also, bone morphogenetic proteins have been shown to downregulate Bcl2 in pulmonary VSMCs (38).

These mechanisms are not mutually exclusive, as recently proposed by Shanahan's group (8, 35). It is likely that VSMCs undergo osteochondrogenic adaptive changes, moving from a "contractile" to a "secretory" phenotype and deposit matrix vesicles to protect the cell from the toxic effects of elevated calcium and phosphate. However, when this system becomes overwhelmed and as matrix calcification propagates, cell death ensues.

Regulation of inducers and inhibitors of hydroxyapatite formation

Phosphate may additionally promote vascular calcification by modulating levels of inducers and inhibitors of hydroxyapatite formation that are synthesised by VSMCs. For example, tissue non-specific alkaline phosphatase (TNAP) is an ectoenzyme present on cell surfaces and in matrix vesicles that is absolutely required for bone formation (7, 39). Inorganic pyrophosphate consists of two molecules of phosphate linked by an ester bond, and is a key inhibitor of hydroxyapatite formation (7). TNAP cleaves pyrophosphate, thus generating more phosphate, and is upregulated *in vitro* in phosphate-driven VSMC calcification (26, 29). Rat aortic rings incubated with elevated phosphate calcify only when subjected to mechanical damage, and express elevated TNAP and decreased pyrophosphate (40). Upregulation of TNAP occurs in dialysis patients (8, 41) and could be partly due to dialysis-induced VSMC injury (8). TNAP also dephosphorylates osteopontin, another inhibitor of medial calcification that is upregulated but inactive in uraemic conditions (17, 26).

Elastocalcinosis

Besides VSMCs, the other major component of the tunica media is the elastic lamellae. Elastin degradation increases its affinity for calcium (42), facilitating epitactic growth of hydroxyapatite along the elastic lamellae. Elastin degradation has been implicated in phosphate-induced AMC by several studies. Uraemic mice fed a high-phosphate showed calcified deposits initiating at sites of elastin degradation (17). Furthermore, Moreau et al. studied elastocalcinosis in cultured rat aortic rings in the presence of elevated phosphate and warfarin treatment (43). They found early expression of matrix metalloproteinase MMP-9, an elastin degrading enzyme, closely followed by transforming growth factor (TGF)- β signalling and osteochondrogenic phenotype transition of VSMCs. Similarly, treatment of rat VSMCs with alpha-elastin ac-

celerated phosphate-induced VSMC transformation (44). Simionescu et al. have shown that peptides derived from elastin degradation bind to elastin laminin receptors (ELRs) on the surface of adjacent VSMCs, and perpetuate proliferation and osteogenic differentiation via TGF- β signalling (45). TGF- β upregulates Runx2/Cbfa1, the major osteoblastic transcription factor (46, 47). Of note, MMP knock-out mice are resistant to elastin degradation and calcification (48). Finally, expression of matrix metalloproteinases MMP-2 and 9, two key elastase enzymes, are upregulated in arteries from diabetic CKD patients and correlate with vascular stiffness (49).

Role of PiT-1 in phosphate-induced vascular calcification

Phosphate transport into cells is primarily mediated by NaPi co-transporters, of which there are three types. The type I and II NaPi co-transporters in kidney and intestinal epithelium play important roles in whole body phosphate homeostasis; they are also present in other tissues including liver and brain (50, 51). The type III NaPi co-transporters, PiT-1 and PiT-2, are ubiquitously expressed. Real-time PCR detects similar RNA levels of PiT-1 and PiT-2 in rat VSMCs, and there is no expression of type I nor type II NaPi co-transporters (52). In contrast, PiT-1 is the predominant NaPi co-transporter in human VSMCs (53).

PiT-1 (also known as gibbon ape leukaemia virus receptor 1 [GlvR-1], leukaemia virus receptor 1 homolog, solute carrier family 20 member 1 [SLC20A1]) has been identified as a pivotal transporter in phosphate-induced VSMC calcification. Jono et al. reported that phosphate uptake in human VSMCs was dependent on a sodium gradient, and treatment with phosphonoformic acid (PFA, a competitive inhibitor of NaPi transport) inhibited phosphate uptake and VSMC osteochondrogenic differentiation (26). Real-time PCR and Northern blot analysis suggested that the NaPi co-transporter involved was PiT-1 (26). Further investigation was done using PiT-1 specific small interfering RNAs (siRNAs) (53). PiT-1 knockdown in human VSMCs suppressed phosphate-induced mineralisation, and subsequent overexpression of mouse PiT-1 rescued phosphate transport and VSMC osteochondrogenic changes. Neither phosphate loading of matrix vesicles nor apoptosis was mediated by PiT-1. Taken together, these findings demonstrate that PiT-1 is required for phosphate-induced calcification of human VSMCs *in vitro*. Of note, it was recently shown that PFA prevents calcification by direct inhibition of hydroxyapatite formation (inhibits nucleation in a similar way to pyrophosphate and bisphosphonates [54]) and is a poor inhibitor of type III NaPi co-transporters (55). Future mechanistic studies involving this class of transporters should therefore be cautious with the use of PFA.

Interestingly, PiT-1 is upregulated by inducers of calcification including BMP-2 (56, 57), calcium (58), and platelet-derived growth factor PDGF (59, 60). Suzuki et al. exposed MC3T3-E1 osteoblast-like cells to BMP-2 and found an increase in the V(max) of

Na-dependent phosphate transport (57). The aforementioned study by Jono et al. showed increasing phosphate uptake by human VSMCs as extracellular phosphate was raised from 0.5 mM to 3 mM (26). In contrast, Villa-Bellosta et al. found that PiT-1 and PiT-2 transporters in rat VSMCs are saturated under physiological phosphate concentrations (52). Indeed, this group has used membrane protein biotinylation to demonstrate that PDGF treatment of rat VSMCs increased PiT-1 abundance at the endoplasmic reticulum and not at the cell surface (59), discussed further in the next section. These disparate findings may be due to species variation. Overall, PiT-1 in vascular calcification likely involves more complex pathways beyond phosphate transport, as described in more detail below.

VSMC proliferation (62, 64). PiT-1 may also exert effects at the endoplasmic reticulum level. Rat aortic VSMCs treated with PDGF show increased PiT-1 expression in the endoplasmic reticulum, as evidenced by co-localisation with markers such as ERp46 and Derlin-1 (59). From these findings, Villa-Bellosta et al. hypothesise that PiT-1 may promote vascular calcification via modulation of anti-calcification proteins (such as MGP) or modification of kinases that phosphorylate secreted matrix proteins (such as osteopontin) (59).

► Figure 1 outlines the putative roles of PiT-1 and the VSMC changes that occur in phosphate-mediated vascular calcification.

PiT-1 signalling involves Erk activation

As noted previously, treatment of calcifying mouse VSMCs with high phosphate results in increased phosphorylation of Erk 1/2 in conjunction with VSMC osteochondrogenic transdifferentiation (32). Inhibition of Erk phosphorylation by the MEK inhibitor U0126 prevented upregulation of Runx2/Cbfa1 and promoted VSMC lineage markers (32). Other studies have implicated Erks in VSMC regulation and osteoblast differentiation (61–63). Erk 1/2 signalling is also activated by the calcification inducer BMP-2 (61) and by platelet-derived growth factor-BB-mediated modulation of

Other roles of PiT-1

The type III NaPi co-transporters were originally described as retroviral receptors with multiple membrane-spanning regions in the early 1990s (65, 66). It later emerged that they were important for phosphate transport in mammalian cells (67, 68). Besides mediating vascular calcification, PiT-1 and PiT-2 may be involved in important physiological pathways in bone, parathyroid glands, kidney, and intestine (69). Given that AMC mimics bone formation, it is not surprising that PiT-1 has been implicated in bone development and mineralisation. PiT-1 can be detected in 17-day-old murine embryos in hypertrophic chondrocytes that are initiating mineralisation (70). In osteoblast-like cells, PiT-1 ex-

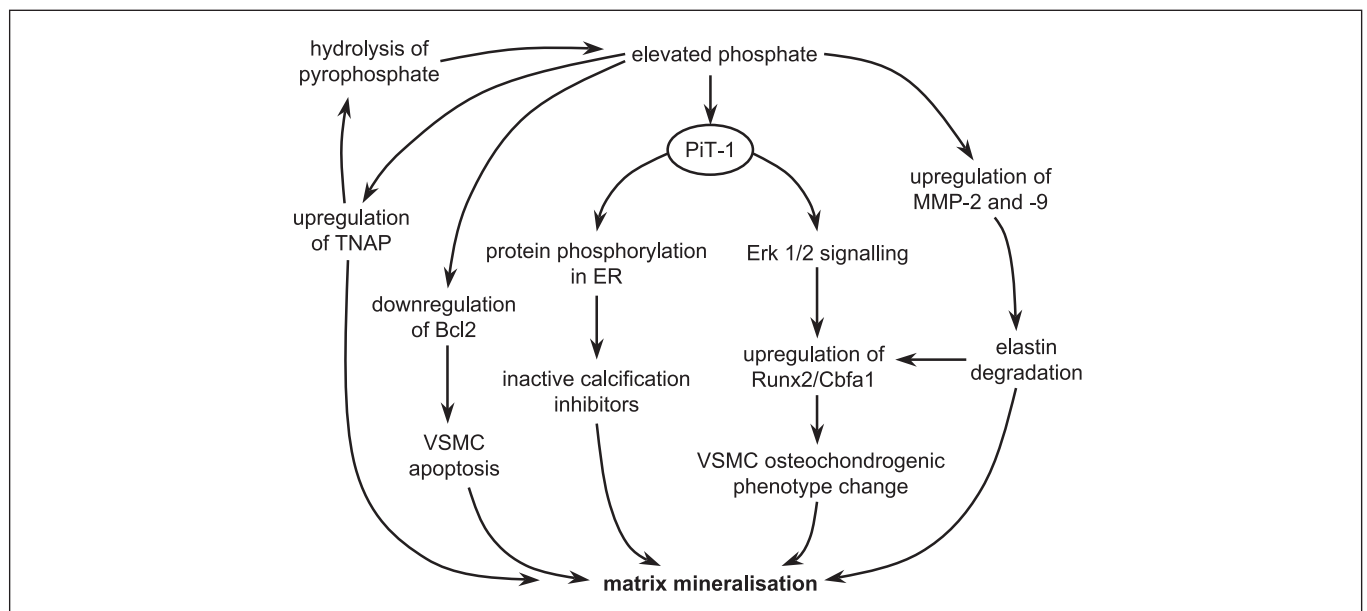


Figure 1: Proposed mechanisms of phosphate-induced vascular calcification include vascular smooth muscle cell (VSMC) phenotype change, VSMC apoptosis, elastin degradation, and dysregulation of pro- and anti-calcification mediators. TNAP cleaves pyrophosphate to generate two molecules of phosphate, perpetuating elevated phosphate conditions. PiT-1 is necessary to induce VSMC osteochondrogenic differentiation, and may additionally play a role in protein modulation at the endo-

plasmic reticulum that results in inactivation of anti-calcification mediators such as osteopontin and matrix Gla protein, though evidence for this is needed. Whether PiT-1 is required for phosphate-driven apoptosis and/or elastin degradation is not yet known. ER endoplasmic reticulum; MMPs matrix metalloproteinases; PiT-1 type III sodium-dependent phosphate co-transporter 1; TNAP tissue non-specific alkaline phosphatase.

pression increases during differentiation and correlates with mineralisation (57).

PiT-1 and cell proliferation

Many processes and structures in the cell require phosphate. These include the phospholipids in the cell membranes, DNA, ATP production, and phosphorylation of proteins. Recently, Beck et al. demonstrated that knockdown of PiT-1 in HeLa and HepG2 cells resulted in decreased cell proliferation (71). Interestingly, the regulation of cell proliferation via PiT-1 did not depend on phosphate transport, as a PiT-1 protein defective in Pi transport rescued the proliferation defect in these cells (71). Thus, PiT-1 may have important roles in addition to cellular phosphate homeostasis.

Knockout of NaPi transporters

Genetic knockouts of various phosphate transporters have been used to understand the physiological role of these transporters. The double knockout of mouse NaPi-IIa and NaPi-IIc is viable, but synergistically results in severe hypophosphataemia and rickets, while the single gene knockouts do not (72). Murine NaPi-IIb knockout results in lethality between embryonic day (E) 8.0 and 10.5 and display defects in growth, somitogenesis, angiogenesis, and failure of the chorionic trophoblasts to form the labyrinth layer in the placenta (73). Considering the role of PiT-1 in VSMC calcification *in vitro*, interest has developed in also studying the role of PiT-1 in an *in vivo* system. To that end, PiT-1 knockout mice have been recently created by two different groups. Surprisingly, like the NaPi-IIb mice, PiT-1 knockout was embryonic lethal, though at a different stage. PiT-1 null embryos arrested between E11.5 and 13.5 and displayed severe anaemia (74, 75). Anaemia might be due to increased apoptosis and reduced proliferation of the fetal liver (75), but a fundamental defect in haematopoiesis has not yet been ruled out. In addition, one group observed abnormal vascular development of the yolk sac (74), though this was not observed by the second group (75). Though the role of PiT-1 in bone development or calcification cannot be addressed in these knockout null embryos, the use of PiT-1 conditional alleles (74, 75) will be invaluable to create tissue-specific knockouts and the use of the hypomorphic allele (75) will allow for the analysis of subtle phenotypes where the expression level of PiT-1 is critical. No PiT-2 knockout mice have yet been reported.

Conclusions

Serum phosphate has emerged as a non-traditional risk factor for vascular disease and mortality in CKD. The VSMCs from dialysis vessels are particularly susceptible to calcification due to prolonged

exposure to the CKD milieu. Awareness of the importance of calcium-phosphate balance has led to renewed interest in comparing phosphate clearance of various dialysis modalities (76–78), and in patient education on dietary phosphorus content (79, 80). More data is needed regarding oral phosphate binders (see [81] for review). The biology of the arterial tunica media is greatly altered in elevated phosphate conditions; there is VSMC transition to bone phenotype and apoptosis, inactivation of local anti-calcification factors, and elastin degradation. The PiT-1 phosphate transporter appears to be a key mediator in phosphate-induced VSMC osteochondrogenic differentiation, and was recently discovered to be important in cell proliferation and embryonic development, indicating more functions for this protein than previously thought.

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