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Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID:	cjpp-2015-0083.R1
Manuscript Type:	Invited Review
Date Submitted by the Author:	15-Apr-2015
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Keyword:	vascular calcification, phosphate, vitamin D, chronic kidney diseases, FGF23



Vitamin D, Phosphate and Vasculotoxicity

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Running title: Phosphate and vascular calcification

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Abstract

Vascular calcification is a complex process that is formed by the ectopic deposition of calcium-phosphate hydroxyapatite. Medial and intimal vascular calcification is frequently present in patients with diabetes mellitus and chronic kidney disease (CKD) which markedly increases the morbidity and mortality of these patients. Increased serum calcium and phosphate levels, along with the use of active vitamin D metabolites, are commonly believed to be related to the evolvement of vascular wall mineralization in CKD patients. Because CKD patients have lower serum levels of vitamin D, they are routinely prescribed with vitamin D supplements which exert a dualistic role that is both healthful and harmful in these patients, perhaps protecting bone health, at the expense of promoting vascular pathology. This brief article explains how reducing phosphate burden in CKD patients could minimize vitamin D-associated vascular wall calcification.

Key words: Phosphate, Blood Vessels, Calcification

Vascular calcification

The underlying molecular mechanisms of vessel wall stiffening and reduced vascular compliance in vascular calcification are not yet clear (El-Abbadi and Giachelli 2007; Lanzer et al. 2014; Razzaque 2013). It is believed that an initial insult to the cellular components of the affected vessels can act as a triggering event, which is further promoted by the presence of calcification-inducing factors such as hypervitaminosis D, hyperphosphatemia, and hypercalcemia. Lanzer et al. in their recent review listed the diseases that are frequently associated with vascular calcification, including atherosclerosis, diabetes mellitus (type 2), CKD, ageing, arterial calcification due to deficiency of CD73 (ACDC), hyperparathyroidism, vitamin D toxicity, osteoporosis, Kawasaki disease, idiopathic basal ganglia calcification (IBGC), rheumatoid arthritis, generalized arterial calcification of infants (GACI), β -Thalassemia, and calciphylaxis (Lanzer et al. 2014). Experimental studies have suggested that reduction of calcification repressor factors such as ectonucleotide pyrophosphatase /phosphodiesterase 1 (NPP-1), ANK (transmembrane protein), and matrix gla protein (MGP) can also promote ectopic calcification (El-Abbadi and Giachelli 2007; Lanzer et al. 2014; Razzaque 2013).

Ectopic calcification is generally divided into two categories: dystrophic calcification, usually occurring in damaged tissues, and metastatic calcification, which is more related to the systemic dysregulation of calcium and phosphate metabolism. Whatever the underlying causes, vascular calcification is a high-risk factor for cardiovascular disease. Which mechanism might be inducing and promoting vascular calcification is not clear, but the increased presence of bone morphogenetic protein

(BMP2) in calcified human atherosclerotic lesions is suggestive of an ectopic skeletogenesis in the vascular wall. Furthermore, the loss of local and/or circulating calcification inhibitory factors like vitamin-K dependent matrix-Gla protein (MGP), pyrophosphate, fetuin A, and osteoprotegerin (OPG) could further contribute to the evolvement of calcification (Lanzer et al. 2014). Vascular calcification in patients with CKD is a good example of how the imbalance between inorganic phosphate (Pi) and pyrophosphate might propagate the calcification process (Razzaque 2013). In addition to the above mentioned calcification-promoting factors, vitamin D supplementation could also promote the calcification process, as evident in patients with CKD undergoing hemodialysis treatment. A few important inducers and suppressors of vascular wall calcification are listed in **Table 1**. As mentioned, vitamin D is believed to play an important role in initiating and promoting vascular calcification.

Vitamin D and vascular calcification

Vitamin D is essential for bone formation, growth and development. It also influences mineral ion homeostasis and affects the immune system (Baeke et al. 2010; Binderup et al. 1991; Daniel et al. 2006; Martineau et al. 2007; Veldman et al. 2000). The synthesis of vitamin initiates in the skin in humans, is processed further in the liver by the enzyme 25 hydroxylase (CYP27A1) to synthesize 25-hydroxyvitamin D [25(OH)D], and in the kidney by the enzyme 1α hydroxylase [1α (OH)ase; CYP27B1] to convert it into active vitamin D metabolite [1,25(OH)₂D₃]. In addition to these activation steps, an inactivation process is mediated by the enzyme 24-hydroxylase (CYP24) that generates 24,25(OH)₂D in the liver or kidney, contributing to normal vitamin D maintenance levels

in the body. The biologically active form, $1,25(\text{OH})_2\text{D}_3$ mostly exerts its functions by interacting with the high-affinity vitamin D receptor (VDR), which is a ligand-dependent transcription factor (Dusso et al. 2005). In addition to interacting with VDR, some studies have suggested that $1,25(\text{OH})_2\text{D}_3$ can also exert functions through cell membrane receptors or its caveolae components (Dusso et al. 2005). Moreover, the presence of VDR is also noted beyond the organs maintaining homeostatic balance of calcium (intestine, bone, kidney, and parathyroid gland). Studies have found an association between hypovitaminosis D and various aging related conditions such as osteoporosis, cancer, diabetes, autoimmune disorders, hypertension, atherosclerosis, muscle weakness, and neuronal disorders (multiple sclerosis, Alzheimer's disease, Parkinson's disease, and schizophrenia) (Annweiler et al. 2010). The National Health and Nutrition Examination Survey III showed that people with vitamin D levels in the lowest quartile had a mortality rate ratio of 1.26 (95% CI, 1.08-1.46) (Melamed et al. 2008).

The role of vascular smooth muscle cells (VSMCs) in medial calcification is rather complex, and the in vivo significance of reported molecular events is not universally agreed upon; it is thought that in the presence of a calcifying microenvironment, VSMCs transformed into osteogenic cells express some of the bone-related genes like Cbfa1/Runx2, Msx2, BMP2, alkaline phosphatase, and osteopontin (OPN). Osteogenic gene expression can be related to the nucleation of calcium phosphate crystals, as studies have found that osteogenic expression could be blocked with calcification inhibiting factors, including pyrophosphate and phosphonoformic acid, even in the presence of high concentrations of calcium or phosphate (Lanzer et al. 2014). Moreover, higher concentrations of extracellular calcium and phosphate could

induce apoptosis, necrosis, or autophagy of the VSMCs to promote calcification process (**Figure 1**). It is relevant to mention that vitamin D can also induce transdifferentiation of VSMCs into osteoblast-like cells, and thereby promote genesis of vascular calcification (Rebsamen et al. 2002; Tukaj 2008; Tukaj et al. 2000). Finally, inflammatory mediators, including TNF α could promote vascular calcification by facilitating the osteogenic programming of VSMCs (Izquierdo et al. 2012). However, further studies are needed to precisely differentiate the primary molecular events from secondary events of vascular calcification in order to design effective therapeutic strategies that prevent or delay its occurrence.

Calcium supplements along with vitamin D are reported to have beneficial effects on progression of osteoporosis, however such benefits are diminished by the potential risk of harmful cardiovascular events (Reid and Bolland 2008; Reid et al. 2011; Reid et al. 2008). Studies have reported that calcium supplements can increase the risk of myocardial infarction and stroke by 30% (Bolland et al. 2008; Bolland et al. 2011). Nevertheless, in healthy postmenopausal women, a daily intake of calcium (1000 mg) with vitamin D (400 IU) supplementation neither increased nor decreased the risk of coronary heart disease or cerebro-vascular disorders (Hsia et al. 2007). The beneficial effects of calcium and vitamin D supplementation to osteoporotic patients is not yet settled, and our experimental studies suggest that detrimental effects of calcium and vitamin D supplementation on cardiovascular calcification may be partly related to phosphate balance (Razzaque 2009, 2011a, 2011b, 2011c). In general, hypovitaminosis-D is indicated when 25(OH)D levels in serum fall below 50 nmol/l; levels between 50 to 25 nmol/l are considered as vitamin-D insufficiency; levels between 25 to 12.5 nmol/l indicate moderate vitamin-D deficiency; and levels below 12.5 nmol/l

indicate severe vitamin-D deficiency (Lips 2001). It is important to note that CKD patients have severe vitamin D deficiency that impairs their bone functions. Studies have shown survival benefits of adequate natural vitamin D levels in CKD patients (Pilz et al. 2011), but treatment often poses a problem, as vitamin D treatment has been linked to subsequent development of vascular calcification in these patients (Kalantar-Zadeh and Kovesdy 2009; Mizobuchi et al. 2009; Parfitt 1969). Of particular importance, we have recently found that reducing the phosphate burden could suppress vitamin D-associated vascular calcification (Ohnishi et al. 2009a) in experimental animal models.

The list of the factors that can influence vitamin D synthesis is growing, and studies have found that phosphate-regulating fibroblast growth factor 23 (FGF23) can suppress $1\alpha(\text{OH})\text{ase}$ activity, thereby suppressing $1,25(\text{OH})_2\text{D}_3$ synthesis (Horst et al. 1997; Tsujikawa et al. 2003).

Fibroblast growth factor 23

FGF23, a ~30 kDa protein, is a master regulator of phosphate metabolism. Genetically manipulated FGF23 gene knockout-mouse and transgenic-mouse models have convincingly shown the phosphaturic functions of FGF23 (Bai et al. 2004; DeLuca et al. 2008; Liu and Quarles 2007; Liu et al. 2006; Shimada et al. 2004b; Shimada et al. 2004c; Sitara et al. 2004), and partly explained the underlying mechanisms of human diseases that are associated with abnormal phosphate balance (Bergwitz and Juppner 2010; Berndt and Kumar 2007; Razzaque 2007; Razzaque and Lanske 2007), including autosomal dominant hypophosphatemic rickets (ADHR) (White et al. 2001), X-linked

hypophosphatemia (XLH) with vitamin D-resistant rickets/osteomalacia (Jap et al. 2011; Tenenhouse 1999), and autosomal recessive hypophosphatemic rickets/osteomalacia (ARHR) (Lorenz-Depiereux et al. 2006). Furthermore, when transgenic mice over-express the FGF23 gene, the mice develop hypophosphatemia and rickets/osteomalacia due to increased urinary excretion of phosphate (Bai et al. 2004; DeLuca et al. 2008; Shimada et al. 2004c). In addition to phosphate dysregulation, FGF23 knockout mice also have extremely high serum levels of $1,25(\text{OH})_2\text{D}_3$ with vascular calcification (Razzaque et al. 2006; Sitara et al. 2006), while FGF23 transgenic mice producing high levels of FGF23 have reduced serum levels of $1,25(\text{OH})_2\text{D}_3$ (Bai et al. 2004; DeLuca et al. 2008; Shimada et al. 2004c). Of particular interest, FGF23 can inhibit the expression of $1\alpha(\text{OH})\text{ase}$ to reduce the production of the active vitamin D metabolite $1,25(\text{OH})_2\text{D}_3$ (Shimada et al. 2004a), while vitamin D can exert stimulating effects on FGF23 (Shimada et al. 2004a; Shimada et al. 2005). Circulatory FGF23 levels are extremely high in patients with CKD, and because FGF23 is a counter regulatory hormone for vitamin D, elevated circulatory levels of FGF23 might contribute to low vitamin D levels in CKD patients.

In CKD patients receiving dialysis treatment, vitamin D treatment has been reported to be associated with an increased occurrence of vascular calcification (Goldsmith et al. 1997); uremic tumoral calcinosis is another complication of vitamin D therapy in dialysis patients (Quarles et al. 1991). Supplementing active vitamin D metabolites is a routine treatment option for CKD patients. Use of a high calcium dialyzer bath and prolonged treatment of vitamin D are believed to cause tumoral calcinosis (Cofan et al. 1999), as use of a low calcium bath and discontinuation of vitamin D therapy could delay or prevent such calcinosis (Franco et al. 2005). In

various genetically modified mouse models, including FGF23 or Klotho knockout mice, extensive vascular calcification is noted with increased vitamin D activities (Nakatani et al. 2009; Razzaque and Lanske 2006; Razzaque et al. 2006). It should also be noted that increased vitamin D activity in FGF23 and Klotho knockout mice is associated with elevated serum levels of phosphate (Ohnishi et al. 2009a, 2009b; Razzaque et al. 2006; Sitara et al. 2006). In a similar line of observations in humans, high phosphate and low vitamin D serum levels are identified as independent risk factors for increased mortality in pre-dialysis CKD patients (Shoji et al. 2004; Voormolen et al. 2007; Wang et al. 2008).

An association between serum phosphate levels and vascular calcification has been reported in the general population. After 15 years in the CARDIA study (Coronary Artery Risk Development in Young Adults) of 3,015 individuals (Foley et al. 2009), a 52% higher risk of coronary artery calcification was documented in participants whose initial serum phosphate levels were >3.9 mg/dl, compared to those with serum phosphate levels <3.3 mg/dl (Foley et al. 2009). Of particular importance, in physiological cytoplasmic concentrations of Pi (~ 1 mM), calcification is usually detected in specific genetic disorders; however, in higher cytoplasmic concentrations of Pi (>2 mM), enhanced calcium-phosphate precipitation on the elastin of the vessel walls can accelerate the calcification process (Lanzer et al. 2014).

Vitamin D deficiency may be an indicator of phosphate dysregulation. A relationship between these two conditions exists in many diseases associated with hypovitaminosis D. For example, assumptions about a causative role played by vitamin D in chronic kidney disease and the need for vitamin D supplementation are contradicted when considering phosphate dysregulation. Monk and Bushinsky (Monk

and Bushinsky 2011) state that vitamin D levels fall in advanced chronic kidney disease because the kidney is unable to convert sufficient amounts of 25(OH)D to calcitriol, thus elevating PTH levels. This vitamin D “insufficiency” is used as an indication to supplement 25(OH)D levels. However, an alternative explanation when considering phosphate dysregulation is that high serum levels of phosphate stimulates secretion of FGF23 from bone which lowers the enzymatic action of $1\alpha(\text{OH})\text{ase}$ needed by the kidneys to synthesize calcitriol (Holick 2007). In addition, hyperphosphatemia lowers serum calcium which increases PTH secretion to rapidly restore serum calcium levels via bone resorption. Hyperphosphatemia is often associated with hyperparathyroidism and causes vascular calcification (Askar 2015), as in patients with CKD, which occurs independently of vitamin D. Interestingly, experiments also suggest that reduction of calcification repressors is triggered by excess PTH levels alone, causing calcification even with normal or low phosphate and calcium levels (Askar 2015).

CKD and vascular calcification

Dysregulation of mineral ion metabolism and skeletal anomalies, accompanied by vascular calcification, are common features of CKD progression. Vascular calcification is strongly associated with mortality in CKD patients receiving hemodialysis treatment (London et al. 2003); occurrence of coronary artery calcification is five times higher than that of non-dialysis CKD patients (Braun et al. 1996). Vascular calcification can be limited to either the intimal or medial regions, but in advanced stages, full thickness of the vessel wall may be mineralized. Medial calcification can be found in large elastic type arteries (ascending aorta), medium-sized visceral arteries, and in small arteries of

the heart, uterus, ovary, breast and other organs. Autopsy analysis has shown a higher degree of intimal calcification in patients with CKD (Lanzer et al. 2014). Using standard radiographic studies, vascular calcification was detected in about 60% of patients with advanced stages of CKD (Meema and Oreopoulos 1986). Recently, employing an electron beam computerized tomography system, vascular calcification affecting coronary arteries has been reported to be higher among CKD patients with diabetes compared with non-diabetic CKD patients (94% vs. 59%) (Merjanian et al. 2003).

Common biochemical abnormalities detected in patients with CKD are hyperphosphatemia, hyperparathyroidism, elevated FGF23, and hypovitaminosis D. Reduced serum 25(OH)D levels are detected even in the earlier stage 2 level of CKD (Rickers et al. 1985). In addition to phosphate dysregulation and other possible causes previously described, reductions in vitamin D levels include the cumulative effect of lack of sunlight exposure, impaired skin synthesis of vitamin D, and altered intestinal absorption of vitamin D (Del Valle et al. 2007; Jacob et al. 1984; Vaziri et al. 1983). Also, severe proteinuria in patients with CKD can reduce vitamin D binding protein (DBP) to reduce vitamin D activities (Sato et al. 1982). Finally, reduction of non-calcified renal mass in patients with CKD reduces $1\alpha(\text{OH})\text{ase}$ activities to impair the generation of active vitamin D metabolites. The prevalence of vitamin D deficiency in the CKD population ranges from 70 to 80% (Wolf and Thadhani 2007). Use of vitamin D replacement therapy in patients with CKD is a routine practice, and various studies have demonstrated reduction of serum PTH levels by such replacement therapy, with possible side effects of hypercalcemia and/or hyperphosphatemia (Kandula et al. 2011).

Phosphate-dependent vascular calcification was shown in both FGF23 and *klotho* knockout mice (Kuro-o et al. 1997; Nakatani et al. 2009; Ohnishi et al. 2009a; Ohnishi and Razzaque 2010; Razzaque 2011c; Razzaque et al. 2006; Sitara et al. 2008); in both these genetically modified mice, increased renal activity of sodium/phosphate co-transporters (NaPi2a) is associated with extremely high serum phosphate levels (Nabeshima 2006; Nakatani et al. 2009; Ohnishi et al. 2009a). Such phosphate toxicity in FGF23 and *klotho* knockout mice caused widespread calcification in kidney, lung, heart and aorta. In addition to developing phosphate toxicity, FGF23 and *klotho* knockout mice also have high serum levels of calcium and $1,25(\text{OH})_2\text{D}_3$ (Kuro-o et al. 1997; Nakatani et al. 2009; Ohnishi et al. 2011a; Ohnishi et al. 2011b; Ohnishi et al. 2009a; Ohnishi and Razzaque 2010, 2012; Razzaque et al. 2006; Sitara et al. 2008). To determine the effect of high serum phosphate in vascular calcification, independent of high serum calcium and vitamin D levels, we generated FGF23 or *klotho* knockout mice with high serum calcium and $1,25(\text{OH})_2\text{D}_3$ levels and relatively normal serum phosphate levels (Ohnishi et al. 2009a; Ohnishi and Razzaque 2010; Sitara et al. 2008). By lowering the serum phosphate levels in FGF23 and *klotho* knockout mice, we have shown markedly suppressed vascular calcification, despite the presence of significantly higher serum calcium and $1,25(\text{OH})_2\text{D}_3$ levels (Ohnishi et al. 2009a; Sitara et al. 2008). These *in vivo* results clearly suggest the beneficial effects of lowering serum phosphate levels to suppress the genesis of vascular calcification, even in the presence of extremely high serum calcium and $1,25(\text{OH})_2\text{D}_3$ levels (Ohnishi et al. 2009a). Our observations in genetically modified mouse models, mimicking CKD patients, suggest that in absence of high serum phosphate levels, vascular calcification cannot be induced by high vitamin D and high calcium levels. Such *in vivo* results imply

that, by minimizing the risk of phosphate burden, the safely margins for vitamin D treatment in patients with CKD could be widened without the risk of inducing vascular calcification, or perhaps the need for vitamin D treatment could be eliminated altogether. Of particular importance, higher mortalities in dialysis patients with CKD are associated with vascular calcification (Mizobuchi et al. 2009), and therapeutic benefits of lowering phosphate burden in patients with CKD has been reported (Pilz et al. 2011).

Concluding remarks

Vitamin D deficiency is more prevalent in elderly populations, and vitamin D supplementation to minimize the risk for osteoporosis and other skeletal disorders is a routine practice. However, uncontrolled use of vitamin D and calcium supplements might increase the risk of developing cardiovascular calcification (Heikinheimo et al. 1992; Jackson et al. 2006; Trivedi et al. 2003). Likewise, vascular wall mineralization is a fatal complication of advanced stages of CKD that markedly increases morbidity and mortality of affected patients. Although a meta-analysis of studies on vitamin D supplementation in patients with CKD found that vitamin D supplementation lowers PTH levels (Kandula et al. 2011), this may be related to hypercalcemia caused by vitamin D supplementation. According to the researchers (p. 59), “hypercalcemia and hyperphosphatemia were not reported in all of the studies, and the reported definitions were heterogeneous.”

Vieth et al. (Vieth 2006a, 2006b, 2012; Vieth et al. 2007; Vieth et al. 2001) examined the levels of vitamin D supplementation that may be “safely” ingested before toxic symptoms appear, but to what purpose? The evidence suggests that any

pharmacological level of excess vitamin D above the normally regulated physiological levels has no benefits and can only begin to increase risk of harm. Citing the U.S. Preventive Services Task Force investigation of vitamin D and calcium supplementation, Nestle et al. pointed out that there is no solid evidence of greater benefit than harm from vitamin D supplementation (Nestle and Nesheim 2013). Several recent systematic reviews and meta-analyses of random control trials found that Vitamin D supplementation had insignificant effects in reducing risk for chronic diseases, fractures, and falls (Autier et al. 2014; Bolland et al. 2014; Theodoratou et al. 2014). Of relevance, in vivo studies have shown that vascular wall calcification could be reduced by lowering serum phosphate levels, even in the presence of extremely high serum calcium and $1,25(\text{OH})_2\text{D}_3$ levels, clearly suggesting that reducing “phosphate toxicity” should be a therapeutic priority in CKD patients to minimize the risk of developing vascular mineralization and associated complications (Brown and Razzaque 2015).

Acknowledgements

Part of the original research that formed the basis of this review article was performed by Razzaque's lab members (Drs. Teruyo Nakatani, Junko Akiyoshi, Shigeko Kato, Satoko Osuka and Mutsuko Ohnishi) at the Harvard School of Dental Medicine, Boston, Massachusetts, USA. We would like to express our gratitude to Dr. Syed K. Rafi, PhD, Department of Psychiatry and Genetics, Kansas University Medical Center, Kansas, USA, for critically reading this manuscript and offering useful suggestions.

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Draft

Table 1

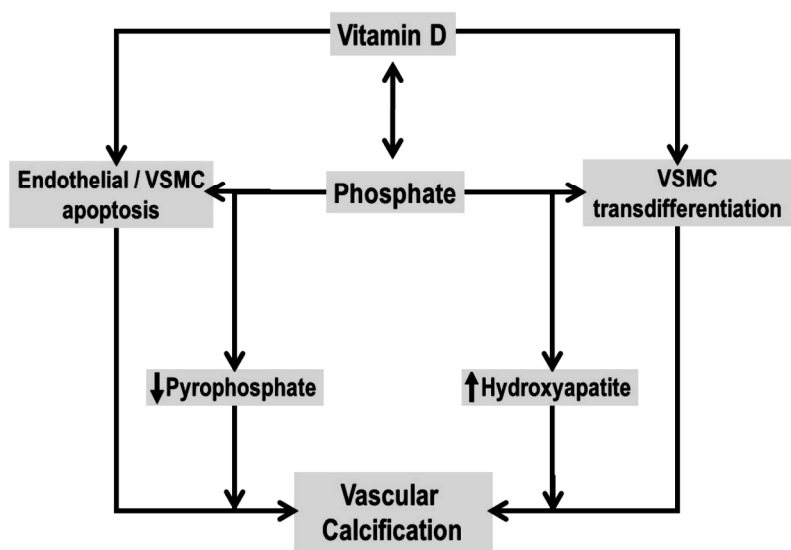
Partial list of known inducers and inhibitors of vascular calcification. Modified from earlier publication (Razzaque 2011a). [**ANK**: transmembrane protein; **BMP**: bone morphogenetic protein; **LDL**: low-density lipoprotein; **HDL**: high-density lipoprotein; **IGF-1**: insulin-like growth factor-1; **NPP-1**: pyrophosphatase/phosphodiesterase; **PTH**: parathyroid hormone; **PTHrP**: PTH-related peptide; **RUNX2**: runt-related transcription factor 2; **TGF- β 1**: transforming growth factor- β 1; **TNAP**: tissue-non-specific alkaline phosphatase]

Inducers	Inhibitors
Vitamin D	Pyrophosphate
Uremic toxins	PTH/ PTHrP
TNAP	Osteoprotogerin
TGF β 1	Osteopontin
RUNX2	Omega-3 fatty acid
Phosphate	NPP-1
Pit-1	Matrix Gla Protein
Osteocalcin	Magnesium
MSX2	Klotho
Leptin	Insulin/ IGF-1
LDL	HDL
Inflammatory cytokines	Fetuin-A
Hyperparathyroidism	BMP-7
Calcium	ANK
BMP-2/4	Adiponectin

Figure legend

Simplified schematic diagram outlining the possible pathological events that are associated with vascular calcification (Razzaque 2011a, 2013, 2014). Increased vitamin D activities can induce both endothelial and VSMC apoptosis, and facilitate phenotypic transformation of VSMCs into the osteoblast-like phenotype to promote vascular calcification. Moreover, phosphate toxicity can not only facilitate essential hydroxyapatite deposition in the calcifying vessels but also initiate the early events by inflicting apoptotic cell death on both endothelial cells and VSMCs. **VSMC**: vascular smooth muscle cell

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267x150mm (300 x 300 DPI)