IN DEPTH REVIEW

The influence of phosphate, calcium and magnesium on matrix Gla-protein and vascular calcification: a systematic review

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Abstract

Vitamin K-dependent matrix Gla protein (MGP) is a key inhibitor of vascular calcification (VC). MGP is synthesized by chondrocytes and vascular smooth muscle cells (VSMC) and the absence or inactivity of MGP results in excessive calcification of both growth plate and vasculature. Apart from its vitamin K dependency little is known about other factors that influence MGP metabolism. Phosphate, calcium and magnesium are involved in bone mineralization and play an important role in VC. In this review we provide a summary of the effect of phosphate, calcium, and magnesium on MGP metabolism. Elevated phosphate and calcium levels promote VC, in part by increasing the release of matrix vesicles (MV) that under the influence of calcium and phosphate become calcification competent. Phosphate and calcium simultaneously induce an upregulation of MGP protein and gene expression, which possibly inhibits calcification. Elevated phosphate levels did not change MGP protein levels in MV. On the contrary, elevated calcium concentrations caused a decrease of MGPloading in MV, which might in part explainthe calcifying effects of MV. Magnesium is a known inhibitor of VC. However, magnesium has been shown to have an inhibitory effect on MGP synthesis induced through downregulation of the calcium-sensing receptor and hereby causing a decrease in calcium induced MGP upregulation. There might also be stimulatory effect of magnesium on MGP in which the TRPM7 channel is involved. In conclusion there is a clear interaction between MGP and phosphate, calcium and magnesium. The upregulation of MGP by phosphate and calcium might be a cellular response that possibly results in the mitigation of VC.

Key words: Carboxylation, ckd-mbd, magnesium, MGP, Minerals, vascular calcification

Introduction

Cardiovascular events account for 50% of all death in patients with end stage renal disease [1] (full text). This increased cardiovascular risk is partly caused by a process known as vascular calcification (VC). VC is characterized by increased calcium salt depositions in the arteries leading to arterial stiffness [1] (full text) [2] (full text). VC is a complex process that is similar to bone formation and involves transdifferentiation of vascular smooth muscle

cells (VSMCs) into osteoblast-like cells [3] (full text) [4] (full text). The transdifferentiation of VSMC results from an imbalance between pro-osteogenic factors and osteogenesis inhibitors [5] (full text). In addition, the precipitation of calcium and phosphate in the form of hydroxyapatite crystalsis an important component of VC. The precipitation of hydroxyapatite is initially mediated by small, membranous vesicles that form a nidus for calcification and contain minerals and substances that fuel this process. These vesicles are termed matrix vesicles (MV) and are released from the cell membrane of resident cells in the vessel wall [6].

Matrix γ-carboxy-glutamate (Gla) protein (MGP) is a protein secreted by chondrocytes as well as VSMC and is a key inhibitor of VC [7] [8] [9] (full text). Knock-out mice lacking MGP develop extensive calcification in arteries and cartilages and die within six to eight weeks after birth as a result of blood-vessel rupture [7]. In humans, mutations in the gene encoding MGP result in the Keutel syndrome, a rare disorder characterized by abnormal calcification of soft tissues [10] [11]. Several mechanisms by which MGP inhibits calcification have been postulated. Firstly, it has been suggested that MGP binds hydroxyapatite crystals thereby shielding nucleation sites for mineral growth. Thus, MGP might act as a competitor to hydroxyapatite precipitation in VC [12] (full text) [13] (full text). Secondly, MGP can bind and inactivate bone morphogenetic protein-2 (BMP-2). BMP-2 is an osteogenic growth factor, found to be active in VSMCs [14]. Besides that, MGP-null mice demonstrated an upregulated expression of the bone-specific transcription factor cbf1a/Runx2 and the osteogenic protein osteopontin. The upregulation of these genes is associated with transdifferentiation of VSMCs into osteoblast-like cells [15] (full text).

Before MGP can exert its calcification inhibitory effects, it has to undergo two posttranslational modifications: γ-glutamate carboxylation and serine phosphorylation (illustrated in Figure 1). Carboxylated and phosphorylated MGP is regarded as the active form of MGP [16] (full text) [17] (full text). The ratio of active MGP compared to inactive MGP in plasma is unknown. Most ELISA-based detection strategies used so far do not differentiate between all different MGP conformations (Figure 1).

The carboxylation of MGP is entirely dependent on the presence of its cofactor vitamin K [17] (full text). Little is known about factors involved in the phosphorylation of MGP. Phosphate, calcium and magnesium are major components of cartilage and bone [18]. In addition, these ions play an important role in the pathogenesis of VC. As a result, many

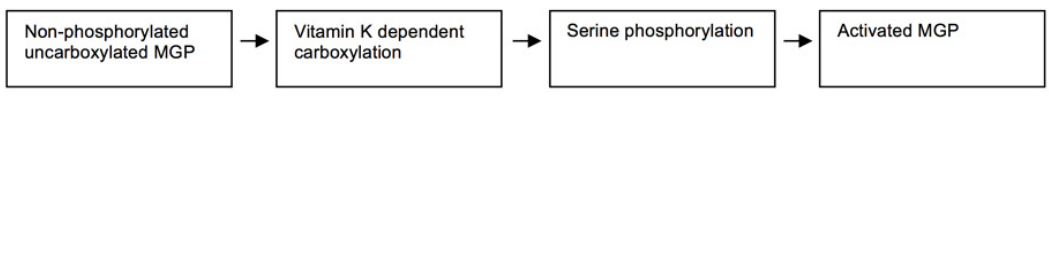

Figure 1.
Post translational modifications of MGP.

studies focused on the role of phosphate, calcium and magnesium in VC [19] [20] (full text) [21] (full text) [22] [23]. However, only a limited number of studies describe the direct interaction of these ions with MGP. Recent literature suggests interaction between MGP and these ions, however data are conflicting. This article aims to provide an overarching conceptual framework of the complex interaction between MGP, phosphate, calcium and magnesium.

Methods

We identified studies from the bibliographic databases of PubMed between the 1st of December 2014 until the 1st of January 2015, using search terms: 'phosphate AND matrix Gla protein', 'calcium AND matrix Gla protein', 'magnesium AND matrix Gla protein', AND 'interaction' AND 'stimulation'. We found a total number of 556 articles. All studies, either *in vitro* or *in vivo*, that focused on the direct interaction of phosphate and/or calcium and/or magnesium with MGP were included. Based on these criteria, 11 studies were classified as eligible; all of which were in vitro studies.

Phosphate

In CKD hyperphosphatemia is a common complication. There is compelling evidence that elevated phosphate levels correlate with VC and result in a higher cardiovascular mortality in CKD patients [24] (full text) [25] (full text) [26] (full text) [27] (full text). The mechanisms by which phosphate induces VC are, however not fully understood. It is known that the presence of high levels of phosphate accelerate the precipitation of calcium and phosphate in the form of hydroxyapatite [26] (full text). In mouse VSMCs, elevated phosphate levels stimulate the expression of osterix, an osteoblastic transcription factor. Osterix is an essential factor in the formation of calcification prone MV [28] (full text). Under physiological conditions these MVdo not calcify since they contain calcification inhibitors such as MGP and Fetuin-A [26] (full text). However, an elevated phosphate level results in an increased release of MV that are mineralization competent and might form a nidus for hydroxyapatite precipitation [26] (full text).

Four studies reported that high phosphate levels increased MGP levels (Table 1) [5] (full text); [29] [30] (full text) [31]. One study found no effect of phosphate on MGP levels [32] (full text). The effect of phosphate on MGP was first investigated in an *in vitro* model using the ATDC5 cell line. ATDC5 cells are derived from teratocarcinoma that differentiate into chondrocytes [33]. An elevated phosphate level induced a statistically significant 6-fold increase in MGP mRNA expression as compared to control [29]. There are however, two important limitations to these studies. Firstly, the concentration of phosphate was unphysiologically high, and secondly no data are presented on the posttranslational modification of MGP, which dictates its activity.

Hyperphosphatemia induces upregulation of the ERK1/2-Fra-1 pathway, which was shown to be essential for upregulation of MGP [30] (full text). The ERK1/2-Fra-1 pathway consists of the ERK1/2 cascade, a member of the MAPK family that communicates extracellular signals to the nucleus, which results in transcription of proteins [34]. Blocking ERK1/2 signal transduction using U0126, a widely used inhibitor of MEK1/2, the upstream kinases of ERK1/2, suppressed Pi-stimulated induction of MGP at both mRNA and protein level [30] (full text). Additionally, Fra-1 expression is stimulated by phosphate [30] (full text). Fra-1 is a key regulator of MGP expression, since overexpression of Fra-1 increased MGP expression in primary osteoblasts [35] (full text). Blocking ERK1/2 activation suppressed the

stimulating effect of phosphate on Fra-1. Besides that, ERK1/2 activity also prevents FRA-1 from degradation. These data strongly suggest that phosphate stimulates MGP by activating Fra-1 through the ERK1/2 pathway [30] (full text). Additionally PiT-1, an active transporter of phosphate into VSMCs [36] (full text), may also play a role in the ERK1/2 pathway. Elevated Pi-induced signaling via ERK1/2 phosphorylation was abrogated in PiT-1 deficient VSMCs [37].

One recent study focused on MV isolated from VSMC that were treated with elevated phosphate. The amount of MGP in MV remained unchanged compared with MV originating from VSMC that were not treated with phosphate [32] (full text). This is not in line with results that phosphate upregulates total MGP in VSMCs. Moreover, it was suggested that phosphate reduces the affinity of MGP for hydroxyapatite [38].

In summary, phosphate stimulates total MGP and MGP transcription through the ERK1/2-Fra-1 pathway. However the amount of MGP in MV is not affected by elevated phosphate levels. In addition phosphate may limit the affinity of MGP to hydroxyapatite crystals. This suggests that in spite of the overall effects of phosphate on VC, phosphate leads to higher levels of MGP and MGP transcription, possibly resulting in some mitigation of VC.

Table 1. An overview of the interactions between phosphate, calcium, magnesium and MGP.

Article	Cell line	Used ion concentrations	Duration	MGP detection	Result
Julien, 2007 (ref.n. 29)	ATDC5 cell line (chondrocytes)	Pi unknown control concentration, 10.0 mM Ca2+ unknown Mg2+. unknown	24 hours	PCR	High phosphate increased MGP gene expression
Julien, 2009 (ref.n. 30)	MC3T3-E1 cell line (osteoblast precursor cells) and primary osteoblasts mice	Pi 1.0, 9.0 mM Ca2+ unknown Mg2+. unknown	24 hours	PCR and western blotting	High phosphate increased MGP gene expression
Louvet L, 2013 (ref. n. 5)	Human aorta VSMC	Pi 0.9, 3.0 mM Ca2+ 1.8 mM Mg2+. 0.8, 1.5, 2.0 mM	21 days	ELISA on cell medium	High phosphate increased MGP protein High magnesiumdecreased MGP protein
Khoshniat, 2011 (ref. n. 31)	MC3T3-E1 cell line (osteoblast precursor cells)	Pi 1.0, 10.0 mM Ca2+ 0, 1.8 mM Mg2+. unknown	24 hours	PCR	High phosphate increased MGP gene expression High calcium increased MGP gene expression
Kapustin, 2011 (ref. n. 32)	Human aorta VSMC	Pi 2.5 mM Ca2+ 5.4, 2.7 mM Mg2+. unknown	5 days	Mass spectrometry on MGP in MV	High phosphate did not change MGP protein High calciumdecreased MGP protein
Farzaneh-Far, 2000 (ref. n. 41)	Rat aorta VSMC	Pi unknown Ca2+ 1.8, 6.0 mM Mg2+ unknown	48 hours	PCR	High calciumincreased MGP gene expression
Nakatani, 2006 (ref. n. 40)	ATDC5 cell line (chondrocytes)	Pi unknown Ca2+ 1.0, 6.0 mM Mg2+ 0.7, 4.2 mM	28 days	PCR and immunohistochemistry	High phosphate increased MGP protein and gene expression High magnesiumdecreased MGP protein and gene expression
Montezano, 2010 (ref. n. 46)	VSMC WKY rats and inbred mice	Pi unknown Ca2+ unknown Mg2+ 2.0, 2.5, 3.0 mM	10 days	PCR	High magnesiumincreased MGP gene expression
Kircelli F, 2012 (ref. n. 48)	Bovine VSMC	Pi 0.9 mM Ca2+ 1.8 mM Mg2+ 1.0, 2.0, 3.0 mM	14 days	ELISA on cell medium	High Magnesiumincreased MGP protein
Montes de OA, 2014 (ref. n. 45)	Human aorta VSMC	Pi 0.9, 3.3 mM Ca2+ 1.8 mM Mg2+ 0.8, 1.4, 2.6 mM	9 days	PCR	High Magnesiumincreased MGP gene expression

Calcium

Calcium is an abundant ion that plays an important role in the pathogenesis of VC. Both *in vivo* and *in vitro* studies showed a correlation between calcification and high calcium levels. This effect is both independent and synergistic with elevated phosphate levels [31] [39]. Multiple mechanisms are responsible for the calcifying effects of calcium ions, including an increased production of mineralization competent MV [26] (full text). If both high phosphate and calcium are present, calcification effects are strongly enhanced [21] (full text) [26] (full text).

However, despite the calcifying effects of calcium, an elevated calcium level can also induce an increase in both MGP transcription and MGP protein level. These effects are described in four studies summarized in Table 1. In three studies it was shown that calcium increased the level of MGP [31] [40] [41], while one study in turn found a decrease in MGP [32] (full text). In one study, it was shown in a MC3T3-E1 cell line that both calcium and phosphate are required to induce a rise in MGP. In comparison to the studies describing the effects of phosphate on MGP metabolism, the concentrations for calcium appear unphysiologically high. However, local tissue calcium levels can exceed plasma concentrations, possibly due to local apoptosis and therefore data from these *in vitro* studies might still be applicable and translated to the clinical situations [21] (full text).

The interaction between calcium and phosphate ions is probably based on the formation of precipitates that stimulate ERK1/2 phosphorylation [31]. As previously mentioned, phosphorylated ERK1/2results in an increase in both protein and mRNA MGP levels. In human aortic VSMC the phosphorylation of ERK1/2 can be mediated through the calcium-sensing receptor (CaSR) [42] (full text). Calcium in rat VSMC stimulates the upregulation of MGP through a pathway that might involve a receptor, that is functionally related to the CaSR. CaSR-agonists induce a significant stimulation of MGP transcription in rat aorta VSMC. However, when rtPCR was carried out on total rat mRNA with a known CaSR primer, the CaSR was not found in this cell line. This suggests the presence of another receptor that is functionally related, but molecularly distinct from the CaSR [41]. However, more recent studies using a more selective calcimimetic do suggest that the CaSR is involved [43]. Calcium does not only increase the amount of MGP, but can even influence the effectiveness of MGP in preventing VC. In the presence of high calcium, MGP shows an increased affinity for hydroxyapatite. It is suggested that calcium induces a conformational change of MGP that promotes MGP-hydroxyapatite binding [38].

In contrast with these data, one study focused on MGP in MV released from VSMC. Under pathologic conditions MV can promote calcification. It was found that when human aortic VSMC are treated with calcium, the amount of MGP in MV initially rises, but after 48 hours treatment MGP diminishes [32] (full text).

In summary, the majority of these data support the hypothesis that MGP is upregulated in response to elevated calcium levels. This might imply an inherent protective mechanism in the formation of VC by high levels of calcium through the up regulation and conformation of MGP. However, in response to elevated calcium levels, the amount of MGP in MV decreased. The overall impact of calcium-mediated effects on MGP metabolism on calcification is not fully elucidated and more research is needed.

Magnesium

Magnesium is widely known as an inhibitor of VC both *in vitro* and *in vivo* [5] (full text) [44]. Magnesium does not only prevent, but may even decrease already existing VC [45] (full

text). Some *in vitro* studies found that its inhibiting function is exerted in the presence of phosphate or calcium [5] (full text) [40] [45] (full text) **[46]** (full text). The mechanisms by which magnesium prevents VC are not fully established. Among others effects, magnesium directly prevents apatite crystallization in vitro **[47]**. Also, magnesium inhibits the Wnt/β-catenin signaling pathway, a pathway involved in VC [45] (full text). Besides that, together with other ions, magnesium directly affects MGP expression, as discussed below.

The interaction between magnesium and MGP was studied in five studies, summarized in Table 1. In two studies magnesium was found to reduce the level of MGP [5] (full text) [40] whereas in three other studies it increased the level of MGP [45] (full text) [46] (full text) **[48]** (full text). In a recent study that investigated human aortic VSMC with phosphate-induced calcification, higher concentrations of magnesium decreased the level of MGP. Magnesium had no effect on MGP levels in the absence of phosphate [5] (full text). Another study investigated an ATDC5 cell line treated with both calcium and magnesium. In the presence of magnesium, calcium-induced elevated MGP levels declined. This study reported that calcium increases MGP through the CaSR and that magnesium downregulates the expression of the CaSR and thereby causes a decrease in calcium induced MGP [40]. Finally, magnesium was found to reduce the affinity of MGP for hydroxyapatite, by competing with MGP for binding sites. This competition in binding site might be explained either by magnesium-hydroxyapatite binding or magnesium-MGP binding. Magnesium might also affect the conformation of MGP [38].

However, other recent studies show that magnesium increases the level of MGP. In calcifying VSMC MGP was increased by magnesium in the absence but also in the presence of other ions. These studies were performed using human, bovine or rat VSMC [45] (full text) [46] (full text) [48] (full text). Incubating calcifying bovine VSMC with β-glycerophosphate, a calcification medium, led to an 86% decrease of MGP secretion. When magnesium was added, MGP first normalized and after the addition of up to 3mM magnesium, MGP even increased to 68% above baseline values [48] (full text). It was found that when the magnesium channel TRPM7, responsible for magnesium entry into the cell was blocked, the induced increase in MGP was reversed [46] (full text).

In summary, whether magnesium upregulates or downregulates MGP remains controversial. There seem to be two pathways for the interaction between magnesium and MGP: an inhibitory effect possibly through the downregulation of the expression of the CaSR and a stimulatory effect in which cellular entry across the TRPM7 channel is involved. The net effect of magnesium on VC is inhibitory, but whether this is despite of or due to the interaction with MGP, still needs to be elucidated. An overview of the interactions of phosphate, calcium and magnesium on both VC and MGP is provided in Figure 2.

Discussion

While phosphate and calcium are both potential inducers of VC, magnesium is generally considered to be protective against VC. In the presence of phosphate and calcium an upregulation of MGP is observed. However in MV elevated calcium concentration caused a decrease in MGP whereas an elevated phosphate level had no effect on MGP. The effect of magnesium on MGP remains controversial.

Data on the interaction between MGP and calcium and/or phosphate were consistent, usually pointing to increased production of MGP by these minerals, except for one study by Kapustin et al. [32] (full text) In that study MGP was investigated in mineralization competent MV in particular. They found that MGP was significantly reduced in MV released from VSMC treated with calcium and remained unchanged after treatment with phosphate.

These contradictory results could possibly be explained by the fact that Kapustin and colleagues focused on MGP in MV instead of total MGP including intracellular MGP.

MGP in MV was shown to represent at least the phosphorylated form of MGP [49] (full text). MGP is a highly insoluble protein that is carried intracellular in vesicular structures which are probably precursors of MV. This fraction contains acidic MGP, which was identified as the phosphorylated form of MGP. This phosphorylated form of MGP was undetectable in the cytosolic fraction of VSMC [49] (full text). Although the carboxylation status of the microsomal MGP is unknown, this might suggest that Kapustin and colleagues detected activated MGP, since this study focused on MGP in MV, in contrast to other studies. Although future research should confirm this hypothesis, this might indicate that calcium and phosphate increase the level of inactive MGP, but reduce the physiologically active form of MGP, present in MV. Furthermore clinical studies showed that circulating uncarboxylated and dephosphorylated MGP increased progressively in a CKD setting and high levels of these inactive metabolites were associated with the severity calcification [9] (full text). Hence, an increase in MGP in the presence of calcification factors does not necessarily indicate an increase in activated MGP and an effective protective mechanism.

Magnesium has a protective effect on VC, but the data on the interaction between magnesium and MGP are contradictory. All reviewed articles that involved magnesium and MGP, focused on total MGP protein or MGP transcription. There was no difference found in methodology, such as: used ion levels, cell lines or MGP detection strategies that could explain the differences in outcomes. We did not find studies with a specific focus on MGP in MV.

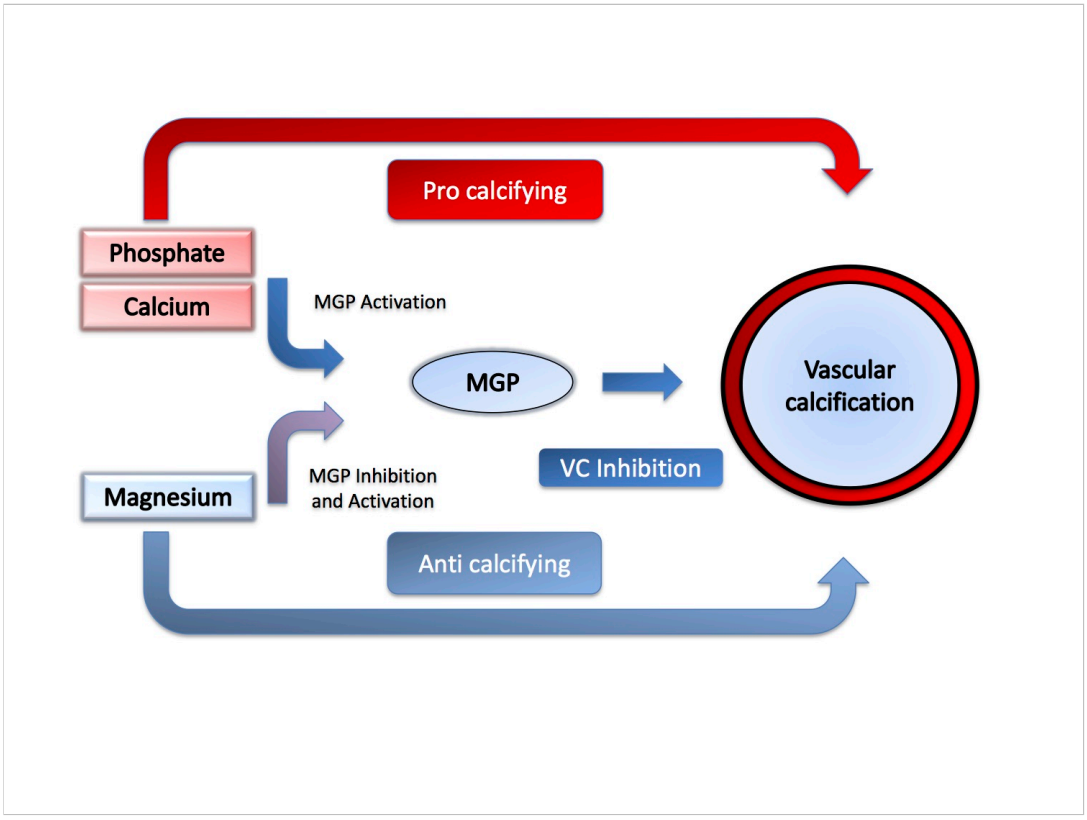

Figure 2.
An overview of the interactions of phosphate, calcium and magnesium on both vascular calcification and MGP. High phosphate increases MGP gene expression and MGP protein. High calcium increases MGP protein and gene expression, and decreases MGP in MV (not shown in the figure). It is unclear if high magnesium increases or decreases MGP gene expression and MGP protein.

A suggested pathway involved in the inhibition of MGP by magnesium is the CaSR. Indeed the presence of CaSR was found in ATDC5 cells and its expression was inhibited by magnesium, limiting upregulation of MGP [40]. However in other studies using rat aorta VSMC or the MC3T3-E1 cell line, the CaSR was not detected [31] [41]. In human aorta VSMC, however, the CaSR protein was detectable [42] (full text). Nevertheless, whether magnesium induces inhibition of MGP through the suppression of the CaSR remains questionable. Future research should elucidate if the CaSR plays a role in MGP regulation in CKD patients.

Future research should focus on the interaction of MGP with all ions together and should differentiate between different forms of MGP, in which recently introduced antibodies that detect specific forms of MGP [22] could be of use. A better understanding of factors that influence MGP, could lead to new insights for future therapies in VC. One such example is the effect of calcimimetics, CaSR agonist that are often used in CKD patients in order to control secondary hyperparathyroidism. One recent clinical trial showed that calcimimetics can prevent VC in ESRD patients and several in vitro studies showed that this effect is possibly achieved by the upregulation of MGP [43] **[50]** **[51]** (full text). Another example is the suggestion that phosphate binders may increase, instead of reduce the risk of VC [52] (full text). Although it is suggested this is due to the alleged detrimental effects of the calcium content of binders used, this is still unproven, and the effects of these binders on MGP unknown [52] (full text). This review should be a call for more in-depth research into the complex mechanisms that underlie vascular calcifications, and the influence of minerals on the cellular protective responses. Especially the role of MGP is far from settled, while being a potent protein to protect the vessel walls.

An important limitation of the articles reviewed, is that all studies used strategies that detected either MGP transcription or total MGP. As previously described, MGP can only exert its inhibiting function after post-translational modifications [16] (full text) **[53]** (full text). Therefore, the detection of MGP transcription does not necessarily reflect active MGP, but the production of its precursor. Antibodies used in protein detection do often not differentiate between active or inactive MGP either. It was also found that antibodies used in ELISA, not only target all forms of MGP, but cross-react with other MGP-like antigens, such as fetuin-A [49] (full text). Therefore, it is uncertain if the results obtained by the reviewed studies indicate biologically effective interactions. Furthermore, in this article we focused on the interaction between MGP, phosphate calcium and magnesium in VSMC, chondrocyte and osteoblast cell lines. Although we did not find clear differences between MGP interactions in different cell lines, this could account for some bias in our review. Some of the differences between studies could be explained by the non-physiological conditions that are inherent of in vitro models. In turn, it is possible that under pathological conditions like severe hyperphosphatemia, the physiological protection from MGP in VSMC is lost, and some models detect this abnormal response.

Conclusion

Calcium and phosphate induce VC, but might simultaneously limit their calcifying effects by the upregulation of MGP. However in MV elevated calcium concentrations caused a decrease in MGP and an elevated phosphate level had no effect on MGP. Magnesium prevents vascular calcification, but its effect on MGP remains unclear. An inhibitory effect of magnesium is possibly induced through the CaSR and a there might also be stimulatory effect in which the TRPM7 channel is involved. More knowledge is required to fully understand the underlying mechanisms of the interactions and possible clinical implications.

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