Vascular Calcification Revisited: A New Perspective for Phosphate Transport

Ricardo Villa-BelOSTa*

Centro Nacional de Investigaciones Cardiovasculares (CNIC) Carlos III, Madrid, Spain

Abstract: Elevated serum phosphorus has emerged as a key risk factor for pathologic calcification of cardiovascular structures, or vascular calcification (VC). To prevent the formation of calcium-phosphate deposits (CPD), the body uses adenosine-5'-triphosphate (ATP) to synthesize inhibitors of calcification, including proteins and inhibitors of low molecular weight. Extracellular pyrophosphate (PPI) is a potent inhibitor of VC, which is produced during extracellular hydrolysis of ATP. Loss of function in the enzymes and transporters that are involved in the cycle of extracellular ATP, including Pi transporters, leads to excessive deposition of calcium-phosphate salts. Treatment of hyperphosphatemia with Pi-binders and Injection of exogenous PPI are the effective treatments to prevent CPD in the aortic wall. The role of sodium phosphate cotransporters in ectopic calcification is contradictory and not well defined, but their important role in the control of intracellular Pi levels and the synthesis of ATP make them an important target to study.

Keywords: ATP, calcium, phosphate, pyrophosphate, vascular calcification.

INTRODUCTION

Ectopic calcification, the deposition of calcium crystals on soft-tissues, is one of the most important factors determining patients’ morbidity and mortality around the world. Pathologic calcification of cardiovascular structures, or vascular calcification (VC), is associated with a number of diseases and is a common consequence of aging [1]. Calcium-phosphate deposition (CPD), in the form of hydroxyapatite or whitlockite, is the hallmark of VC and can occur in blood vessels, myocardium, and cardiac valves [1, 2]. In blood vessels, calcified deposits are found in distinct layers of the aortic wall and are associated with specific pathologies. Intimal calcification occurs in atherosclerotic lesions, whereas medial calcification (so-called “Monckeberg’s medial sclerosis”) occurs in the medial layer of the aortic wall and is associated with the elastic lamina [3-5].

Despite major advances in recent years, our understanding of the calcification pathogenesis is far from complete. Different mechanisms on the pathogenesis of vascular calcification have been proposed, including 1) loss of inhibitions, 2) osteocondrogenes differentiation of vascular cells, 3) apoptosis, 4) calcium and phosphorus homeostasis, 5) circulating nucleation complexes/paracrine factors and 6) matrix degradation [2, 6].

During the past decade, elevated serum phosphorus (in the form of inorganic phosphate, Pi) has emerged as a key risk factor for vascular calcification in the general population and in patients with chronic kidney disease (see Fig. 1) [6].

In this case, different factors play an important role in the control of phosphate (Pi) and calcium (Ca) homeostasis, including Pi/Ca excretion and absorption by the kidneys, intestines, and bone. An increased absorption or decreased excretion of phosphate can induce a relative small elevation in serum Pi, which has been correlated with the presence of calcified vessel due to an increase in the CPD formation and saturation in the inhibition [7, 8]. Several diseases have been correlated with the dysregulation of Pi homeostasis, including hyperparathyroidism, vitamin D (hyper-and hypovitaminosis), chronic renal disease, osteoporosis, and diabetes mellitus. Treatment of hyperphosphatemia with phosphate binders is associated with slow progression of cardiovascular calcification in hemodialysis patients (see Fig. 1) [9, 10].

In vitro experiments show CPD in vascular smooth muscle cells (VSMCs) incubated with high Pi [11]. This observation was interpreted logically as the consequence of an increase in the intracellular Pi [12]. However, old and new studies [7, 8, 13-16] show that the formation of CPD is a passive physicochemical process that does not require any cellular activity, suggesting an important role of Ca/Pi homeostasis.

There are two major consequences regarding the fate of VSMCs in phosphate-induced vascular calcification. The first involves apoptosis-dependent matrix mineralization, which has been detected both in cultured human VSMCs [17, 18] and in arteries from pediatric dialysis patients [19]. The second consequences invokes a profound transition to a bone-forming phenotype, that results in the loss of VSMCs markers (SM α-actin, SM22α) and the expression of osteochondrogenic markers (Runx2/Cbfa1; BMP-2) [20-22]. Recent studies show that calcium-phosphate deposits can induce both the transition to a bone-forming phenotype and...
involved in the extracellular ATP/PPi metabolism could provide potential future therapeutic targets to prevent ectopic calcification. The purpose of this manuscript is to analyze the contribution of phosphate and extracellular pyrophosphate homeostasis during vascular calcification, including the formation/deposition of hydroxyapatite and the synthesis of inhibitors, with special mention of the contribution of phosphate transport during this process.

1. ON BIOLOGICAL CALCIFICATION

Biological mineralization, or biomineralization, is the formation and deposition of inorganic minerals (biominerals) within or outside the cells of a various organisms. Biomineralization in specific sites of hard tissues (such as in bone, antlers, or dentine) is considered a physiological process; however, the accumulation of biominerals in soft tissues (such as in blood vessels, extracellular matrix of articular cartilaginous tissues of the joints, internal organs, and muscles) is considered ectopic biomineralization, or pathological calcification. Under normal conditions, the soft tissues are not mineralized, but due to aging and other pathological conditions, soft tissues become calcified, which leads to morbidity and mortality.

The main biomineral found in mineralized vertebrate connective tissue are calcium-phosphate salts. In an aqueous system of calcium and phosphate, there are several known non-ion-substituted calcium phosphates; however, not all have been found in biological tissues (see Table I). The phosphate ion is a polyatomic ion that consists of one central phosphorus atom surrounded by four oxygen atoms in a tetrahedral arrangement. In biological systems, it is found as a
of human dental and urinary calculi [53], has been strongly suggested as a precursor to hydroxyapatite, which is found in natural and prosthetic heart valve [54] (although OCP has not been observed in vascular calcification).

The deposition of these calcium-phosphate salts, both in vitro and in vivo, takes place on extracellular matrix proteins, such as elastin and collagens. According to the charge neutralization theory of calcification, the high glycine content of these matrix proteins favors the formation of beta-turns that are known to interact with calcium ions. In bone and connective tissues, these salts are predominantly deposited on the type I collagen and elastic fibers, respectively. Elastin, the main component of the elastic fibers in the medial layer of the aortic wall, is synthesized early in life and its expression and organization affects arterial aging. A recent study showed that a mouse model of elastin haploinsufficiency had a significant reduction in arterial calcification [56]. Moreover, the products derived from elastin degradation accelerate the phosphate-induced mineralization in vitro and in vivo via up-regulation of alkaline phosphatase [57].

2. ON ECTOPIC CALCIFICATION INHIBITORS

Extracellular fluids (such as serum, urine and synovial fluids) in vertebrates are supersaturated with phosphate and calcium, resulting in a tendency for spontaneous calcium phosphate precipitation. The synthesis of CPD inhibitors is therefore essential for survival. In the extracellular fluids, there are a range of endogenous low and high molecular weight inhibitors, including 1) low molecular weight (such as pyrophosphate and citrate) and 2) small and medium-sized proteins (such as, Matrix Gla Proteins, Fetuin A, and osteopontin) [2].

Extracellular pyrophosphate (PPi) is a potent physicochemical inhibitor of hydroxyapatite crystal formation and growth [14, 58–60]. Recent studies have shown that endogenous production of PPi and daily injections of exogenous PPi prevent medial vascular calcification in rats and mice (see Fig. 1) [28–30]. In hemodialysis patients, plasma PPi is reduced by 32% after standard hemodialysis [61, 62]; in a mouse model of progeria, PPi in plasma was decreased 4-fold [30]. In both cases, this decrease in PPi leads to exces-

| Abbreviation | Compound | Formula | Molar Ca/P ratio | Presence in calcified tissues |
|--------------|----------|---------|------------------|-----------------------------|
| MCPA         | monocalcium phosphate anhydrous | Ca(HPO₄)₂ | 0.5 | NO |
| MCPM         | monocalcium phosphate monohydrate | Ca(HPO₄)₂H₂O | 0.5 | NO |
| DCPA         | dicalcium phosphate anhydrous | CaHPO₄ | 1 | NO |
| DCPD         | dicalcium phosphate dihydrate | CaHPO₄·2H₂O | 1 | YES |
| β-TCP        | β-tricalcium phosphate | β-Ca₃(PO₄)₂ | 1 | NO |
| OCP          | octocalcium phosphate | Ca₈(HPO₄)₆(PO₄)₂·5H₂O | 1.33 | YES |
| ACP          | amorphous calcium phosphates | Ca₈(PO₄)₆nH₂O | 1.5 | YES |
| HA           | hydroxyapatite | Ca₁₀(PO₄)₆(OH) | 1.67 | YES |

Table 1. Existing Calcium Phosphates.

In the presence of calcium, various phosphates are obtained by charge neutralizing these different inorganic phosphate ions [41]: 1) monocalcium phosphate anhydrous (MCPA), 2) dicalcium phosphate anhydrous (DCPA), and 3) β-tricalcium phosphate (β-TCP). MCPA and DCPA are hydrated to form monocalcium phosphate monohydrate (MCPM) and dicalcium phosphate dihydrate (DCPD), respectively (see Fig. 2). DCPD, also called Brushite, is often found in calcified tissues [42–44], whereas MCPA, MCPM, DCPA, and β-TCP never appear in calcifications. The Mg-substituted β-TCP form, called whitlockite, is found in some calcified tissues [45, 46], such as in the aorta [47, 48]; whitlockite is not formed under physiological conditions unless magnesium ions are present. The final product of the calcium and phosphate salts reaction in neutral or basic solutions is crystalline hydroxyapatite (HA; Ca₁₀(PO₄)₆(OH)), the main component of bone [49]. The process of normal calcification occurs during the growth of hydroxyapatite; however, precipitation of biological hydroxyapatite in aberrant is also possible; which may lead to “HA deposition disease.”

There are two precursors of HA: amorphous calcium phosphate (ACP; Ca₈(PO₄)₆nH₂O) and octocalcium phosphate (OCP; Ca₈H₂(PO₄)₆·5H₂O). ACP consists mainly of roughly spherical Ca₈(PO₄)₆ clusters (called Posner’s clusters) that appear to be energetically favored compared to Ca₁₀(PO₄)₆ and Ca₈(PO₄)₆ clusters [50]. The structure of HA, which was first reported in 1964 [51], can be interpreted as an aggregation of Posner’s clusters [52]. OCP is often found as an unstable transient intermediate during the precipitation of the thermodynamically more stable HA [53]. Ion-substituted ACPs are found in soft-tissue pathological calcifications [43], whereas OCP, one of the stable components

free phosphate ion in solution and is called inorganic phosphate (Pi), to distinguish it from phosphates bound with different biological molecules. Aqueous Pi exists in four forms (see Fig. 2) according to its triprotic equilibrium: 1) trihydrogen phosphate (H₃PO₄), 2) dihydrogen phosphate ion (H₂PO₄⁻), 3) hydrogen phosphate ion (HPO₄²⁻), and 4) phosphate ion (PO₄³⁻). Inorganic phosphate is quite strong with respect to the first dissociation (pKₐ=2.1), moderately weak with respect to the second (pKₐ=6.9), and very weak with respect to the third (pKₐ=12.4).

There are two precursors of HA: amorphous calcium phosphate (ACP; Ca₈(PO₄)₆nH₂O) and octocalcium phosphate (OCP; Ca₈H₂(PO₄)₆·5H₂O). ACP consists mainly of roughly spherical Ca₈(PO₄)₆ clusters (called Posner’s clusters) that appear to be energetically favored compared to Ca₁₀(PO₄)₆ and Ca₈(PO₄)₆ clusters [50]. The structure of HA, which was first reported in 1964 [51], can be interpreted as an aggregation of Posner’s clusters [52]. OCP is often found as an unstable transient intermediate during the precipitation of the thermodynamically more stable HA [53]. Ion-substituted ACPs are found in soft-tissue pathological calcifications [43], whereas OCP, one of the stable components
sive vascular calcification in the medial layer of the aortic wall (see Fig. 1).

PPi is degraded to Pi enzymatically by tissue non-specific alkaline phosphatase (TNAP) in extracellular fluids (see Fig. 3). Over-expression of TNAP in cells is sufficient to cause medial vascular calcification in aortic rings ex vivo; and addition of alkaline phosphatase to culture media is sufficient to cause matrix calcification [63, 64]. Moreover, TNAP activity is increased in models of medial vascular calcification, such as in uremic rats or in a mouse model of Hutchinson-Gilford Progeria Syndrome [30, 65]. A recent study showed that in vivo over-expression of TNAP increases skeletal mineralization [66]; however, other studies have shown that phosphatase inhibitors can prevent vascular smooth muscle calcification in vitro [67, 68] (see Fig. 1) and that the ablation of phosphatase function produces a loss of skeletal mineralization [69].

The major generator of endogenous extracellular PPi in cartilage (and in a variety of other tissues) is the enzyme ectonucleotide pyrophosphatase/phosphodiesterase (eNPP), which hydrolyzes extracellular ATP to generate PPi and AMP (see Fig. 3). Members of the eNPP family include NPP1, NPP2, and NPP3; they exist both as membrane proteins, with an extracellular active site, and as soluble proteins in body fluids (such as, PC-1, autotaxin, and B10). In vascular smooth muscle cells and in the aorta, eNPP1 is the main source of extracellular Pi [31, 32, 64]. Mutations in eNPP1 result in generalized arterial calcification of infancy (GACI), which is characterized by calcification of the internal elastic lamina of large and medium-sized arteries [70, 71]. Moreover, eNPP1-null mice develop ectopic artery calcification [72-74].

A direct competitor of the substrate for eNPP is the family of ectonucleoside triphosphate diphosphohydrolase (known as eNTPD, also called Apyrase). Members of the apyrase family, including eNTPD1 or CD39, can hydrolyze ATP and ADP with varying preferences for the individual type of nucleotide. eNTPD1 is the major ecto-enzyme expressed in the rat aorta which hydrolyzes 90% of ATP [64] and releases small amount of ADP due to its high affinity for ADP (in an ATP/ADP ratio of 1:0.8) [32]. CD39 has the most evidence linking it directly to the regulation of signaling through purine receptors, but has not been linked to ectopic calcification directly. A recent study indirectly linked
CD39 with vascular calcifications [30]. Smooth muscle cells derive from aortas of a new mouse model of Hutchinson-Gilford Progeria syndrome, characterized by excessive vascular calcification, shown a high expression and activity of eNTPD1. These data suggests that CD39 could potentially limit the availability of ATP for Ppi conversion by eNPP1. The expression and regulation of this enzyme in the context of ectopic calcification should be further explored.

Another ecto-enzyme involved in extracellular ATP metabolism is the membrane-bound ecto-5’nucleotidase (NT5E, called CD73). This enzyme preferentially binds AMP and converts it to adenosine (Ado) and Ppi (see Fig. 3). Mutations in CD73 induce medial arterial calcification of the lower extremity arteries with periaricular calcification [75]. Serum calcium and phosphate levels are normal; however, cells from these patients showed high levels of TNAP activity that are significantly reduced by adenosine supplementation or by CD73 over-expression, suggesting that adenosine inhibits TNAP activity. Nevertheless, the increase in TNAP activity in CD73-deficient cells could be a compensatory mechanism used to regulate hydrolysis of AMP to Ado and Ppi. TNAP is a non-specific ecto-phosphohydrodrolase with broad substrate specificity [76]. It releases Ppi from a variety of organic compounds, including adenosine 5’-triphosphate, 5’-diphosphate (ADP) or 5’-monophosphate (AMP) respectively. The last step in the degradation of extracellular ATP is the membrane-bound ecto-5’-nucleotidase (CD73) that preferentially binds AMP and converts it to adenosine (Ado) and Ppi. To generate ATP by mitochondria or another metabolic pathway, Ado and Ppi are recovered from the extracellular space by equilibrative nucleoside transporter 1 (ENT1) and sodium-phosphate transporter (NPT), respectively. To close the cycle of extracellular ATP metabolism, ATP is released by cells via exocytotic mechanisms and via multiple types of membrane channels, including connexin hemichannels (Cx), pannexin (Panx), cystic fibrosis transmembrane conductance regulator (CFTR), and the sulfon fyurea receptor (SUR). (*) Indicates the ectoenzymes and transporters involved in extracellular ATP metabolism that induce ectopic calcification by loss of its function.

Like TNAP, CD73 is a GPI-anchored ectonucleotidase. In both enzymes, a soluble form cleaved from GPI-anchor has previously been described [27]. Both enzymes can catalyze the final step of extracellular nucleotide degradation [77, 78], but CD73 is the major enzyme responsible for the formation of extracellular adenosine from released adenine nucleotides. This last step in the degradation of extracellular nucleotides is very important for cell viability, as only the transport of nucleosides (such as adenosine) has been demonstrated, not nucleotides (such as AMP) across the cell membranes. Adenosine should be recovered from the extracellular space to generate ATP by mitochondria or another metabolic pathway. There are two known types of nucleoside transporters involved in the transport of adenosine into cells: concentrative nucleoside transporters (CNTs; SLC28)
and equilibrative nucleoside transporters (ENTS; SLC29). In a recent study, the loss of the equilibrative nucleoside transporter 1 (ENT1, SLC29A1) in mice could explain the diffuse idiopathic skeletal hypertosis (DISH) in humans, which is characterized by the ectopic calcification of spinal tissues [36]. In this study, the authors found a significant reduction in the expression of eNPP1, ANK, and TNAP in the intervertebral discs from ENT1⁻/⁻ null mice compared to wild-type mice. This is the first report of a role for ENT1 in regulating the calcification of soft tissues and closes the cycle of extracellular ATP metabolism (see Fig. 3).

The extracellular ATP metabolism cycle begins with the transport of ATP to the extracellular space (see Fig. 3). ATP release by cells occurs through at least two mechanisms. Multiple types of membrane channels have been shown to mediate ATP release, including connexin hemichannels (Cx), pannexin (Panx), cystic fibrosis transmembrane conductance regulator (CFTR), multidrug resistance gene product mdr (P-glycoprotein), and the sulfonlurea receptor (SUR) [79-83]; the last three proteins mentioned use the energy of ATP hydrolysis to facilitate the movement of a large array molecules across the cell plasma membrane, such that they are members of a class of integral membrane proteins so-called ABC transporters. In addition to ATP released through these channels, cells can release ATP via exocytotic mechanisms [84].

In 2000, two new genes were identified that play an important role in the control of tissue calcification and arthritis [85-87]; however, the molecular mechanisms remain, in part, unknown (see Table 2). First, it was reported that mutations in ABCC6 cause Pseudoxanthoma elasticum (PXE), a heritable disorder of connective tissue characterized by testicular microlithiasis [88] and calcification of the elastic fibers in skin, arteries, and retina [85, 86]. ABCC6 (ATP-binding cassette sub-family C member 6) is a member of the super family of ABC transporters, also known as multidrug resistance-associated protein 6 (MRP6). The MRP family is composed of several related pumps that are able to transport various molecules across extra- and intra-cellular membranes including glutathione-S-conjugates, bilirubin glucuronide, glycocholic acid, and cyclic nucleotides [89, 90]. ABCC6 may acts as a pump that releases endogenous low molecular weight inhibitors of calcium phosphate deposits in fluids outside cells, such as ATP or citrate; however, this has not been demonstrated.

The second gene reported is known as progressive ankylosis (ANK) [87]. Mutations in the ANK gene cause a severe form of generalized joint calcification and arthritis when studied in a mouse model. Loss of ANK function cause excessive hydroxyapatite formation in ANK⁻/⁻ null mice. Cells from the ANK mutant have a reduction in extracellular Pi and levels, and overexpression of ANK in tissue cultured cells results in an increase in extracellular Pi [87]. Using several helix prediction programs was proposed for the ANK protein 7-12 membranes-spanning helices and a central channel [87, 91, 92]. Therefore, it seems as though ANK regulates Pi transport from the cytoplasm to the extracellular milieu [87, 91, 93]; however, recent studies showed that ANK could be a channel or regulator of adjacent channels, which release ATP outside the cells [31, 64, 94].

In humans, mutations in the channel core of ANK cause craniometaphyseal dysplasia (CMD) [91, 92], and mutations in the N- and C-terminus of the ANK protein cause chondrocalcinosis (CC) [95-97]. CMD is a rare skeletal condition of abnormal bone formation characterized by an increased density of craniofacial bones and abnormal modeling of the metaphyses of the tubular bones. CC is a disease of articular cartilage that is radiographically characterized by the deposition of calcium pyrophosphate dihydrate crystals in the joint. Like mouse progressive ankylosis, CMD is associated with a decrease in extracellular Pi levels [91, 92] whereas CC is associated with an increase in the amount of Pi in the extracellular space which induce the spontaneous formation of calcium pyrophosphate crystals [95-97].

Recent reports suggest that the Pi/Pi ratio is strictly controlled by a complex interplay of genes that regulate Pi and

| Protein Affected | Role                  | Genetic Disease                | Symbol | Ectopic Calcification          | Ref               |
|------------------|-----------------------|--------------------------------|--------|-------------------------------|------------------|
| eNPP1            | Synthesis of Pi       | Generalized Arterial Calcification of Infancy | GACI   | Medial Arterial                | Rutsch F., 2003. |
| CD73             | Hydrolisis of AMP     |                                |        | Medial Arterial and Periarticular | St Hilaire C., 2011. |
| ENT1             | Ado Transporter       | Idiopathic Skeletal Hypertosis  | DISH   | Spinal Tissues                 | Warrach S., 2013. |
| Pit-2            | Pi Transporter        | Familial Idiopathic basal Ganglia Calcification | Basal Galgla and cortex | LeGeros RZ. 2007. |
| ANK              | ?                     | Cranioanetaphyseal dysplasia    | CMD    | Craniofacial Bones             | Foster BL., 2006. |
| ANK              | ?                     | Condrocalcinosis                | CC     | Articular cartilage            | Wang J, 2009.    |
| ABCC6            | ?                     | Pseudoxanthoma elasticum        | PXE    | Elastic fibers in skin, arteries and retine. Testicular calcification. | Gurley KA, 2006. Costello JC, 2011. Williams CJ, 2002. |

Table 2. Existing Genetic Disease involved in extracellular ATP/Pi metabolism that produces ectopic Calcification.
PPI concentrations [98-100], ANK could play a key role in this complex process by regulating ATP excretion by different channels, regulating phosphate transport and regulating both eNPP1 and TNAP activities [101-104]. In addition, transfection of eNPP1 in osteoblasts enhances extracellular PPI levels only when wild-type ANK is present [100]; overexpression of wild-type ANK proteins result in downregulation of TNAP activity in chondrogenic cells [102].

3. ROLE OF PHOSPHATE TRANSPORT IN ECTOPTIC CALCIFICATION

Classically, ATP has been considered the major energy source in the cell and is produced by a wide variety of enzymes from adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine (Ado). In mammalian cells, substrate level phosphorylation and oxidative phosphorylation are two major mechanisms of ATP biosynthesis. Metabolic processes that use ATP as an energy source convert it back into its precursors. Therefore, ATP is continuously recycled in the cell. However, over the last few years, a novel role for ATP as a potent extracellular signaling molecule and as a source of extracellular PPI has emerged [105]. Several ectoenzymes use extracellular ATP for this purpose (as mentioned in the previous section) and the final products of extracellular ATP hydrolysis by these enzymes are Ado and Pi [27, 105], which need to be transported into the cell to synthesize new ATP. Therefore, a loss in the transport of Ado (see adobe) or Pi (see below) can result in a decrease in the synthesis of ATP, which can in turn decrease the synthesis of inhibitors and lead to excessive calcium-phosphate deposition.

Cellular Pi levels are controlled by sodium phosphate co-transporters (NPT) [106]. The roles of NPT in human clinical disease and physiology processes have not been defined. Two families of sodium phosphate (NaPi) co-transporters have been principally identified, each with multiple members [107]; Type II (also called SLC34 or NaPi-II) and type III (SLC20 or NaPi-III). Type I (SLC17 or NaPi-I) phosphate transporters mediate the transmembrane transport of organic anions. Originally identified as Pi transporters, the relatively low affinity of NaPi-I family members for phosphate suggested that they transported more readily organic and inorganic anions than phosphate. Unlike Type I, Type II and type III transporters transport phosphate with high affinity (Km = 0,1 or less) but show differences in their affinities for H2PO4− and HPO42− ions [108-110].

The SCL34 family [111] comprises three members (also called NaPi-II), which are expressed in the small intestine (NaPi-Ib) and the kidney (NaPi-Iia and NaPi-Iic), two important sites for the control of phosphate homeostasis. NaPi-Ia is expressed predominantly both on renal proximal tubules and on the osteoclast basolateral membrane. Under normal conditions, NaPi-Iia is the NPT responsible for 95% of phosphate reabsorption in the proximal tubule [109]. Expression of NaPi-Iic was found exclusively in kidney and was described as being growth related [112, 113]. Interestingly, mice deficient in NaPi-Iia exhibit nephrocalcinosis and kidney stone formation but these defects are not observed in people with a loss of NaPi-Iia function [114]. Instead, mice deficient in NaPi-Iic exhibit hypercalciuria and hypercalcemia, but not renal calcification or osteomalacia [115-117]. Moreover, expression of NaPi-Iib has been detected in a number of tissues, including small intestine, lung, mammary glands, testis, and liver [111]. The NaPi-Iib knockout is lethal [118], but NaPi-Iib loss-of-function is associated with alveolar calcification in middle age [119, 120], and sometimes calcification in other organs, such as the testis [119] (see Table 3).

Type III sodium-phosphate co-transporters are represented by Pit-1 and Pit-2, which are members of the SLC20

| Transporter | Expression | Loss of Function | Ref. |
|-------------|------------|-----------------|------|
| Pit-1 (SLC20A1) | VSMCs, Chondrocytes | Not produced ectopic calcification | Crouthamel MH, 2013. Cecil DL, 2005. |
| Pit-2 (SLC20A2) | Brain, Liver, Heart, VSMCs | Brain Calcification | Wang C, 2012. |
| NaPi-Iia (SLC34A1) | Kidney, Bone (osteoclast) | Nephrocalcinosis, stone formation | Beck L, 1998. |
| NaPi-Iib (SLC34A2) | Small Intestine, Testis, Liver, Lung, Mammary Glands | Testicular Calcification, Pulmonary microlithiasis | Corut A. 2006. Huqun IS, 2007. |
| NaPi-Iic (SLC34A3) | Kidney | ? | | |
family of solute carriers [121]. Both co-transporters mediate the movement of Pi ions across the cell membrane [40, 122] and are ubiquitously expressed, suggesting a “housekeeping” function. More precise localization studies revealed different levels of Pit-1 and Pit-2 expression. Expression of Pit-1 mRNA is highest in osteoblasts, vascular smooth muscle cells (VSMCs), and bone marrow [12, 40, 123]. Pit-1 plays a critical role in cartilage calcification and regulation of apoptosis and cell proliferation [124–127]. Deletion of Pit-1 expression in the mouse showed it played an essential function in liver development [128]. In a recent study, the targeted deletion of Pit-1 in VSMCs in mouse did not induce aortic calcification due to compensatory regulation by Pit-2 [129]. Moreover, the expression of Pit-2 is highest in VSMCs [40], liver, heart, and brain [121]. An association has recently been found between loss-of-function of Pit-2 and familial idiopathic basal ganglia calcification in humans [38]. In addition, a recent study [130] has showed that knockout of Pit-2 in mice causes calcification in the thalamus, basal ganglia, and cortex, demonstrating that reduced Pit2 expression alone can cause brain calcification (see Table 3).

CLINICAL PERSPECTIVE

This association between los of function in NPT and ectopic calcification complete the cycle of extracellular ATP metabolism (see Fig. 3) and suggest that the role of sodium phosphate co-transporters in the initiation and progression of ectopic calcification should be reinterpreted as a key piece in the synthesis of calcification inhibitors. Moreover, in clinical practice, is important to evaluate both phosphate and pyrophosphate homeostasis in order to respond two important questions 1) Does Pi in serum is high? and 2) Does P Pi in serum is low?. In the case of positive response the first treatment that we should think in order to designer the cor-
trect therapeutic strategic to prevent vascular calcification is, respectively: 1) phosphate binders in order to reduce the amount of Pi in blood; and 2) injections of P Pi in order to increase the availability of P Pi. Both types of treatment men-
tioned in this review have been used in clinical trials in pa-
tients receiving chronic hemodialysis (for example, www.clinicaltrial.gov identifiers: NCT01755078 and NCT01503021).

LIST OF ABBREVIATIONS

| Abbreviation | Description |
|--------------|-------------|
| ACP          | amorphous calcium phosphate |
| Ado          | adenosine |
| ADP          | adenosine-5′-diphosphate |
| AMP          | adenosine-5′-monophosphate |
| ATP          | adenosine-5′-triphosphate |
| β-TCP        | β-tricalcium phosphate |
| CC           | chondrocalcinosis |
| CMD          | craniometaphyseal dysplasia |
| CPD          | Calcium-phosphate deposition |
| DCPA         | dicalcium phosphate anhydrous |
| DCPD         | dicalcium phosphate dehydrate |
| eNPP         | ecto-nucleotide pyrophosphatase/phosphodiesterase |
| ENT1         | equilibrative nucleoside transporter 1 |
| HA           | hydroxyapatite |
| MCPA         | monocalcium phosphate anhydrous |
| MCPM         | monocalcium phosphate monohydrate |
| NaPi         | sodium phosphate co-transporters |
| OCP          | octocalcium phosphate |
| P Pi         | Pyrophosphate |
| TNAP         | Tissue non-specific alkaline phosphatase |
| VC           | Vascular calcification |
| VSMCs        | vascular smooth muscle cells |

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by a Junior Grant “Juan de la Cierva” postdoctoral contract from Ministerio de Economía y Competitividad (MINECO), Spain (JCI-2011-09663).

DISCLOSURES

None

REFERENCES

[1] Shanahan CM, Crouthamel MH, Kapustin A, Giachelli CM. Arterial calcification in chronic kidney disease: key roles for calcium and phosphate. Circ Res 2011; 109:697-711.
[2] Rutsch F, Nitschke Y, Terkeltaub R. Genetics in arterial calcification: pieces of a puzzle and cogs in a wheel. Circ Res 2011; 109:578-592.
[3] Monckeberg JG. Uber die reine mediaverkalkung der extremitatenarterien und ihr verhalten zur arteriosklerose. Virchows Arch Pathol Anat 1902; 171:141-167.
[4] Rocha-Singh KJ, Zeller T, Jaff MR. Peripheral arterial calcification: Prevalence, mechanism, detection, and clinical implications. Catheter Cardiovasc Interv 2014.
[5] Urry DW. Neutral sites for calcium ion binding to elastin and collagen: a charge neutralization theory for calcification and its relationship to atherosclerosis. Proc Natl Acad Sci U S A 1971; 68:810-814.
[6] Giachelli CM. The emerging role of phosphate in vascular calcification. Kidney Int 2009; 75:890-897.
[7] Villa-Belotta R, Millan A, Sorribas V. Role of calcium-phosphate deposition in vascular smooth muscle cell calcification. Am J Physiol Cell Physiol 2011; 300:C210-220.
[8] Villa-Belotta R, Sorribas V. Phosphonoformic acid prevents vascular smooth muscle cell calcification by inhibiting calcium-phosphate deposition. Arterioscler Thromb Vasc Biol 2009; 29:761-766.
[9] Ketteler M, Biggar PH. Use of phosphate binders in chronic kidney disease. Curr Opin Nephrol Hypertens 2013; 22:413-420.
[10] Malberti F. Hyperphosphataemia: treatment options. Drugs 2013; 73:673-688.
[11] Jono S, McKee MD, Murry CE et al. Phosphate regulation of vascular smooth muscle cell calcification. Circ Res 2000; 87:E10-17.
[12] Li X, Yang HY, Giachelli CM. Role of the sodium-dependent phosphate co-transporter, Pit-1, in vascular smooth muscle cell calcification. Circ Res 2006; 98:905-912.
Vascular Calcification Revisited

MacGregor J, Robertson WG, Nordin BE. Octocalcium phosphate: the phase governing the solubility equilibrium in apatitic calculi. Br J Urol 1965; 37:518-524.

Schibler D, Russell RG, Fleisch H. Inhibition by pyrophosphate and polyphosphate of aortic calcification induced by vitamin D3 in rats. Clin Sci 1968; 35:363-372.

Schinke T, Karsenty G. Vascular calcification—a passive process in need of inhibitors. Nephrol Dial Transplant 2000; 15:1272-1274.

O’Neill WC. Vascular calcification: not so crystal clear. Kidney Int 2007; 71:282-283.

Proudfoot DJ, Skepper JN, Hegyi L et al. Apoptosis regulates human vascular calcification in vitro: evidence for inhibition of vascular calcification by apoptotic bodies. Circ Res 2000; 87:1055-1062.

Ewence AE, Bootman M, Roderick HL et al. Calcium phosphate crystals induce cell death in human vascular smooth muscle cells: a potential mechanism in atherosclerotic plaque destabilization. Circ Res 2008; 103:e28-34.

Shreff EC, McNair R, Figg N et al. Dialysis accelerates medial vascular calcification in part by triggering smooth muscle cell apoptosis. Circulation 2008; 118:1748-1757.

Steitz SA, Speer MY, Curinga G et al. Smooth muscle cell phenotypic transition associated with calcification: upregulation of Cbfα 1and downregulation of smooth muscle lineage markers. Circ Res 2001; 89:1147-1154.

Li X, Yang HY, Giachelli CM. BMP-2 promotes phosphate uptake, phenotypic modulation, and calcification of human vascular smooth muscle cells. Atherosclerosis 2008; 199:271-277.

Speer MY, Li X, Hiremath PG, Giachelli CM. Runx2/Cbfα 1, but not loss of myocardium, is required for smooth muscle cell lineage reprogramming toward osteohedrosclerosis. J Cell Biochem 2010; 110:935-947.

Sage AP, Lu J, Tintut Y, Demer LL. Hyperphosphatemia-induced nanocrystals upregulate the expression of bone morphogenetic protein-2 and osteopentin genes in mouse smooth muscle cells in vitro. Kidney Int 2011; 79:414-422.

Lei Y, Sinha A, Nosoudi N et al. Hydroxypatite and calcified elastic induce osteoblast-like differentiation in rat aortic smooth muscle cells. Exp Cell Res 2014.

Villa-Bellotta R, Sorribas V. Calcium phosphate deposition with normal phosphate concentration. -Role of pyrophosphate. Circ J 2011; 75:2705-2710.

Fish RS, Klootwijk E, Tam FW et al. ATP and arterial calcification. Eur J Clin Invest 2013; 43:405-412.

Zimmermann H. Extracellular metabolism of ATP and other nucleotides. Naunyn Schmiedebergs Arch Pharmacol 2000; 362:299-309.

O’Neill WC, Lamoshvili KA, Malluche HH et al. Reduced plasma calcium phosphate in dialysis patients treated with vitamin D3: a pilot study in Sprague-Dawley rats. Circ J 2007; 71:1152-1156.

Lamoshvili KA, Monier-Faugere MC, Wang X et al. Effect of bisphosphonates on vascular calcification and bone metabolism in experimental renal failure. Kidney Int 2009; 75:617-625.

Warraich S, Bone DB, Quinonez D et al. Loss of equilibrative nucleoside transporter 1 in mice leads to progressive ectopic mineralization of spinal tissues resembling diffuse idiopathic skeletal hyperostosis in humans. J Bone Miner Res 2013; 28:1135-1149.

Sugita A, Kawai S, Hayashibara T et al. Cellular ATP synthesis mediated by type III sodium-dependent phosphate transporter Pit-1 is critical to chondrogenesis. J Biol Chem 2011; 286:3094-3103.

Wang C, Li Y, Shi L et al. Mutations in SLC20A2 link familial idiopathic basal ganglia calcification with phosphate homeostasis. Nat Genet 2012; 44:254-256.

Lohman AW, Billaud M, Isakson BE. Mechanisms of ATP release and signalling in the blood vessel wall. Cardiovasc Res 2012; 95:269-280.

Villa-Bellotta R, Bogaert YE, Levi M, Sorribas V. Characterization of phosphate transport in rat vascular smooth muscle cells: implications for vascular calcification. Arterioscler Thromb Vase Biol 2007; 27:1030-1036.

Johnsson MS, Nancollas GH. The role of brushite and octacalcium phosphate in apatite formation. Crit Rev Oral Biol Med 1992; 3:61-82.

LeGeros RZ. Calcium phosphates in oral biology and medicine. Monogr Oral Sci 1991; 15:1-201.

LeGeros RZ. Formation and transformation of calcium phosphates: relevance to vascular calcification. Z Kardiol 2001; 90 Suppl 3:116-124.

O’Neill WC. The fallacy of the calcium-phosphorus product. Kidney Int 2007; 72:792-796.

Kodaka T, Debari K, Higashi S. Magnesium-containing crystals in human dental calculus. J Electron Microsc (Tokyo) 1988; 37:73-80.

Scottichord CA, Ali SY. Magnesium whitlockite deposition in articular cartilage: a study of 80 specimens from 70 patients. Ann Rheum Dis 1954; 54:339-344.

Reid JD, Anderssen ME. Medial calcification (whitlockite) in the aorta. Atherosclerosis 1993; 101:213-224.

PNg C H, Boadile R, Horton M et al. Magnesium whitlockite of the aorta. Pathology 2008; 40:539-540.

Lee JS, Morrissett JD, Tung CH. Detection of hydroxyapatite in calcified cardiovascular tissues. Atherosclerosis 2012; 224:340-347.

Kanzaki N, Treboux G, Onuma K et al. Calcium phosphate clusters. Biomaterials 2001; 22:2921-2929.

Kai MI, Young RA, Posner AS. Crystal Structure of Hydroxyapatite. Nature 1964; 204:1050-1052.

Posner AS, Betts F. Synthetic amorphous calcium phosphate and its relation to bone mineral structure. Acc. Chem. Res. 1975; 8:273-281.

Nancollas GH, Johnsson MA. Calculus formation and inhibition. Adv Dent Res 1994; 8:307-311.

Tomazic BB, Brown WE, Schoen FJ. Physicochemical properties of calcific deposits isolated from porcine bioprosthetic heart valves removed from patients following 2-13 years function. J Biomed Mater Res 1994; 28:35-47.

Price PA, Chan WS, Jolson DM, Williamson MK. The elastic lamellae of devitalized arteries calcify when incubated in serum: evidence for a serum calcification factor. Arterioscler Thromb Vase Biol 2006; 26:1079-1085.

Khavanidar Z, Romans H, Li J et al. Elastin Haploinsufficiency Impedes the Progress of Arterial Calcification in MGP-Deficient Mice. J Bone Miner Res 2014; 29:327-337.

Hosaka N, Mizobuchi M, Ogata H et al. Elastin degradation accelerates phosphate-induced mineralization of vascular smooth muscle cells. Calcif Tissue Int 2009; 85:529-532.

Aflrey AC, Ibels LS. Role of phosphate and pyrophosphate in soft tissue calcification. Adv Exp Med Biol 1978; 103:187-193.

Fleisch H. Inhibitors and promoters of stone formation. Kidney Int 1978; 13:361-371.

Schleiper G, Westenfeld R, Brandenburg V, Ketteler M. Inhibitors of calcification in blood and urine. Semin Dial 2007; 20:113-121.

Lamoshvili KA, Khaswani W, O’Neill WC. Reduced plasma calcium phosphate levels in hemodialysis patients. J Am Soc Nephrol 2005; 16:2495-2500.

O’Neill WC, Sigrist MK, McIntyre CW. Plasma pyrophosphate and vascular calcification in chronic kidney disease. Nephrol Dial Transplant 2010; 25:187-191.

Lamoshvili KA, Cobbs S, Hemingar RA et al. Phosphate-induced vascular calcification: role of pyrophosphate and osteopontin. J Am Soc Nephrol 2004; 15:1392-1401.
Villa-Bellosa R, Wang X, Millan JL et al. Extracellular pyrophosphate metabolism and calcification in vascular smooth muscle. Am J Physiol Heart Circ Physiol 2011; 301:H61-68.

Lomashvili KA, Garg P, Narisawa S et al. Upregulation of alkaline phosphatase and pyrophosphate hydrolysis: potential mechanism for uremic vascular calcification. Kidney Int 2008; 73:1024-1030.

Narisawa S, Yadav MC, Millan JL. In vivo overexpression of tissue-nonspecific alkaline phosphatase increases skeletal mineralization and affects the phosphorylation status of osteopontin. J Bone Miner Res 2013; 28:1587-1598.

Narisawa S, Harmey D, Yadav MC et al. Novel inhibitors of alkaline phosphatases suppress vascular smooth muscle cell calcification. J Bone Miner Res 2007; 22:1700-1710.

Kiffer-Moreira T, Yadav MC, Zhu D et al. Pharmacological inhibition of PHOSPHO1 suppresses vascular smooth muscle cell calcification. J Bone Miner Res 2013; 28:881-91.

Yadav MC, Simao AM, Narisawa S et al. Loss of skeletal mineralization by the simultaneous ablation of PHOSPHO1 and alkaline phosphatase function: a unified model of the mechanisms of initiation of skeletal calcification. J Bone Miner Res 2011; 26:286-297.

Rutsch F, Ruf N, Vaingankar S et al. Mutations in ENPP1 are associated with ‘idiopathic’ infantile arterial calcification. Nat Genet 2003; 34:379-381.

Ruf N, Uhlenberg B, Terkelbaub R et al. The mutational spectrum of ENPP1 as arising after the analysis of 23 unrelated patients with generalized arterial calcification of infancy (GACI). Hum Mutat 2005; 25:98.

Koshizuka Y, Ikegawa S, Sano M et al. Isolation of novel mouse genes associated with ectopic ossification by differential display method using tw, a mouse model for ectopic ossification. Cytogenet Cell Genet 2001; 94:163-168.

Cecil DL, Terkelbaub RA. Arterial calcification is driven by RAGE in Enpp1−/− mice. J Vac Res 2011; 48:227-235.

Johnson K, Polewski M, van Etten D, Terkeltaub R. Chondrogenesis mediated by PPi depletion promotes spontaneous tissue mineralization by the simultaneous ablation of PHOSPHO1 and TNAP genes in chondrocalcinosis. Rheumatology (Oxford) 2007; 459:499-508.

Costello JC, Rosenthal AK, Kurup IV et al. Parallel regulation of extracellular ATP and inorganic pyrophosphate: roles of growth factors, transcription modulators, and ANK. Connect Tissue Res 2011; 52:139-146.

Wang X, Xu J, Du B, Kirsch T. Role of the progressive ankylosis gene (ank) in cartilage mineralization. Mol Cell Biol 2005; 25:312-323.

Addison WN, Azari F, Sorensen ES et al. Pyrophosphate inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating osteopontin, and inhibiting alkaline phosphatase activity. J Biol Chem 2007; 282:15872-15883.

Zhang Y, Brown MA, Peach C et al. Investigation of the role of ENPP1 and TNAP genes in chondrocalcinosis. Rheumatology (Oxford) 2007; 46:586-589.

Costello JC, Rosenthal AK, Kurup IV et al. Parallel regulation of extracellular ATP and inorganic pyrophosphate: roles of growth factors, transcription modulators, and ANK. Connect Tissue Res 2011; 52:139-146.

Addison WN, Azari F, Sorensen ES et al. Pyrophosphate inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating osteopontin, and inhibiting alkaline phosphatase activity. J Biol Chem 2007; 282:15872-15883.

Zhang Y, Brown MA, Peach C et al. Investigation of the role of ENPP1 and TNAP genes in chondrocalcinosis. Rheumatology (Oxford) 2007; 46:586-589.

Costello JC, Rosenthal AK, Kurup IV et al. Parallel regulation of extracellular ATP and inorganic pyrophosphate: roles of growth factors, transcription modulators, and ANK. Connect Tissue Res 2011; 52:139-146.

Addison WN, Azari F, Sorensen ES et al. Pyrophosphate inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating osteopontin, and inhibiting alkaline phosphatase activity. J Biol Chem 2007; 282:15872-15883.
[111] Wagner CA, Hernando N, Forster IC, Biber J. The SLC34 family of sodium-dependent phosphate transporters. Pflugers Arch 2014; 466:139-153.

[112] Silverstein DM, Barac-Nieto M, Murer H, Spitzer A. A putative growth-related renal Na(+)-Pi cotransporter. Am J Physiol 1997; 273:R928-933.

[113] Segawa H, Kaneko I, Takahashi A et al. Growth-related renal type II Na/Pi cotransporter. J Biol Chem 2002; 277:19665-19672.

[114] Beck L, Karaplis AC, Amizuka N et al. Targeted inactivation of Npt2 in mice leads to severe renal phosphate wasting, hypercalciumia, and skeletal abnormalities. Proc Natl Acad Sci U S A 1998; 95:5372-5377.

[115] Ichikawa S, Sorensen AH, Imel EA et al. Intronic deletions in the SLC34A3 gene cause hereditary hypophosphatemic rickets with hypercalciumia. J Clin Endocrinol Metab 2006; 91:4022-4027.

[116] Bergwitz C, Roslin NM, Tieder M et al. SLC34A3 mutations in patients with hereditary hypophosphatemic rickets with hypercalciumia predict a key role for the sodium-phosphate cotransporter NaPi-IIc in maintaining phosphate homeostasis. Am J Hum Genet 2006; 78:193-201.

[117] Lorenz-Depiereux B, Benet-Pages A, Eckstein G et al. Hereditary hypophosphatemic rickets with hypercalciumia is caused by mutations in the sodium-phosphate cotransporter gene SLC34A3. Am J Hum Genet 2006; 78:193-201.

[118] Sabbagh Y, O’Brien SP, Song W et al. Intestinal npt2b plays a major role in phosphate absorption and homeostasis. J Am Soc Nephrol 2009; 20:2348-2358.

[119] Corut A, Senyigit A, Ugur SA et al. Mutations in SLC34A2 cause pulmonary alveolar microlithiasis and are possibly associated with testicular microlithiasis. Am J Hum Genet 2006; 79:650-656.

[120] Huqn, Izumi S, Miyazawa H et al. Mutations in the SLC34A2 gene are associated with pulmonary alveolar microlithiasis. Am J Respir Crit Care Med 2007; 175:263-268.

[121] Collins JF, Bai L, Ghishan FK. The SLC20 family of proteins: dual functions as sodium-phosphate cotransporters and viral receptors. Pflugers Arch 2004; 447:647-652.

[122] Bottger P, Hede SE, Grunnet M et al. Characterization of transport mechanisms and determinants critical for Na+-dependent Pi symport of the PiT family paralogs human PiT1 and PiT2. Am J Physiol Cell Physiol 2006; 291:C1377-1387.

[123] Yoshiko Y, Candeliere GA, Maeda N, Aubin J-E. osteoblast autonomous Pi regulation via PiT1 plays a role in bone mineralization. Mol Cell Biol 2007; 27:4465-4474.

[124] Beck L, Leroy C, Salaun A et al. Identification of a novel function of PiT1 critical for cell proliferation and independent of its phosphate transport activity. J Biol Chem 2009; 284:31363-31374.

[125] Salaun C, Leroy C, Rousseau A et al. Identification of a novel transport-independent function of PiT1/SLC20A1 in the regulation of TNF-induced apoptosis. J Biol Chem 2010; 285:34408-34418.

[126] Cecil DL, Rose DM, Turkeltaub R, Liu-Bryan R. Role of interleukin-8 in PiT1 expression and CXCR1-mediated inorganic phosphate uptake in chondrocytes. Arthritis Rheum 2005; 52:144-154.

[127] Mansfield K, Teixeira CC, Adams CS, Shapiro IM. Phosphate ions mediate chondrocyte apoptosis through a plasma membrane transporter mechanism. Bone 2001; 28:1-8.

[128] Beck L, Leroy C, Beck-Cormier S et al. The phosphate transporter PiT1 (Slc20a1) revealed as a new essential gene for mouse liver development. PLoS One 2010; 5:e9148.

[129] Crouthamel MH, Lau WL, Leaf EM et al. Sodium-dependent phosphate cotransporters and phosphate-induced calcification of vascular smooth muscle cells: redundant roles for PiT-1 and PiT-2. Arterioscler Thromb Vase Biol 2013; 33:2625-2632.

[130] Jensen N, Schroder HD, Hejbol EK et al. Loss of function of Slc20a2 associated with familial idiopathic Basal Ganglia calcification in humans causes brain calcifications in mice. J Mol Neurosci 2013; 51:994-999.