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REVIEW ARTICLE

Tissue Non-Specific Alkaline Phosphatase and Vascular Calcification: A Potential Therapeutic Target

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Abstract: Vascular calcification is a pathologic phenomenon consisting of calcium phosphate crystal deposition in the vascular walls. Vascular calcification has been found to be a risk factor for cardiovascular diseases, due to its correlation with cardiovascular events and mortality, and it has been associated with aging, diabetes, and chronic kidney disease.

Studies of vascular calcification have focused on phosphate homeostasis, primarily on the important role of hyperphosphatemia. Moreover, vascular calcification has been associated with loss of plasma pyrophosphate, one of the main inhibitors of calcification, thus indicating the importance of the phosphate/pyrophosphate ratio.

Extracellular pyrophosphate can be synthesized from extracellular ATP by ecto-nucleotide pyrophosphatase/phosphodiesterase, whereas pyrophosphate is hydrolyzed to phosphate by tissue-nonspecific alkaline phosphatase, contributing to the formation of hydroxyapatite crystals.

Over the last decade, vascular calcification has been the subject of numerous reviews and studies, which have revealed new agents and activities that may aid in explaining the complex physiology of this condition. This review summarizes current knowledge about alkaline phosphatase and its role in the process of vascular calcification as a key regulator of the phosphate/pyrophosphate ratio.

Keywords: Vascular calcification, TNAP, alkaline phosphatase, pyrophosphate, hemodialysis, CKD, aging.

1. INTRODUCTION

Vascular calcification is a pathologic phenomenon consisting of the deposition of calcium phosphate crystals in vascular walls [1, 2]. Vascular calcification has been found to be a risk factor for cardiovascular diseases due to its correlation with cardiovascular events and mortality, and it has been associated with aging, diabetes, and Chronic Kidney Disease (CKD) [3-5]. The clinical characteristics of vascular calcification include vessel thickening and the presence of hydroxyapatite crystals in vessel walls [6].

Physiologically, mineralization occurs in hard tissues, mainly bones and teeth, during development. Under pathological conditions, however, soft tissues such as vascular vessels may also undergo mineralization [7, 8], with calcium crystals deposited primarily in large elastic and muscular arteries [9], especially the aorta and the coronary and carotid arteries [1, 10], as well as in heart valves [11, 12], increasing the risk of cardiovascular infarction.

Pathological calcifications have been classified into two major types, both of which prevent correct vessel function, and both often coexisting [2, 13]. The first type is intimal calcification, which is associated with atherosclerotic plaque and is dependent on lipid and cholesterol accumulation [14]. The second type of calcification, known as Mönckeberg's sclerosis, takes place on the medial layer of the aortic wall and involves the deposition of minerals within Vascular Smooth Muscle Cells (VSMCs) [8, 15, 16].

Extracellular pyrophosphate is one of the main inhibitors of calcification, preventing the formation of hydroxyapatite crystals [17]. Extracellular pyrophosphate may be synthesized from extracellular ATP by ecto-nucleotide pyrophosphatase/phosphodiesterase [18, 19] and may be subsequently hydrolyzed to phosphate by phosphatases [20], mainly by Tissue-Nonspecific Alkaline Phosphatase (TNAP), thereby promoting calcification [6, 21-23].

2. ALKALINE PHOSPHATASE TYPES

Alkaline Phosphatases (APs) (E.C. 3.1.3.1) are ubiquitous ectoenzymes that are widely distributed in nature, from bacteria to humans, and are found in nearly all living organisms, except for some plants. Despite their wide distribution, their sequences are highly conserved, with ~57.8% of amino acids conserved in all mammals, suggesting that this family of enzymes plays a key role in physiological processes throughout evolution [24]. Although the main features of

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these enzymes are conserved from bacteria to mammals, mammalian APs have specific characteristics that enable them to adapt to different environments, such as higher activity and K_m values and lower heat stability [25].

This family of enzymes was first described in 1923 as phosphatases present in the skeleton that are responsible for generating the phosphate required for bone formation [26].

In humans, APs are encoded by four homologous genes. Three of these enzymes, Placental AP (PLAP), Germ Cell AP (GCAP), and Intestinal AP (IAP), are tissue-specific, with highly restricted expression. By contrast, the fourth isozyme, tissue non-specific AP (TNAP), is present in numerous tissues but is especially abundant in mineralizing tissues, the kidneys, and the Central Nervous System (CNS) [24].

3. SYNTHESIS

APs are synthesized as 66-kDa proteins, which are modified in the endoplasmic reticulum by the addition of carbohydrate chains through O- and N-linked sugar chains. These modified proteins are subsequently processed in the Golgi apparatus and finally localize to the outer membrane *via* Glycosylphosphatidyl Inositol (GPI) anchors. Post-transductional events allow these proteins to anchor to the membrane *via* GPI motifs, enabling movement of the enzymes in the membrane. TNAP is also detected in the systemic circulation and other biological fluids due to the action of phospholipases that cleave GPI from the membrane and release TNAP. The sugar chains of all AP isoforms differ from each other, and although all APs have the same peptide sequence, each of these isoforms has different sites of glycosylation [27].

4. STRUCTURE

All APs are homodimers consisting of two monomers (Fig. **1**), which are anchored to the cytoplasmic membrane *via* GPI [28]. These anchors consist of an ethanolamine phosphate, three residues of mannose, a glucosamine, and a phosphatidylinositol. Each monomer contains 484 amino acid residues, four metal atoms, one phosphate ion, and 603 water molecules. The central core is formed by an extended β-sheet flanked by α-helices [27]. The active site of each monomer consists of two Zn binding sites, an Mg^{2+} binding site, and a serine residue, which binds phosphate and enables monophosphate hydrolysis. The two monomers are connected by a two-fold crystallographic axis called the crown domain, which is a flexible loop formed by the insertion of a 60-residue segment from each monomer. This crown domain is formed by two small b-sheets and is surrounded by six large, flexible loops containing a short α-helix. This region is responsible for isozyme properties, such non-competitive inhibition, heat stability, and interactions with extracellular matrix proteins [27].

Each of these monomers contains three metal-binding sites (M1 and M2 for Zn^{2+} and M3 for Mg^{2+}), one phosphate-binding site, and one calcium-binding site (M4 site), all of which are necessary for enzymatic activity (Fig. **1**). Other molecules such as iron have been shown to interact with APs, decreasing its activity in a dose-dependent manner, suggesting that other molecules could interact with the metal binding sites, thereby further regulating TNAP activity [29]. Moreover, a recent study suggested that the Mg^{2+}/Ca^{2+} ratio can affect TNAP activity in the aortic wall and, therefore, affect pyrophosphate hydrolysis and the process of calcification [30].

5. ENZYME REACTIONS

Among their many activities, the role played by APs in monophosphate ester hydrolysis suggests their potential influence on phosphorus-associated pathologic conditions, such as vascular calcification. APs have many substrates and participate in many metabolic and biosynthetic pathways [31], as well as being involved in diverse microbial survival mechanisms.

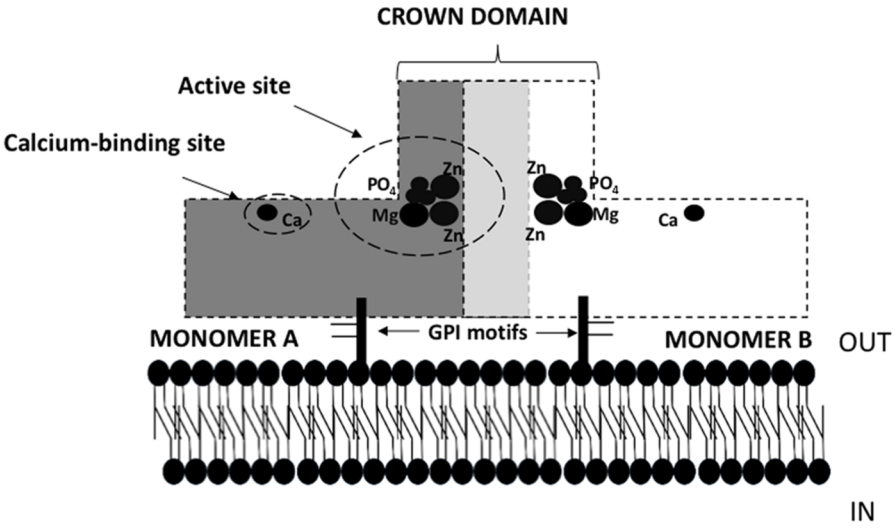

Fig. (1). Schematic representation of tissue non-specific alkaline phosphatase. Each monomer contains an active site (metal binding site) where phosphorus binds, and a calcium binding site. The calcium and metal union site allow interactions with Ca, Mg or Zn, which are necessary for the enzyme to express activity. The two monomers are connected in the crowd domain by flexible sequences of each. Both monomers are anchors to the membrane *via* GPI motifs.

The main role of AP is in the hydrolysis of phosphoric monoesters, which releases inorganic phosphate. The reaction involves the attack of a serine alkoxide on a phosphorus of the substrate to form an enzyme-phosphate complex, followed by hydrolysis of the serine phosphate [32]. Table **1** shows some of the main reactions of and products produced by APs.

Table 1. Alkaline phosphatase substrates and reactions.

Substrate	Product
R-Phosphate + H_20	R + Phosphate (Pi)
Pyrophosphate	Pi+Pi
Nucleoside triphosphate	Nucleoside diphosphate + Pi
Nucleoside diphosphate	Nucleoside monophosphate + Pi
Pyridoxal 5´-phosphate	Pyridoxal + Pi
Ethylphosphate	Ethanol + Pi
Thiamin monophosphate	Thiamine + Pi
Glycerona phosphate	Glycerone + Pi
4-nitrpphenyl phosphate	4-Nitrophenol + Pi

6. HYPOPHOSPHATASIA

One of the most common manifestations of the loss of TNAP activity is hypophosphatasia [33, 34], a systemic bone disease characterized by hypomineralization of hard tissues like teeth and structural bones. Severe forms of hypophophatasia can result in respiratory failure and death. Akp2 [35] is one of the genes encoding TNAP in mice, and knockout of this gene results in a suitable animal model of hypophosphatasia. Akp2 knockout mice transfected with a gene encoding human TNAP have also been used to assess the potential role of enzyme replacement therapy in patients with hypophophatasia [36, 37].

7. ROLE OF ALKALINE PHOSPHATASE IN VASCULAR CALCIFICATION

Extracellular pyrophosphate is a potent physicochemical inhibitor of hydroxyapatite crystal formation and growth [17, 38, 39]. Recent studies suggest that plasma pyrophosphate deficiency is associated with vascular calcification [40]. For example, in hemodialysis patients, plasma pyrophosphate concentration is reduced after standard hemodialysis [41, 42]. Moreover, plasma pyrophosphate concentration was 4-fold lower in a mouse model of progeria than in wild-type mice [43]. These reductions in plasmatic pyrophosphate were associated with excessive vascular calcification in the medial layer of the aortic wall. Moreover, daily injections of exogenous pyrophosphate were found to prevent vascular calcification in rats and mice [43-45].

TNAP hydrolyzes pyrophosphate to phosphate (Fig. **2**) in extracellular fluids [6, 21]. The addition of AP to culture media is sufficient to cause matrix calcification [46]. Moreover, over-expression of TNAP in cells is sufficient to induce medial vascular calcification in rat aortic rings *ex vivo* [21]. TNAP activity is increased in models of medial calcification, including uremic rats [23] and a mouse model of Hutchison-Gilford Progeria Syndrome [43]. Moreover, dialysis was recently shown to affect both plasma AP activity [42] and pyrophosphate hydrolysis [47] . Over-expression of TNAP *in vivo* increases skeletal mineralization [48]. However, phosphatase inhibitors have been shown to prevent vascular smooth muscle calcification *in vitro* [49, 50] and ablation of phosphatase function induces a loss of skeletal mineralization [51]. Finally, TNAP stimulates vascular smooth muscle cell trans-differentiation into chondrocytes through calcium deposition and BMP-2 activation [52]. Taken together, all of these findings suggest that TNAP could represent a target for the treatment of ectopic calcification in blood vessels [53, 54].

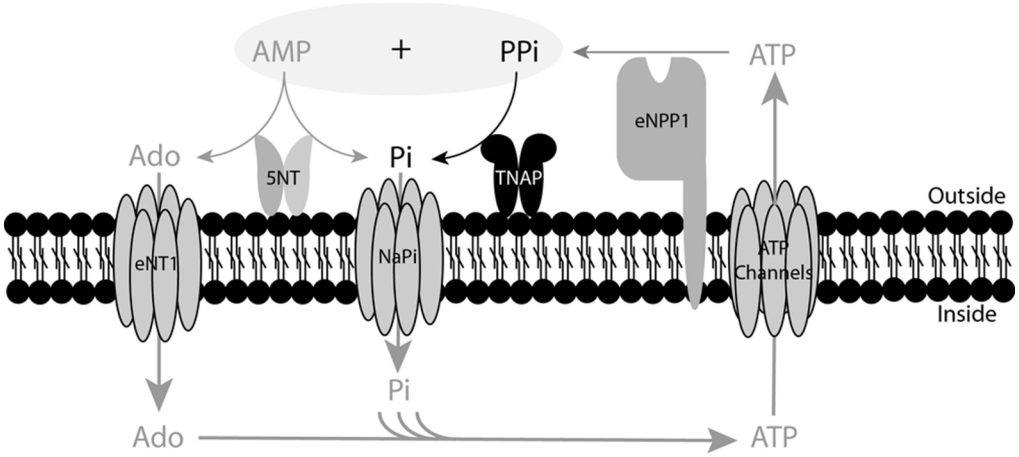

Fig. (2). Role of TNAP in the extracellular pyrophosphate metabolism. ATP is released by cells via exocytotic mechanisms and through multiple types of membrane channels. Ectonucleotide pyrophosphatase phosphodiesterase (eNPP) hydrolyzes ATP, releasing pyrophosphate (PPi) and adenosine-5'-monophosphate (AMP). PPi is degraded to phosphate (Pi) by tissue non-specific alkaline phosphatase (TNAP). AMP is degraded to adenosine (Ado) and Pi *via* ecto-5´nucleotidase (5NT). Ado and Pi are recovered from the extracellular space by equilibrative nucleoside transporter 1 (ENT1) and sodium phosphate transporter (NaPi), respectively. ATP is generated in the mitochondria or through another metabolic pathway.

8. GENETICS

Human APs are encoded by four genes, each of which encodes one of the four isozymes, with the *ALPL*, *ALPP*, *ALPP2*, and *ALPI* genes encoding TNAP, placental AP, germ cell AP and intestinal AP, respectively. *ALPL*, the gene encoding TNAP, is located on the short arm of chromosome 1 (1p36.1-34), whereas the other genes are present on the long arm of chromosome 2 (2q34-37) [25].

The *ALPL* gene extends over approximately 40–50 kb of DNA [55] and consists of 12 exons. Exons 2–12 are coding exons, whereas exon 1 consists of two alternative noncoding exons and regulatory motifs in the 5'-untranslated regions, allowing two transcripts to originate from the same coding region. In contrast to *ALPL*, the genes encoding the tissue specific APs are very compact, occupying less than 5 kb each, and show highly homologous organization [27].

CLINICAL PERSPECTIVE AND CONCLUSION

AP activity has been associated with the occurrence of vascular calcification. The potential role of APs in calcification suggests that inhibition of APs may constitute a potential therapeutic approach to prevent calcification. Studies assessing the effects of inhibitors of AP expression and activity in diseases that manifest excessive vascular calcification are warranted.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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