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Vascular calcification is dependent on plasma levels of pyrophosphate

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Abstract

Plasma levels of pyrophosphate, an endogenous inhibitor of vascular calcification, are reduced in end-stage renal disease and correlate inversely with arterial calcification. However, it is not known whether the low plasma levels are directly pathogenic or are merely a marker of reduced tissue levels. This was tested in an animal model in which aortas were transplanted between normal mice and Enpp1−/− mice lacking ectonucleotide pyrophosphatase phosphodiesterase, the enzyme that releases extracellular pyrophosphate. Enpp1−/− mice had very low plasma pyrophosphate and developed aortic calcification by 2 months that was greatly accelerated with a high-phosphate diet. Aortas of Enpp1−/− mice showed no further calcification after transplantation into wild type mice fed a high phosphate diet. Aorta allografts of wild type mice calcified in Enpp1−/− mice but less so than the adjacent recipient Enpp1−/− aorta. Donor and recipient aortic calcium contents did not differ in transplants between wild type and Enpp1−/− mice, demonstrating that transplantation per se did not affect calcification. Histology revealed medial calcification with no signs of rejection. Thus, normal levels of extracellular pyrophosphate are sufficient to prevent vascular calcification and systemic Enpp1 deficiency is sufficient to produce vascular calcification despite normal vascular extracellular pyrophosphate production. This establishes an important role for circulating extracellular pyrophosphate in preventing vascular calcification.

Introduction

Vascular calcification is a common occurrence in chronic kidney disease (CKD) and end-stage renal disease (ESRD) that likely contributes to high burden of cardiovascular disease in these conditions. Although the pathogenesis is multifactorial, it is clear that deficiency of endogenous inhibitors of hydroxyapatite formation such as extracellular pyrophosphate (ePPi) play an important role. Inhibitory concentrations of ePPi are present in the circulation1,2 and are reduced in ESRD2 and correlate inversely with arterial calcification in CKD and ESRD3. However, it is not known whether this systemic deficiency plays a
direct role in calcification or is merely a marker of decreased tissue production. Although exogenous PPi can prevent vascular calcification in uremic animals, this requires very large doses that result in supraphysiologic plasma levels.

Ectonucleotide pyrophosphatase phosphodiesterase (NPP1) is the enzyme that synthesizes ePPi, using ATP released by cells as a substrate. The skeleton is a major site of NPP1-mediated synthesis of ePPi but NPP1 is also present in vascular smooth muscle. Deficiency in humans results in severe, fatal arterial calcification in infancy and arterial calcification also occurs in mice lacking this enzyme when fed a high-phosphate diet. NPP1 also has important roles in purinergic signaling and insulin action independent of ePPi synthesis but their role in vascular calcification remains unclear. A potential alternate source of ePPi is release of PPi from cells, which may occur through the membrane protein ANK. Although deficiency of ANK can promote vascular calcification, the relative importance of NPP1 and ANK and their contribution to plasma ePPi remain unclear.

To determine the role of systemic versus vascular production of PPi, plasma ePPi and aortic calcification were examined in NPP1-deficient (Enpp1−/−) mice, and aortic calcification was compared in aortas transplanted between normal and Enpp1−/− mice. The results not only establish an important role for systemic ePPi but also demonstrate that NPP1 is the major source of plasma ePPi and that PPi production can account for the effect of NPP1 on vascular calcification.

**Results**

Mice homozygous for the Enpp1 null mutation (Enpp1−/−) spontaneously developed aortic calcification by age 2 months (Fig. 1). This calcification was variable and frequently not apparent by staining with alizarin red, and was quite focal so that examination of multiple sections was required for histologic detection. Calcification was greatly accelerated by increasing the phosphorus content of the diet from 0.4% to 1.5%. Quantitative data are provided in Fig. 2 and show that on a 0.4% diet, calcium content of the abdominal aorta was approximately 4-fold higher in Enpp1−/− mice compared to wild-type mice, but was approximately 40-fold higher on a 1.5% phosphorus diet. Dietary phosphorus had no effect on aortic calcium content in wild-type mice and there was no significant effect of age between 2 and 4 months on aortic calcium content in either type of mice (not shown).

As shown in Table 1, plasma PPi, measured on the high-phosphorus diet, was approximately 30-fold lower in the Enpp1−/− mice (p=0.0013), consistent with the absence of the synthetic enzyme. Other potential mechanisms for the calcification were also explored. Specifically, plasma phosphate and calcium, also shown in Table 1, were not elevated in Enpp1−/− mice. Plasma phosphate tended to be lower in Enpp1−/− mice, probably explained by the phosphaturia in these mice that may be due to elevated fibroblast growth factor-23 levels. Activity and content of tissue non-specific alkaline phosphatase (TNAP) did not differ between wild-type and Enpp1−/− aortas from mice aged 2–4 months, an age at which some calcification is present (Fig. 3A,B). To assess osteogenic trans-differentiation, immunohistochemistry for osterix, an osteogenic transcription factor, was performed in 6 sections from 6 different calcified Enpp1 aortas (age 4–6 months). There was no cellular...
staining (Fig. 3C) while nuclear staining was readily apparent in neonatal spine used as a positive control (Fig. 3D). Non-specific staining of the calcifications was present in some of the sections.

To determine the extent to which this difference in plasma ePPi influenced vascular calcification, calcification was examined in aortas transplanted between Enpp1−/− and wild-type mice. Aortas were transplanted orthotopically from 2 month-old mice into the infrarenal portion of the abdominal aorta of littermates, after which the mice were fed a 1.5% phosphorus diet for another 2 months. Operative mortality was as follows: WT allografts into WT, 2 out of 10; Enpp1−/− allografts into WT, 0 out of 8; Enpp1−/− allografts into Enpp1−/−, 3 out of 15; WT allografts into Enpp1−/−, 1 out of 8. In total, WT recipients had an 11% mortality while Enpp1−/− recipients had a 17% mortality, due entirely to graft thrombosis. There was no delayed mortality. The mice were otherwise healthy and transplantation did not affect aortic structure (Fig. 4). Specifically, there was no intimal thickening, cellular infiltration, or fibrosis suggestive of rejection. Alizarin red stains of representative aortic allografts and recipient aortas 2 months after transplantation are shown in Figure 5 while quantitative data are provided in Figure 6A. There was no calcification of wild-type allografts placed into wild-type recipients, indicating that transplantation alone does not induce calcification. Wild-type allografts had a significantly greater calcium content when placed into Enpp1−/− recipients than into wild-type recipients (49.3 ± 15.0 nmol/mg vs. 14.4 ± 3.1 nmol/mg; p=0.039) but the content was far lower than in the adjacent recipient Enpp1−/− aorta or in Enpp1−/− allografts placed in Enpp1−/− mice. Enpp1−/− allografts transplanted into WT mice showed a small amount of calcification but this did not differ from that present in Enpp1−/− aortas at 2 months of age, the time of harvest for transplantation (32.7 ± 7.2 nmol/mg vs. 28.9 ± 9.0 nmol/mg). Thus there was no increase after transplantation. There was extensive calcification of Enpp1−/− allografts in Enpp1−/− mice that did not differ from the recipient aortas, indicating that transplantation did not suppress calcification. As shown in Figures 6B and 6C, the histologic pattern of calcification was identical in Enpp1 allografts and recipient aortas. Thus, transplantation per se did not affect calcification.

Discussion

Mice lacking NPP1 spontaneously developed aortic calcification that was exclusively medial in its pattern. This could not be explained by differences in systemic calcium or phosphate levels though the calcification was greatly augmented by a high-phosphate diet as previously described \(^9\). There was also no increased expression of TNAP or the osteogenic transcription factor osterix that could explain the greater calcification. However, plasma ePPi was almost undetectable in Enpp1−/− mice, consistent with deficiency of ePPi as a cause of the calcification and indicating that NPP1 is responsible for almost all of the circulating ePPi. This is in contradistinction to cultured cells, in which NPP1 and the putative transporter ANK appear to contribute equally to ePPi \(^11\). The tissue source of circulating ePPi remains to be identified. Current data point to the skeleton as the major source of ePPi \(^6\) and the vasculature is unlikely to contribute since isolated aortas do not release net amounts of ePPi \(^7\).
Calcification of Enpp1−/− aortas was completely arrested after transplantation into Enpp1+/+ littermates, indicating that systemic levels of ePPi present in normal mice are sufficient to prevent further vascular calcification. Although another circulating factor distinct from ePPi cannot be ruled out, this is unlikely since arterial calcification in NPP1 deficiency can be prevented in mice by eliminating TNAP\textsuperscript{11} and reduced in humans by treatment with bisphosphonates\textsuperscript{13}, which are PPi analogs. Interestingly, there was no reduction in calcium content, suggesting that the calcification is not spontaneously reversible.

The importance of systemic PPi was also demonstrated by the calcification of wild-type aortas transplanted into Enpp1−/− mice. Although this was far less than adjacent Enpp1−/− aortas or Enpp1−/− aortas transplanted into Enpp1−/− mice, some of this difference might be explained by the calcification that existed in the Enpp1−/− aortas at the time of transplantation and can serve as a nidus for further calcification. Thus the near absence of circulating ePPi can result in vascular calcification despite the presence of normal vascular NPP1 activity.

Other potential determinants of tissue ePPi include the membrane transporter ANK and the ecto-enzyme tissue-nonspecific alkaline phosphatase (TNAP). While the extremely low plasma PPi in Enpp1−/− mice indicates that ANK does not contribute significantly to circulating levels, it may contribute to ePPi within the vessel wall since mice lacking ANK develop arterial calcification on a high-phosphate diet\textsuperscript{11}. Such a local effect of ANK is supported by the finding that aortas cultured from these mice show increased calcification\textsuperscript{7}. However, it is not clear whether ANK controls ePPi levels directly by facilitating transport of PPi\textsuperscript{10} or indirectly by transporting and providing ATP\textsuperscript{14} for NPP1. TNAP hydrolyzes ePPi and its robust expression in bone allows mineralization to occur in the face of circulating levels of ePPi that can completely inhibit hydroxyapatite formation\textsuperscript{15,16}. TNAP activity in vascular smooth muscle is increased in uremia\textsuperscript{17} and could compound the systemic ePPi deficiency.

NPP1 has other actions that could influence vascular calcification independent of ePPi. Purinergic metabolism and signaling is dependent on NPP1\textsuperscript{18} since it is an initial first step in the synthesis of extracellular adenosine and is an important pathway for removal of ATP. Given the importance of purinergic signaling in bone and the abundance of receptors in vascular smooth muscle, perturbations in purinergic signaling could also explain or contribute to the vascular calcification that occurs in the absence of NPP1. It has recently been shown that deficiency of CD73, the final ecto-enzyme in the generation of adenosine, leads to extensive arterial calcification in humans\textsuperscript{19}. However, purinergic signaling is autocrine or paracrine in nature and any alterations due to NPP1 deficiency should not be corrected by transplantation into normal mice. While it is possible that paracrine signaling could extend into the allograft, it is unlikely to encompass the entire allograft and no gradient of calcification was observed. This indicates that purinergic signaling is either not important in vascular calcification or is not substantially altered by NPP1 deficiency.

Additionally, NPP1 interacts with the insulin receptor to inhibit insulin signaling\textsuperscript{20} but again this deficiency should not be restored or created by transplantation. Thus the results
strongly imply that NPP1 influences vascular calcification exclusively through generation of ePPi.

The results provide further evidence for the central role of ePPi in vascular calcification and demonstrate an important contribution from circulating ePPi. Thus plasma ePPi could be a measurable risk factor for vascular calcification, which is consistent with our previous findings of low plasma PPI in hemodialysis patients and of an inverse relationship between plasma PPI and vascular calcification in patients with renal disease. The ability of exogenous PPI to inhibit vascular calcification in animal models suggests that plasma PPI may also be a modifiable risk factor although direct administration is limited by its short half-life. Identification of the tissue source will be useful in this regard as will other approaches directly aimed at increasing ePPi within the vessel wall such as inhibitors of TNAP.

**Methods**

**Animals**

Enpp1−/− mice on a C57/black6 background have previously been described. Homozygous and wild-type (Enpp1+/+) mice were obtained by breeding heterozygous mice and genotyping was performed by PCR on tail DNA using GAGAAATTCAACCCTTTGTGG as the common primer and CAGCCACTGCATACCCATTA and GGGTGAGAACAGAGTACCTAC as primers for wild-type and Enpp1−/− respectively. To induce vascular calcification, the phosphorus content of the diet was increased to 1.5% by the addition of neutral sodium phosphate.

**Aortic transplantation**

Segments (4–5 mm) of infrarenal aortas were carefully dissected from mice aged 2 months and transplanted orthotopically into littermates of the same sex after removal of a similar segment of aorta, using 11-0 monofilament sutures for end-to-end anastomoses. Allografts and ends of recipient aortas were flushed with heparin (200 units/ml) prior to anastomosis to prevent thrombosis. All protocols were approved by the Emory University Institutional Animal Care and Use Committee.

**Assays**

To measure systemic PPI levels, blood was obtained by vena caval puncture using a 23-gauge needle and immediately heparinized and centrifuged at 4°C to obtain plasma. This was then immediately centrifuged through a 30 kDa molecular weight cut-off filter (Amicon Ultra, Millipore Corp., Billerica, MA) to remove residual platelets and interfering enzymes and then assayed as previously described without storage. Briefly, PPI is assayed as conversion of [14C]UDPglucose to [14C]glucose-1-phosphate with subsequent conversion to 6-[14C]phosphogluconate (using phosphoglucomutase and glucose-6-phosphate dehydrogenase) to drive the reaction to completion. Unreacted substrate is removed by charcoal precipitation. Aortic calcium was measured by the cresolphthalein method after extraction in 0.5 M HCl as previously described. Plasma calcium and phosphate were measured as previously described using cresolphthalein and acid-molybdate methods.
respectively. TNAP was measured as hydrolysis of p-nitrophenyl phosphate by intact aortas in Hanks buffered salt solution as previously described. Immunoblots of TNAP were performed as previously described. Briefly, lumbar and thoracic aortas from 3 month old mice were thoroughly cleaned of adipose tissue and extracted with RIPA buffer containing 2% SDS. Blots from SDS-PAGE were developed with rat anti-TNAP monoclonal antibody (R&D Systems, Minneapolis, MN). The membrane was stripped and re-probed with rabbit anti-α actin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Histology

Aorta were fixed in formalin and 5 micron sections were prepared and stained according to standard protocols. Staining for osterix was performed in formalin-fixed tissues with a rabbit polyclonal antibody (Abcam, Cambridge, MA) using an alkaline phosphatase-linked, anti-rabbit secondary antibody.

Statistics

Errors are presented as standard errors and means were compared using 2-tailed Student’s t-test. Due to the variability of calcium content in heavily calcified aortas, calcium content was logarithmically transformed for analyses involving these aortas. Significance was assigned at p <0.05.

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Figure 1.
Aortic calcification in Enpp1−/− mice, alizarin red stain. A. Aorta from 4 month-old wild-type mouse fed a 0.4% phosphorus diet. B. Aorta from 4 month-old Enpp1−/− mouse fed a 0.4% phosphorus diet. C. Aorta from 4 month-old Enpp1−/− mouse fed a 1.5% phosphorus diet for 6 weeks.
Figure 2.
Aortic calcium content in wild-type and Enpp1−/− mice. Mice were between 2 and 4 months old. Error bars, standard errors. Numbers in parentheses indicate the number of animals. *p<0.001 vs. wild-type.
Figure 3.
Calcification factors in calcified Enpp1−/− aortas. A. Activity of TNAP in intact mouse aortas. WT: wild-type. B. Immunoblots of TNAP. Each lane was loaded with 100 μg protein. Samples from one male and one female of each genotype are shown. Recombinant human TNAP (approximately 8 ng) was used as positive control. The faster mobility is due to the lack of glycosylation. C and D. Immunohistochemistry of osterix in calcified Enpp1−/− aorta and neonatal mouse spine.
Figure 4.
Histology of aortic allografts. Left-hand column: normal mouse aorta. Right-hand column: wild-type allograft in wild-type mouse 5 months after transplantation. Top row: hematoxylin and eosin stain. Middle row: elastin stain. Bottom row: trichrome stain.
Figure 5.
Calcification of transplanted aortas, alizarin red stain. Aortas were harvested 2 months after transplantation. A. Wild-type aorta transplanted into a wild-type mouse. There is no staining of donor or recipient aorta. B. Enpp1−/− aorta transplanted into an Enpp1−/− mouse. There is extensive staining of both the donor and recipient aorta. C. Enpp1−/− aorta transplanted into a wild-type mouse. There is no staining of the recipient aorta with scattered staining of the donor aorta (inset). D. Wild-type aorta transplanted into an Enpp1−/− mouse. There are a few foci of staining in the donor aorta (inset). Arrows indicate suture lines.
Figure 6.
Calcium content and histology in aortas transplanted between wild-type and Enpp1−/− mice. Aortas were harvested 2 months after transplantation. A. Calcium content. Error bars, standard errors. *p <0.001 vs. recipient aorta and p = 0.039 vs. WT into WT; **p = 0.078 vs. recipient aorta and p = 0.002 vs. Enpp1−/− into Enpp1−/−. Numbers in parentheses indicate the number of transplantations. B. von Kossa stain of a representative section of an Enpp1−/− aorta. C. von Kossa stain of a representative section of an Enpp1−/− aorta transplanted into an Enpp1−/− mouse. Calcifications are in the media and along the internal...
elastic lamina (arrows). The latter is often adjacent to the lumen due to loss of the overlying intima during tissue sectioning.
Table 1

Plasma phosphorus and calcium levels.

| Mice      | Dietary P (%) | Plasma P (mM) | Plasma Ca (mM) | Plasma Pi (μM) |
|-----------|---------------|---------------|----------------|----------------|
| Wild-type | 0.4           | 2.50 ± 0.28 (7) | 1.99 ± 0.08 (7) |                |
| Wild-type | 1.5           | 3.42 ± 0.16 (7) | 2.02 ± 0.04 (7) | 2.18 ± 0.33 (8) |
| Enpp1−/−  | 0.4           | 1.84 ± 0.21 (7) | 1.95 ± 0.04 (7) |                |
| Enpp1−/−  | 1.5           | 2.56 ± 0.32* (7) | 1.96 ± 0.03 (7) | 0.065 ± 0.032** (4) |

* p<0.05, ** p<0.001 vs. WT