Vascular calcification is dependent on plasma levels of pyrophosphate

Koba A. Lomashvili[1], Sonoko Narisawa[2], Jose L. Millán[2] and W. Charles O'Neill[1]

[1]Renal Division, Emory University School of Medicine, Atlanta, Georgia, USA and [2]Sanford-Burnham Medical Research Institute, Atlanta, Georgia, USA

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 synthesizes 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.

Kidney International (2014) **85,** 1351–1356; doi:10.1038/ki.2013.521; published online 9 April 2014

KEYWORDS: alkaline phosphatase; ectonucleotide pyrophosphorylase pyrophosphatase; pyrophosphate; transplantation; vascular calcification

Correspondence: *Koba A. Lomashvili, Renal Division, Emory University School of Medicine, WMB 338, 1639 Pierce Dr, Atlanta, Georgia 30322, USA. E-mail: klomash@emory.edu*

Received 10 May 2013; revised 22 October 2013; accepted 31 October 2013; published online 9 April 2014

Vascular calcification is a common occurrence in chronic kidney disease and end-stage renal disease (ESRD) and likely contributes to a 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), has an important role in this process. Inhibitory concentrations of ePPi are present in the circulation[1,2] and are reduced in ESRD[2] and correlate inversely with arterial calcification in chronic kidney disease and ESRD.[3] However, it is not known whether this systemic deficiency has a direct role in calcification or is merely a marker of decreased tissue production. Although exogenous PPi can prevent vascular calcification in uremic animals,[4,5] 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,[6] but NPP1 is also present in vascular smooth muscle.[7] Deficiency in humans results in severe, fatal arterial calcification in infancy[8] and arterial calcification also occurs in mice lacking this enzyme when fed a high-phosphate diet.[9] NPP1 also has important roles in purinergic signaling and on insulin action, independent of ePPi synthesis, but its 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.[10] Although deficiency of ANK can promote vascular calcification,[11] the relative importance of NPP1 and ANK and their contribution to plasma ePPi remain unclear.

To determine the role of systemic vs. 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 (Figure 1). This calcification was variable and frequently not

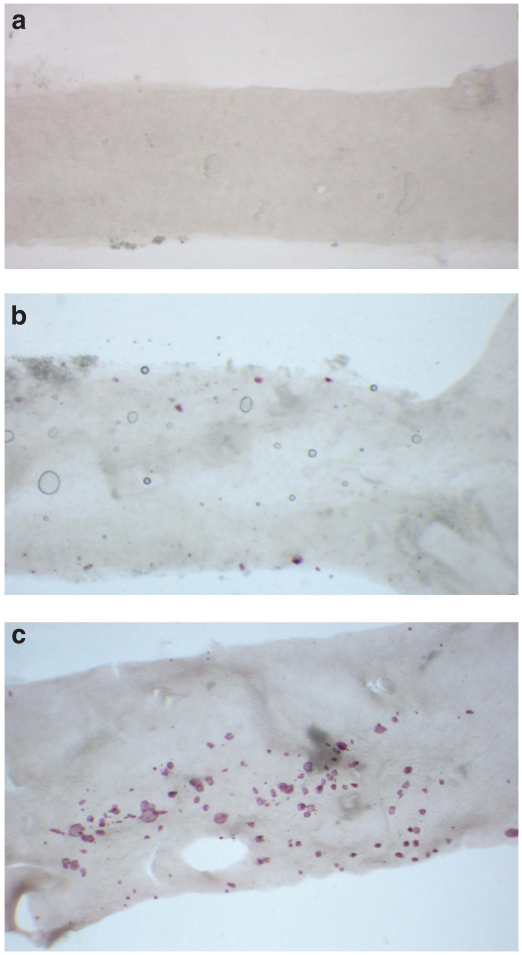

Figure 1 | Aortic calcification in Enpp1$^{-/-}$ mice, alizarin red stain. (**a**) Aorta from 4-month-old wild-type mouse fed a 0.4% phosphorus diet. (**b**) Aorta from 4-month-old Enpp1$^{-/-}$ mouse fed a 0.4% phosphorus diet. (**c**) Aorta from 4-month-old Enpp1$^{-/-}$ mouse fed a 1.5% phosphorus diet for 6 weeks.

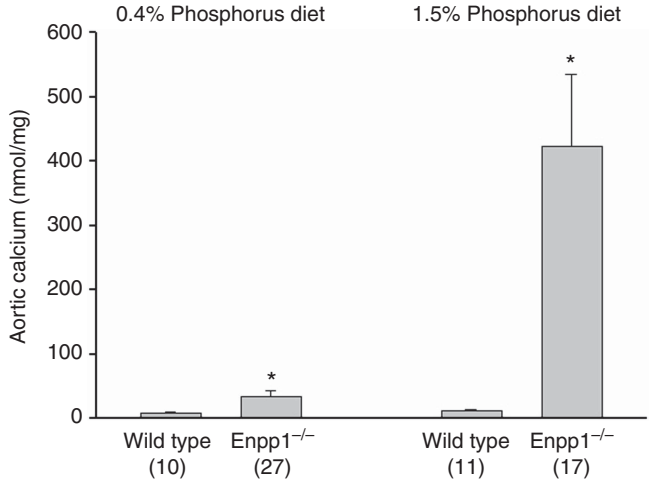

Figure 2 | Aortic calcium content in wild-type and Enpp1$^{-/-}$ mice. Mice were between 2 and 4 months old. Error bars, s.e. Numbers in parentheses indicate the number of animals. *$P<0.001$ vs. wild type.

Table 1 | Plasma phosphorus and calcium levels

Mice	Dietary P (%)	Plasma P (mmol/l)	Plasma Ca (mmol/l)	Plasma PPi (umol/l)
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.

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 Figure 2 and show that, on a 0.4% diet, calcium content of the abdominal aorta was ~4-fold higher in Enpp1$^{-/-}$ mice compared with wild-type mice, but was ~40-fold higher on a 1.5% phosphorus diet. Dietary phosphorus had no effect on the 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 mouse (not shown).

As shown in Table 1, plasma PPi, measured on the high-phosphorus diet, was ~30-fold lower in the Enpp1$^{-/-}$ mice ($P=0.0013$), consistent with the absence of the synthetic enzyme. Other potential mechanisms for 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.[12] Activity and content of tissue-nonspecific alkaline phosphatase (TNAP) did not differ between wild-type and Enpp1$^{-/-}$ aortas from mice aged 2 to 4 months, an age at which some calcification is present (Figure 3a and b). To assess osteogenic trans-differentiation, immunohistochemistry for osterix, an osteogenic transcription factor, was performed in six sections from six different calcified Enpp1 aortas (age 4–6 months). There was no cellular staining (Figure 3c), although nuclear staining was readily apparent in neonatal spine used as positive control (Figure 3d). Nonspecific 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$^{-/-}$