Dietary Pyrophosphate Modulates Calcification in a Mouse Model of Pseudoxanthoma Elasticum: Implication for Treatment of Patients

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Pseudoxanthoma elasticum is a heritable disease caused by ABCC6 deficiency. Patients develop ectopic calcification in skin, eyes, and vascular tissues. ABCC6, primarily found in liver and kidneys, mediates the cellular efflux of ATP, which is rapidly converted into inorganic pyrophosphate (PPI), a potent inhibitor of calcification. Pseudoxanthoma elasticum patients and Abcc6−/− mice display reduced PPI levels in plasma and peripheral tissues. Pseudoxanthoma elasticum is currently incurable, although some palliative treatments exist. In recent years, we have successfully developed therapeutic methodologies to compensate the PPI deficit in animal models and humans. Here, we inadvertently discovered that modulating dietary PPI can also be an effective approach to reducing calcification in Abcc6−/− mice. Our findings were prompted by a change in institutional rodent diet. The new chow was enriched in PPI, which increased plasma PPI, and significantly reduced mineralization in Abcc6−/− mice. We also found that dietary PPI is readily absorbed in humans. Our results suggest that the consumption of food naturally or artificially enriched in PPI represents a possible intervention to mitigate calcification progression in pseudoxanthoma elasticum, that dietary preferences of patients may explain pseudoxanthoma elasticum heterogeneous manifestations, and that animal chow has the potential to influence data reproducibility.


INTRODUCTION

Physiological mineralization is a multifactorial metabolic process generally restricted to the extracellular matrix of bones and teeth. The intra- and extracellular mechanisms regulating mineralization rest upon a delicate balance between calcification inhibitors and promotors. In normal circumstances, calcium and inorganic phosphate concentrations are near saturation in most soft tissues, which necessitates potent calcification inhibition systems (Atzeni et al., 2006).

Our understanding of calcification inhibition processes in soft tissues has evolved in recent years with the identification of mutations in ABCC6 (Bergen et al., 2000; Le Saux et al., 2000; Ringspeil et al., 2000) and the characterization of the molecular function of this ABC transporter (Jansen et al., 2013). ABCC6 mediates the cellular efflux of ATP, which is rapidly converted into inorganic pyrophosphate (PPI) and adenosine by ENPP1 and CD73 at the cellular surface (Jansen et al., 2013, 2014; Markello et al., 2011; Miglionico et al., 2014). PPI is a key molecule in the prevention of mineralization in soft tissues (Heinonen, 2001; Orriss et al., 2016). The liver, where ABCC6 is mainly expressed, is one of the main sources of PPI in the circulation and the overall ABCC6 function accounts for approximately 60% of the PPI plasma levels in mice and humans (Jansen et al., 2014; Pomozi et al., 2017b). Inactivating mutations in genes encoding enzymes participating in PPI homeostasis result in inherited disorders characterized by soft tissue calcification. ABCC6 deficiencies underlie the calcification disorder pseudoxanthoma elasticum (PXE) (OMIM #264800) (Bergen et al., 2000, 2001), some cases of generalized arterial calcification of infancy (OMIM #614473), which is otherwise associated with ENPP1 mutations (OMIM #208000) (Le Boulanger et al., 2010; Nitschke et al., 2012) and the dystrophic cardiac calcification (DCC) phenotype of Abcc6-deficient mice (Aherrahrou et al., 2008; Brampton et al., 2014; Meng et al., 2007). The clinical manifestations related to the ABCC6→ENPP1→CD73 pathway are, in fact, a spectrum of related diseases with similar calcification phenotypes, PXE, generalized arterial calcification of infancy, and also calcification of joints and arteries (CALJA also known as ACDC; OMIM #211800). The latter rare disease is associated with CD73 (encoded by NT5E) deficiency (Markello et al., 2011). The explication of these genetic conditions places ABCC6 as an upstream regulator of a
purinergic/adenosinergic pathway that notably inhibits mineralization (Kauffenstein et al., 2018; Pomozi et al., 2017b). PXE, generalized arterial calcification of infancy, and DCC in Abcc6−/− mice result from a deficit in PPI production, whereas CALJA (Markello et al., 2011; Migliorino et al., 2014) is caused by enhanced PPI degradation, resulting from the activation of tissue non-specific alkaline phosphatase (ALPL) (Migliorino et al., 2014; Ziegler et al., 2017).

We and others have recently shown that PPI supplementation either by injection or oral delivery via drinking water and tissue non-specific alkaline phosphatase inhibition restores mineralization inhibition in Abcc6−/−, Enpp1−/−, and Nt5e−/− animals (Dedinszki et al., 2017; Pomozi et al., 2017b; Ziegler et al., 2017). In this study, we inadvertently tested the hypothesis that dietary PPI can be an effective approach to reducing ectopic calcification associated with ABCC6 deficiency. Polyphosphates and especially PPI are common additives in the food industry. Our results suggest that the phenotypic heterogeneity and intrafamilial variation in PXE manifestations could be at least partly explained by dietary preferences. Dietary PPI could be an easy, safe, and inexpensive approach to mitigate the progression of symptoms of PXE patients.

**RESULTS**

**Elevated levels of PPI in the standard rodent diet**

In the last 2 years, we have initiated follow-up studies of the DCC phenotype using Abcc6−/− mice (Brampton et al., 2014; Pomozi et al., 2017a). However, we were quickly confronted with the inability to reproduce the level of cardiac calcification that we previously characterized and used (Figure 1). In search of plausible solutions to these inexplicable results, we hypothesized that the absence of dystrophic calcification post-cryoinjuries to cardiac tissues could be related to exogenous factors. The possibility of genetic changes or breeding errors in exogenous factors. The possibility of genetic changes or breeding errors in Abcc6−/− mice was quickly ruled out with a review of our systematic genotyping records. As we have shown that PPI supplementation, in small or intermittent injection or oral delivery through water, was very effective at suppressing DCC (Dedinszki et al., 2017; Pomozi et al., 2017b), we evaluated the possibility of PPI contamination in water and/or diet. For this purpose, we developed a protocol to measure the presence of PPI in standard chow (cf. Materials and Methods section).

The PPI concentration of the water supply was negligible (Figure 2). However, we found a relatively high PPI content (0.30 μmol/g dry weight) in the standard chow provided by the Animal Veterinary Services of the University of Hawaii. This diet is manufactured by Envigo/Teklad (Madison, WI) and designated 2920 (Supplementary Table S1 online). If one assumes a daily consumption of about 4 g of food for an average mouse of 25 g, and a PPI bioavailability of 0.5% (estimate from Pomozi et al., 2017b), the 2920 standard chow would provide a cumulative daily intake of PPI of about 6 nmol/d. This value largely exceeds (~4.3 fold) the minimal PPI effective dose to suppress DCC (Figure 2, red line) we previously estimated at approximately 1.4 nmol (Pomozi et al., 2017b). Remarkably, the University of Hawaii Animal Veterinary Services had changed supplier of animal chow in the course of 2015 after our first experiments using DCC (Brampton et al., 2014; Pomozi et al., 2017a). We suspected that the chow formulation used prior to 2015 probably had less PPI. To verify this assumption, we obtained a sample of this animal chow from the manufacturer (designated 5053) and determined its PPI content. Even though the food formulations of the pre- and post-2015 diets (5053 vs. 2920) were comparable (Supplementary Table S1), our results showed that indeed the 2920 diet had 7.1 times more PPI than the previous 5053 diet (0.30 μmol/g vs. 0.042 μmol/g dry weight). To confirm these results, samples of both 5053 and 2920 chow were sent to one of the authors’ laboratory (AV) at the Hungarian Academy of Sciences, in Budapest. Similar PPI results were obtained, which validated our initial results (Figure 2). As control, the standard rodent chow used at the Hungarian Academy of Sciences was measured and showed low PPI concentrations, similar to the 5053 diet (Figure 2).

Remarkably, the formulations of the 2920 and 5053 diets were similar (Supplementary Table S1) notably the phosphorus content. The main source of phosphorus is from dicalcium phosphate, whereas corn and wheat provide additional sources in this diet. The manufacturer has indicated that heat treatment to dry their product is insufficient to generate PPI. As the manufacturer does not specifically add or even account for PPI, we tested whether the irradiation could be the cause of the elevated pyrophosphate using a sample of the same diet but non-irradiated (2020x). The PPI content was found to be similarly elevated (0.28 μmol/g dry weight), suggesting that one or more of the basic ingredients is enriched in PPI.

**PPI in rodent diet is sufficient to suppress ectopic calcification and raise plasma PPI**

To further test the hypothesis that the elevated PPI content of the standard 2920 diet is indeed capable of inhibiting ectopic calcification development in Abcc6−/− mice, we compared calcification data in vibrissae from Abcc6−/− animals on the current 2920 diet and results obtained prior to 2015 with the 5053 chow. In a previous study, we quantified the development of vibrissae mineralization over the lifespan of Abcc6−/− mice (Brampton et al., 2011). This work notably established that vibrissae calcification increased rapidly within the first 6 months of age and slowed thereafter, reaching a plateau in 12-month-old mice. Therefore, we compared data from two age groups that reflect these critical phases in calcification development. The results shown in Figure 3 revealed a significant decrease of 57% in calcification at 6 months of age and a slightly more modest decline (44%) in 1-year-old mice (P < 0.0001). This confirmed that dietary PPI efficiently counteracts the development of chronic mineralization. Further, we verified that the presence of PPI in the diet raised plasma PPI significantly, which is consistent with results published previously (Dedinszki et al., 2017; Pomozi et al., 2017b).

**Crystalline PPI added to plain food is readily absorbed in humans**

Because the elevated level of PPI present in our standard animal chow was readily absorbed and lowered the susceptibility to ectopic calcification in Abcc6−/− mice, we tested
Figure 1. The effect of dietary PPI on the acute dystrophic cardiac calcification phenotype of Abcc6−/−mice. (a) Abcc6−/−mice consuming the post-2015 diet (2920) with high PPI levels developed limited dystrophic cardiac calcification compared to animals that were fed the pre-2015 diet (5053) with low PPI. The level of calcification was measured as total ventricular calcium and normalized to the weight of the tissue. The number of mice per group is shown and results are mean ± standard error of the mean. *P < 0.05; ****P < 0.0001. (b) Representative images of the surface lesions (outlined) and calcification in wild-type and Abcc6−/−mice fed either the post-2015 diet (2920) with high PPI levels or the pre-2015 diet (5053) with low PPI. The calcified deposits (white) were visibly reduced with post-2015 diet (2920). PPI, inorganic pyrophosphate.

whether a dry crystalline form of PPI could also be absorbed in healthy human volunteers consuming regular food. The food chosen was plain boiled potatoes, to which 50 mg/kg body weight of disodium PPI was added. Blood samples were taken before to establish a baseline and at 30, 60, 120, and 240 minutes after the start of the meal (Figure 4). The average plasma PPI rose significantly above baseline levels and peaked at 60 minutes (differential of 1.52 ± 0.30 μmol/L or ~2.3-fold) with an average total concentration of 2.70 ± 0.29 μmol/L (P < 0.001, n = 8). The concentration decreased progressively thereafter, but was still significantly elevated 4 hours (P < 0.01) post ingestion. The average absorption of PPI from solid food was estimated at 0.05% and we found no significant difference between male and female volunteers (P = 0.31, n = 4).

DISCUSSION

The present study shows that dietary PPI is readily absorbed in humans and is also an effective approach to reducing ectopic calcification associated with ABCC6 deficiency in a recognized mouse model of PXE. The clinical manifestations of PXE are highly varied and the heterogeneity in symptoms, occurrence, presentation, and severity is compounded by the absence of clear-cut genotype-phenotype correlation (Le Saux et al., 2001; Legrand et al., 2017; Pfendner et al., 2007). This phenotypic heterogeneity of PXE and the lack of correlation complicates diagnosis and prognosis. The inadvertent discovery of substantial amounts of PPI in our standard animal chow and its consequences on ectopic calcification has three main implications.

PPI as a possible treatment

PXE is an incurable disease. Some symptomatic treatment exists for patients (Finger et al., 2011), and a recent clinical trial with a PPI analog (etidronate) only reported mixed results (Kranenburg et al., 2018). In recent years, we have developed and demonstrated therapeutic methodologies that could be applied to treat PXE patients. In a series of studies with a humanized mouse model, we have shown that 4-phenylbutyrate, a drug used to treat urea cycle disorders, can be repurposed to restore function to ABCC6 proteins having specific amino acid substitutions (Le Saux et al., 2011; Pomozi et al., 2014, 2017a). The other approach addressed the root problem of PXE (Jansen et al., 2013, 2014), by compensating the PPI deficit through various methods of delivery and was proven to be effective in animals with limited testing done in humans (Dedinszki et al., 2017; Pomozi et al., 2017b). Both 4-phenylbutyrate and PPI methods have limitations, with the former being restricted to certain genotypes, whereas the latter lacks approved formulation and clinical guidelines for PPI administration. Although gene therapy has encountered many obstacles from its inception, it has now matured enough along with emerging genome editing technologies that clinical applications can be envisaged in the not so distant future (Dunbar et al., 2018). Indeed, gene therapy would only need to target a few tissues (liver, kidneys) in PXE. However, as these therapeutic concepts are slowly coming of age, defining biomarkers and criteria to determine treatment efficacy in PXE is becoming the next priority in the field.

The consumption of solid food, naturally or artificially enriched in PPI, might be recommended one day as part of a treatment strategy to slow or mitigate progression of ectopic calcification and resulting signs and symptoms. The World Health Organization considers PPI as a nontoxic physiological metabolite with a high maximal tolerable daily intake value of 70 mg/kg (http://www.inchem.org/documents/jecfa/jec2259.htm), which compares favorably to the median lethal dose (LD50) of 2,600 mg/kg reported in rodents (Seo et al., 2011). In the United States, PPI is classified as GRAS (generally recognized as safe) by the US Food and Drug Administration and is designated as additive E450(a) in
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Figure 2. Estimated daily consumption of PPI from the pre-2015 (5053) and post-2015 (2920) chow in Hawaii (HI), the Hungarian chow in Budapest (Hu), and drinking water (HI). The chow used in Hawaii (HI) post-2015 (2920 diet) and in Hungary had significantly higher PPI content (7.1-fold) than the chow used before 2015 (5053 diet) which led to a superior cumulative daily intake. These results were confirmed by the authors located at the Hungarian Academy of Sciences, Budapest, Hungary. The PPI content of the drinking water was comparatively negligible. The dashed red line represents the minimal effective amount of PPI to prevent the development of dystrophic cardiac calcification as previously determined (Pomozi et al., 2017b). The results are mean ± standard error of the mean. ****P < 0.0001. PPI, inorganic pyrophosphate.

Europe. PPI is thus widely used in the food industry as a preservative in, for example, canned seafood, baking soda, and cured meat. Given the level of oral absorption we observed with human volunteers, which is comparable to absorption from water (Dedinszki et al., 2017), PPI derived from food sources or even from toothpaste might have broader application towards other conditions that also manifest ectopic calcification, such as atherosclerosis (Alexopoulos and Raggi, 2009), diabetes (Chen and Moe, 2003), chronic renal insufficiency (Schlieper et al., 2016), β-thalassemia (Aessopos et al., 2002), or heterotopic ossification of traumatized muscle (Jackson et al., 2009).

Dietary PPI as a modulator of the PXE phenotype

The role of ABCC6 in the pathogenesis of PXE, generalized arterial calcification of infancy and DCC is now well established in humans (Le Saux et al., 2012) and several independent animal models (Li et al., 2014, 2017). The development of ectopic calcification is clearly related to PPI deficit (Dedinszki et al., 2017; Jansen et al., 2013; Pomozi et al., 2017b; Ziegler et al., 2017). However, many facets of the phenotypes associated with ABCC6 dysfunction remain obscure, notably the heterogeneous presentation and severity of calcification in PXE (Hosen et al., 2013, 2014, 2017a, 2017b), the potential effects of variation in “normal” animal diet is not often considered in biomedical research. The discovery that an apparently innocuous change in the supplier of normal standard chow had such a profound effect on our results is a good illustration of actual variability between “identical” chow formulations. Besides the fact that PPI is not a reported ingredient in chow, our data suggest that animal diet could have a significant impact on data reproducibility between laboratories at different institutions and that more consideration should be given to what animals eat.

The unexpected results of this study have not only provided a fresh insight into how the immediate environment affects the progression of this rare disease in an animal model, but also furthers the prospect of dietary intervention as a possible treatment for PXE. Importantly, the collective findings that PPI treatment does not reverse established calcification (Dedinszki et al., 2017; Pomozi et al., 2017b) and that the extent of skin lesions correlate with severe cardiovascular and/or ophthalmologic complications in PXE (Navasiolava et al., 2018) suggest that any PPI-based intervention should be initiated as early as possible, at diagnosis or soon thereafter.

MATERIAL AND METHODS

Animals

C57BL/6j mice, designated herein as wild type, were derived from mice purchased from Jackson Laboratories (Bar Harbor, ME). Abcc6<sup>−/−</sup> mice were generated on 129/Ola background (Gorgels et al., 2005) and backcrossed into a C57BL/6j more than 10 times. These mice are herein designated Abcc6<sup>−/−</sup>. Both male and female,
age-matched Abcc6<sup>e<sup/>e<sup/> mice and wild-type mice were used, as sex had no significant impact on results. All animals were housed in approved animal facilities at the University of Hawaii School of Medicine. Mice were kept under routine laboratory conditions with 12-hour light-dark cycle with ad libitum access to water and chow. The University of Hawaii Institutional Animal Care and Use Committees approved these studies. Experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Human volunteers**

To confirm that crystalline PPI can be readily absorbed from solid food by humans, eight healthy subjects (four males, four females) consumed 2 g/kg of boiled potato. Disodium pyrophosphate salt was

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**Figure 3. The effect of dietary inorganic pyrophosphate on the chronic (pseudoxanthoma elasticum–like) calcification phenotype of Abcc6<sup>e<sup/>e<sup/> mice.**

(a) Vibrissae calcification from Abcc6<sup>e<sup/>e<sup/> mice fed either low (5053) or high (2920) inorganic pyrophosphate chow was quantified. The data were collected from 6- and 12-month-old animals when whiskers calcification is optimal. Results pre- and post-dating the change of institutional rodent chow in 2015 were compiled to generate these results. The number of mice per group is shown and results are mean ± standard error of the mean. ****<sup>P</sup> < 0.0001.

(b) Representative images of vibrissae calcification (chronic pseudoxanthoma elasticum–like phenotype) of Abcc6<sup>e<sup/>e<sup/> mice visualized by Alizarin Red S staining (red to dark red color) are shown. Scale bar = 250 μm.

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**Figure 4. Uptake of dietary PPI in plasma of Abcc6<sup>e<sup/>e<sup/> mice and healthy human volunteers.**

(a) Plasma PPI concentration was determined in 3- to 6-month old Abcc6<sup>e<sup/>e<sup/> (−/−) and wild type mice (+/+) that were fed standard chow with low PPI content (5053) prior to 2015 or high PPI levels (2920) after 2015. The number of mice per group is shown and results are mean ± standard error of the mean. ***<sup>P</sup> < 0.001; ****<sup>P</sup> < 0.0001.

(b) Oral uptake of dietary PPI in humans. Volunteers ingested boiled potatoes mixed with 50 mg/kg (body weight) of disodium pyrophosphate. The average plasma PPI level was determined before (0 minutes), and 30, 60, 120, and 240 minutes after food ingestion. Results are mean ± standard error of the mean, n = 8; **<sup>P</sup> < 0.01; ***<sup>P</sup> < 0.001. (c) Individual variations of plasma PPI levels after oral uptake. Results are mean ± standard error of the mean. PPI, inorganic pyrophosphate.
added to the levels of 50 mg/kg of body weight to mashed potatoes, stirred, and consumed within 2 minutes. Blood samples were obtained before food ingestion and at 30, 60, 120, and 240 minutes thereafter. Blood samples were collected in three separate tubes. Plasma samples were divided in three aliquots and frozen at −80°C until use. The human uptake studies were approved by the National Review Board of the Ministry of Health, Hungary (ETT TUKEB). Written informed consent was obtained from each volunteer prior to the study and experiments conformed to the principles of the Declaration of Helsinki, which was indicated in the informed consent. All human samples were de-identified at collection time.

Myocardial cryoinjury
At 72 hours post tail vein injection, cardiac injury was instilled through trans-diaphragm cryoinjury, as described previously (Aherrahrou et al., 2004; Ivandic et al., 2001). Briefly, 10-second freeze-thaw injuries using a liquid nitrogen cooled—probe are applied to the heart through the diaphragm from a 10- to 12-mm incision on the abdomen. This approach limits the area of cardiac injury to a single cardiac location and offers a relative uniform size of the necrotic tissue and a very high survival rate (>90%). Sham-operated Abcc6−/− mice underwent the same surgical procedure using a room-temperature probe. Mice were killed by CO2 asphyxiation 7 days after injury to ensure that the cardiac calcification phenotype was fully developed. Hearts were removed quickly, rinsed in phosphate buffered saline, minced, and placed into 0.15N HCl for 48 hours and then the calcium content of the supernatant was determined by a colorimetric assay (Calcium LiquiColor Test, Stanbio Laboratory, TX).

Histochromy and calcification measurements
Direct histologic visualization of calcium deposition following Alizarin Red S staining on paraffin-embedded sections was carried out on the left muzzle skin of each mouse as described previously (Brampton et al., 2011). The level of mineralization in whiskers or the heart was quantified following several methodologies. We first used the Calcium LiquiColor colorimetric assay (McGee-Russell, 1958) that directly measures the amount of excess calcium, which is normalized to the weight of the excised tissues, as described previously (Brampton et al., 2014) and expressed in mg/dL per gram of tissue.

PPI
Food-grade PPI in disodium form was generously provided by FOSFA Life Science (Breclav, Czech Republic). The dosages referenced therein for human volunteers were based on this disodium form. The concentration of PPI in plasma (whether human or animal) was measured as described previously (Jansen et al., 2013). The levels of PPI in rodent chow were determined with the following method: 1.25 g of ground chow was dissolved in 7.5 mL of sterile Milli-Q water. The sample was agitated at 4°C overnight. After low-g centrifugation to pellet debris, PPI concentration was measured from the supernatant using the same procedure as described by Jansen et al., 2013). To validate our results, coded samples of the diet were sent to the Hungarian Academy of Sciences (AV) to perform identical but blind measurements.

Data analysis
Data were compared by Student t test. Values are expressed as mean ± standard error of the mean. A P value <0.05 was considered statistically significant. Animal numbers used for individual sets of data varied and are shown on the figures.

CONFLICTS OF INTEREST
AV filed a patent entitled “Oral pyrophosphate for use in reducing tissue calcification” to the Netherland patient office (P32885NL00/RKI).

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.10.040.

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