Extracellular pyrophosphate metabolism and calcification in vascular smooth muscle

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Extracellular pyrophosphate metabolism and calcification in vascular smooth muscle. Am J Physiol Heart Circ Physiol 301: H61–H68, 2011. First published April 13, 2011; doi:10.1152/ajpheart.01020.2010.—Extracellular inorganic pyrophosphate (ePPi) is a potent endogenous inhibitor of vascular calcification, but it is not known whether systemic or local vascular PPi metabolism controls calcification. To determine the role of ePPi in vascular smooth muscle, we identified the pathways responsible for ePPi production and hydrolysis in rat and mouse aortas and manipulated them to demonstrate their role in the calcification of isolated aortas in culture. Rat and mouse aortas contained mRNA for ectonucleotide pyrophosphatase/phosphodiesterases (NPP1–3), the putative PPi transporter ANK, and tissue-nonspecific alkaline phosphatase (TNAP). Synthesis of PPi from ATP in aortas was blocked by β,γ-methylene-ATP, an inhibitor of NPPs. Aortas from mice lacking NPP1 (Enpp1−/−) did not synthesize PPi from ATP and exhibited increased calcification in culture. Although ANK-mediated transport of PPi could not be demonstrated in aortas, aortas from mutant (ank/ank) mice calcified more in culture than did aortas from normal (ANK/ANK) mice. Hydrolysis of PPi was reduced 25% by β,γ-methylene-ATP and 50% by inhibition of TNAP. Hydrolysis of PPi was increased in cells overexpressing TNAP or NPP3 but not NPP1 and was not reduced in Enpp1−/− aortas. Overexpression of TNAP increased calcification of cultured aortas. The results show that smooth muscle NPP1 and TNAP control vascular calcification through effects on synthesis and hydrolysis of ePPi, indicating an important inhibitory role of locally produced PPi. Smooth muscle ANK also affects calcification, but this may not be mediated through transport of PPi. NPP3 is identified as an additional pyrophosphatase that could influence vascular calcification.

medial calcification; alkaline phosphatase; ectonucleotide pyrophosphatase; adenosine 5'-triphosphate; ankyrin

EXTRACELLULAR INORGANIC PYROPHOSPHATE (ePPi) is a potent suppressor of vascular calcification both in vitro (8) and in vivo (13, 18) through its ability to inhibit hydroxyapatite formation (10). The importance of endogenous ePPi homeostasis in human cardiovascular function is demonstrated by the severe arterial calcification that occurs in the absence of a synthetic enzyme (17). Cells contain ectoenzymes capable of synthesizing and hydrolyzing ePPi, and our previous studies in cultured vascular smooth muscle cells have implicated them in the control of ePPi levels (15). However, it is not clear whether ePPi levels in vessels in vivo are controlled by local activity of these enzymes rather than by systemic activity via circu-

lating ePPi levels and whether this affects vascular calcification. Although circulating PPi levels are inversely proportional to arterial calcification in patients with advanced chronic kidney disease (14), the correlation is weak and the role of PPi metabolism in the arterial wall has never been investigated.

PPi is synthesized from extracellular ATP by the ectoenzyme nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1), and absence of this enzyme leads to extensive and fatal arterial calcification in children (17) and aortic calcification in mice (6, 11). The ATP analog α,β-methylene-ATP (α,β-meATP) is a competitive inhibitor of this reaction that reduces ePPi production in vascular smooth muscle cells (15), suggesting that NPP1 is a major source of PPi in vascular smooth muscle. However, cultured cells may not be representative of intact tissue and the high inhibitor concentrations raise questions about the specificity, particularly for other members of this enzyme family (NPP2 and NPP3), the role of which in ePPi metabolism is unknown.

An alternative source of ePPi may be transport out of cells through the putative transporter transporter ANK. Mice that are homozygous for a mutation in ANK (ank/ank) develop aortic calcification on a high-phosphate diet (11). Expression in oocytes induces PPi transport (4), and expression in cultured cells decreases intracellular PPi levels and increases PPi levels in the medium (5, 15). However, transport of PPi has never been directly demonstrated in cultured cells or in tissues.

Tissue-nonspecific alkaline phosphatase (TNAP) hydrolyzes PPi to orthophosphate and is a major determinant of hydroxyapatite formation in bone or ectopically by virtue of eliminating inhibitory PPi (11). Calcification of rat aortas in culture is induced by adding alkaline phosphatase (8) and prevented by TNAP inhibitors (12), and TNAP is upregulated in aortas from uremic rats, resulting in increased PPi hydrolysis (9). Whether increased activity of TNAP is sufficient to produce vascular calcification is unknown. The fact that only 50% of ePPi hydrolysis is prevented by TNAP inhibitors is consistent with the fact that PPi hydrolysis is reduced only 50% in aortas from mice lacking TNAP (9) and indicates that additional, but as yet unknown, enzymes are responsible in aorta (9). The recent demonstration that NPP1 can also hydrolyze PPi (1, 20) suggests that its role in ePPi homeostasis is more complex.

Despite the importance of ePPi in arterial calcification, its metabolism in intact vessels has never been studied and the relative importance of the different components in the local control of vascular calcification is unknown. This study examined the expression and activity of the different components of
ePPi metabolism in rat and mouse aortas and determined their local role in vascular calcification using an organ culture model.

**METHODS**

**Animals.** Male Sprague-Dawley rats (200–400 g) were obtained from Charles River Laboratories (Wilmington, MA), and ank/ank mice were obtained from Jackson Laboratories (Bar Harbor, ME). The *Enpp1<sup>−/−</sup>* mice have been described previously (6, 11). All protocols were approved by the Institutional Animal Care and Use Committee at Emory University.

**Vessel studies.** Aortas were perfused with saline and removed, followed by gentle removal of the adventitia. Endothelium was removed by gentle rubbing with a cotton swab except when the vessels were used for culture. Aortic rings were cultured in serum-free DMEM containing 3.8 mM phosphate, and calcification was measured as 45Ca incorporation as previously described (8).

**Transfections.** Aortas were infected with an adenovirus (Ad-TNAP) containing full-length human TNAP cDNA with enhanced alkaline phosphatase activity. Transfections were performed by cloning cDNAs into the pCDNA3 plasmid and transfection with Effectene transfection reagent (Qiagen, Valencia, CA) following the manufacturer’s instructions. The eNPP3 plasmid was obtained from Applied Biosystems (Carlsbad, CA).

**PPi assay.** PPi was measured enzymatically as previously described using either uridine-diphosphoglucose (UDP-glucose) pyrophosphorylase and UDP[14C]glucose (14) or ATP-sulfurylase and adenosine phosphosulfate to generate ATP, which was measured by a coupled luciferin/luciferase reaction (15).

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**RT-PCR.** Total RNA was isolated from rats or mice aorta using RNAzol RT reagent (Molecular Research Center, Cincinnati, OH). Following DNase treatment, reverse transcription was done by 1 µg of RNA samples using Thermoscript RT-PCR kit (Invitrogen). Expression of each sample, 18S rRNA was used as an internal control. PCR was performed with Platinum PCR SuperMix (Invitrogen) and using the following cycle parameters: 94°C for 2 min and 30 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 40 s with final extension at 72°C for 10 min. Products were separated by 1% (wt/vol) agarose gel electrophoresis and visualized by ethidium bromide fluorescence. The primer pairs used for rat have been previously reported (7). Primers for mouse were designed to cross intron-exon boundaries using Clone Manager Suite 7 software (Sci Ed Software, Durham, NC) and are listed in Table 1.

**Transfections.** Aortas were infected with an adenovirus (Ad-TNAP) containing full-length human TNAP cDNA with enhanced GFP cDNA. The cDNA coding human TNAP (AB011026) was generated by PCR using primers 1) 5'-Aaa AAGCTT GCC ACC atg ATT TCA CCA TTC-3'; 2) 5'-GTTTTGGCGCTTTGGATCTCG GCT CAG GG-3' from plasmid pDNA3.TNAP, and cloned into the adenovirus vector pAd.track-CMV at the Hind III and Kpn I restriction sites. Purified vector was linearized using Pme I and cotransformed with 100 ng pAdEasy-1 supercoiled vector (100 ng/µl) into BJ5183 cells by electroporation. Clones containing the pAd-easy-TNAP were selected using kanamycin and verified by sequencing.

**Statistics.** Results are expressed as means ± SE. Significance was determined by Student’s t-test or by ANOVA. A P value of ≤ 0.05 was considered to be significant.

### Table 1. Primer sequences for RT-PCR

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NTPD1-3, nucleotide triphosphate diphosphohydrolase-1–3; ENPP1–3, ectonucleotide pyrophosphatase/phosphodiesterase-1–3; TNAP, tissue-nonspecific alkaline phosphatase; F, forward; R, reverse.

The virus was then amplified in HEK293H cells (Invitrogen) and purified by adeno-X virus purification kit (Clontech, Mountain View, CA). Virus titer was determined by serial dilution and calculated as transduction units (TU/ml) as follows: (infected cells/field) × (field/well)/volume virus (ml) × dilution factor. Rat aortas with endothelium removed were cultured as above with 10<sup>6</sup> TU/ml Ad-TNAP virus.

Transfections of HEK cells for enzyme studies were performed by cloning cDNAs into the pcDNA3 plasmid and transfection with Effectene transfection reagent (Qiagen, Valencia, CA) following the manufacturer’s instruction. The eNPP3 plasmid was obtained from Applied Biosystems (Carlsbad, CA).

**PPi assay.** PPi was measured enzymatically as previously described using either uridine-diphosphoglucose (UDP-glucose) pyrophosphorylase and UDP[14C]glucose (14) or ATP-sulfurylase and adenosine phosphosulfate to generate ATP, which was measured by a coupled luciferin/luciferase reaction (15).

PPi metabolism. Aortas or cells were incubated with [γ<sup>32</sup>P]ATP or 32PPi in a physiologic salt solution (142 mM Na<sup>+</sup>, 121 Cl<sup>−</sup>, 5.4 mM K<sup>+</sup>, 1.8 mM Ca<sup>2+</sup>, 0.8 mM Mg<sup>2+</sup>, 5 mM glucose, and 24 mM HEPES pH 7.4). Orthophosphate was separated from ATP or PPi, in the medium by the acid-molybdate method as previously described (9). PPi production was measured by chromatography on PEI-cellulose plates developed with 650 mM KH<sub>2</sub>PO<sub>4</sub> pH 3. After autoradiography, spots were scraped and counted by liquid scintillation. Uptake of PPi was measured after the aortas were washed six times in cold salt solution without 32PPi. After assays, the rings were dried and weighed, or the protein content of cultured cells was measured.

**Inhibitors.** β,γ-methylene-ATP (β,γ-mATP) and α,β-meATP were obtained from Sigma-Aldrich (St. Louis, MO) and used at 300–μM final concentration. Probenecid was from the same source and used at 2.5 mM. Compound MLS38949 (PubChem CID 2931234) or 2,5-dimethoxy-N-(quinolin-3-yl)benzenesulfonamide is a potent TNAP inhibitor with excellent selectivity over other alkaline phosphatases recently identified through high-throughput screening (3, 19). MLS38949 was used at a final concentration of 30 μM.

**Statistics.** Results are expressed as means ± SE. Significance was determined by Student’s t-test or by ANOVA. A P value ≤ 0.05 was considered to be significant.
RESULTS

Expression of mRNA. Normal rat aortas were probed for the presence of mRNA for proteins involved in extracellular ATP and PP_i metabolism. The relevant ectonucleotidases include the NPPs, which generate PP_i from ATP, the nucleotide triphosphate diphosphohydrolases (NTPDs), which cleave ATP to generate P_i, and TNAP, which hydrolyzes ATP and PP_i to P_i. In addition, the ANK protein may mediate PP_i efflux from cells. As shown in Fig. 1, there was significant expression of NPP1, NPP3, NTPD3, and ANK, and lower levels of NTPD1 and TNAP. There was no significant expression of NPP2 or NTPD2.

PP_i synthesis. Despite numerous attempts with sensitive assays, we were unable to detect release of PP_i from rat aortas into incubation medium under basal conditions. Synthesis of PP_i could only be detected when exogenous ATP was added and only as 32PP_i synthesized from [γ-32P]ATP. ATP hydrolysis was rapid, with 50% hydrolyzed after 20 min and 100% hydrolyzed after 1 h, corresponding to initial rates of 17.4 ± 0.41 and 2.08 ± 0.05 pmol·mg⁻¹·min⁻¹ from 1 and 0.1 μM ATP, respectively. Thin-layer chromatography revealed that only a small proportion (7.3 ± 0.9%) of the ATP was converted to PP_i, with the remainder appearing as orthophosphate (Fig. 2). Some of this PP_i is a contaminant of the [γ-32P]ATP preparation, since a small amount was present in medium incubated without aorta. The rate of PP_i production from 1 μM ATP was 1.27 ± 0.03 pmol·mg⁻¹·min⁻¹. Synthesis of PP_i was completely prevented by the addition of 300 μM β,γ-meATP, an inhibitor of NPP1 (7), or by α,β-meATP, an inhibitor of NPPs and NTPDs (7), but was not affected by inhibition of TNAP. The additional spot probably represents α,β-me[γ-32P]ATP derived from the transfer of 32P from ATP to α,β-meADP via nucleoside diphosphokinases (7). As shown in Fig. 3, A and B, similar findings were obtained in normal mouse aortas, but in aortas from Enpp1⁻/⁻ mice, PP_i levels did not increase above baseline and were not affected by β,γ-meATP. Because the meATPs are substrates for NPPs and NTPDs and are rapidly hydrolyzed, they are not suitable for long-term inhibition of NPP1. Therefore, aortas from Enpp1⁻/⁻ mice were used to examine the role of NPP1 in vascular calcification. Calcification of these aortas was modestly but significantly greater than of aortas from wild-type littermates (Fig. 3C). The variability in calcification is due to the difficulty
in isolating and preparing the mouse aortas without injury, which can induce calcification by itself.

**PPi transport.** The inability to load sufficient quantities of PPi into smooth muscle coupled with intracellular hydrolysis precluded measurement of PPi efflux. Since influx of PPi has been reported in oocytes expressing ANK (4), measurement of PPi influx was attempted in aortas. To distinguish cellular uptake from binding or trapping of PPi in the extracellular matrix, uptake was also measured in devitalized (frozen and thawed) aortas. As shown in Fig. 4A, there was rapid uptake of 32PPi in the first 15 min that was also present in devitalized aortas and remained constant thereafter, consistent with extracellular binding or trapping. However, uptake continued in a linear fashion in normal aortas and presumably represented cellular uptake. As shown in Fig. 4B, uptake was similar in rat and mouse aortas and was not reduced in aortas from ank/ank

![Diagram](image)

**Fig. 3. Synthesis of PPi and calcification in aortas from mice lacking NPP1.** A: autoradiogram of a representative thin-layer chromatogram of medium after incubation of aortic rings with [γ32P]ATP in 1 μM ATP with or without 30 μM MLS38949, 300 μM α,β-meATP, or 300 μM β,γ-meATP. B: quantification of PPi production. Results are the means of 6 experiments. *P < 0.01 vs. control; **P < 0.01 vs. wild-type (WT). C: calcification of aortas from wild-type mice and mice lacking NPP1 (Enpp1−/−). Symbols represent individual rings and means ± SE. Results are from 1 representative experiment. Similar results were obtained in 2 additional experiments. *P < 0.05.

**Fig. 4. PP transport and calcification in aortas.** A: uptake of PPi in normal and devitalized (frozen and thawed) rat aortas. Results are means ± SE of triplicate measurements; PPi concentration was 5 μM. B: uptake of PPi in rat aortas and in aortas from ank/ank mice and wild-type (ANK/ANK) littermates. Results are means ± SE of triplicate measurements for rat aortas and 8 measurements in mouse aortas; time was 180 min; PPi concentration was 1 μM. C: calcification of aortas from ank/ank mice and wild-type (ANK/ANK) littermates. Each symbol indicates a separate animal. *P < 0.01.
mice lacking a functional ANK protein. However, calcification was significantly greater in aortas from ank/ank mice compared with ANK/ANK littermates (Fig. 4C).

**PPi hydrolysis.** Hydrolysis of ePPi was measured by incubating aortas with 1 or 5 μM PPi containing tracer 32PPi, with resulting rates of 0.24 ± 0.01 and 1.27 ± 0.05 pmol·mg⁻¹·min⁻¹. Approximately 50% was inhibited by MLS38949, a potent and highly specific inhibitor of TNAP that inhibits <10% of NPP1 activity (3). Half of the remaining hydrolysis was inhibited by α,β-meATP or β,γ-meATP (Fig. 5A). The effect of the inhibitors was additive. The role of TNAP in calcification was tested in cultured aortas. Since normal aortas do not calcify in culture, even in the presence of high-phosphate concentrations, it was necessary to induce calcification to test the effect of TNAP inhibition. This was accomplished by injuring the aortas through rubbing with a cotton swab as previously described (8). The subsequent calcification was inhibited by the TNAP inhibitor MLS38949 (Fig. 5B). The effect of increased TNAP activity was tested by viral transduction of TNAP into cultured aortas using a GFP-labeled construct, which resulted in extensive cellular labeling (Fig. 5C). This produced an increase in activity compared with transduction of the empty virus (Fig. 5D) that ranged from 2.08-fold to 3.43-fold in three experiments and a significant increase in calcification (Fig. 5E).

The inhibition of PPi hydrolysis by the meATPs suggested involvement of an NPP, and this was investigated by overexpressing these enzymes in HEK cells (Fig. 6A). Hydrolysis of PPi was significantly increased after transfection of TNAP or NPP3 but not NPP1. To ensure that transfection of NPP1 resulted in increased activity, hydrolysis of [γ32P]ATP was examined, using treatment of the medium with inorganic pyrophosphatase before extraction to identify PPi (Fig. 6B).

![Fig. 5. Hydrolysis of PPi and calcification in aortas. A: hydrolysis of PPi (1 μM) by rat aorta with or without 30 μM MLS38949, 300 μM α,β-meATP, or 300 μM β,γ-meATP. Results are the means ± SE of 3–5 experiments. *P < 0.001 vs. control; **P < 0.001 vs. MLS38949, by paired analysis. B: calcification of injured aortas cultured without or with 30 μM MLS38949. Results are the means ± SE of 8 aortic rings. *P < 0.005. C: fluorescence microscopy of rat aorta transduced with GFP-containing adenovirus showing expression of GFP in individual cells. Line indicates 500 μm. D: alkaline phosphatase activity in aortas exposed to Ad-TNAP or empty virus. Concentration of levamisole was 1 mM. Results from a representative experiment are shown. E: calcification of aortic rings cultured with Ad-TNAP or empty virus. Symbols represent individual rings and means ± SE. *P < 0.05.](http://www.ajpheart.org/doi/abs/10.1152/ajpheart.00225.2010)
Production of orthophosphate from ATP did not vary with the transfections, but PPi production was significantly greater in cells transfected with NPP1 or NPP3, confirming successful transfection. PPi hydrolysis did not differ between aortas from Enpp1−/− mice and wild-type littermates and was still inhibited by β,γ-meATP in the Enpp1−/− aortas (Fig. 6C).

**DISCUSSION**

This is the first examination of ePPi and ATP metabolism and its role in smooth muscle calcification in intact vessels. The results indicate that ectoenzymes and transporters capable of producing and clearing ePPi are present in vascular smooth muscle (Fig. 7) and influence vascular calcification in a manner consistent with an important, local inhibitory role of PPi.

Analysis of mRNA transcripts from aorta indicated expression of NPP1, TNAP, and ANK, all of which have been implicated in ePPi metabolism. There was also expression of NPP3 but not NPP2. The NPP family consists of seven single-span transmembrane proteins except for NPP2, which is secreted (21), and only NPPs 1–3 have been implicated in the hydrolysis of nucleotides. Expression of NTPD1 and NTPD3 was also detected. Members of the NTP (apyrase) family can hydrolyze nucleoside 5'-triphosphates and diphosphates with varying preferences for the type of nucleotide (16). Of these, NTPDs 1–3 are ectoenzymes. Although they do not directly participate in ePPi metabolism, they could influence it by reducing the availability of extracellular ATP. The pattern of expression of these ectoenzymes in rat and mouse aorta parallels that in vascular smooth muscle cells in culture (15).

We (15) have previously demonstrated that extracellular ATP is the major source of ePPi in vascular smooth muscle cells, presumably mediated by NPP1 based on inhibition by β,γ-meATP. This inhibitor also prevented PPi production from ATP in aortas, and the absence of PPi synthesis from ATP in Enpp1−/− aortas definitively demonstrates that NPP1 is the sole source of PPi from extracellular ATP in vascular smooth muscle. The results also indicate that NPP1 was completely inhibited at the concentration of β,γ-meATP that was used. Since neither [γ^32]P]ATP nor β,γ-meATP is likely to enter cells, intracellular NPP1 would not be detected or inhibited. However, this enzyme is not known to function intracellularly and this would not contribute to extracellular PPi levels. The rate constant of PPi hydrolysis in aortas was fivefold less than the rate constant of ATP hydrolysis, and, since the concentration of PPi produced from ATP was far less than the starting ATP concentration, hydrolysis of PPi should not have interfered with the measurement of PPi synthesis. This is borne out by the fact that PPi synthesis was not altered by inhibition of TNAP.

Aortas exhibited a progressive uptake of PPi that could not be accounted for by binding to the extracellular matrix and presumably represents cellular uptake. This is the first demonstration of PPi transport in a tissue and the first in vascular...
cells. However, uptake did not differ between aortas from Ank/ank and ank/ank mice, indicating that it is not mediated by ANK. Although it is possible that ANK mediates only efflux and not influx of PPi, its expression in oocytes resulted in measurable influx (4), consistent with bidirectional transport. However, it is possible that ANK is misoriented in oocytes and does not mediate influx under normal conditions. Despite having similar PPi uptake, aortas from ank/ank mice exhibited significantly greater calcification than wild-type aortas, indicating an important local role for ANK in vascular calcification. In addition to ANK mediating efflux but not influx of PPi, another possible explanation is that ANK transports another compound involved in ePPi metabolism such as ATP. The latter is supported by recent data showing that overexpression of ANK in chondrocytes leads to an increase in extracellular ATP (2).

Based on inhibition by MLS38949, a potent and specific inhibitor of TNAP (3, 19), this enzyme is responsible for half the PPi hydrolysis in vascular smooth muscle. This is consistent with our previous results (9) using the less potent and specific inhibitor levamisole and the measurement of PPi hydrolysis in aortas from mice lacking TNAP. Approximately 25% of PPi hydrolysis was blocked by α,β-meATP and β,γ-meATP, both in absence and presence of TNAP inhibitor, suggesting involvement of one of the NPPs. The fact that NPP3 but not NPP1 increased PPi hydrolysis in HEK cells and the fact that PPi hydrolysis was not altered in aortas from Enpp1−/− mice suggest that NPP3 rather than NPP1 contributes to PPi hydrolysis in vascular smooth muscle. Although purified NPP1 has been shown to hydrolyze PPi; at millimolar concentrations in osteoblast-derived matrix vesicles (1) and in proteoliposomes (20), the results in aorta indicate that NPP1 does not hydrolyze PPi in vascular smooth muscle under physiologic conditions. Expression of NPP3 in HEK cells also increased PPi production from ATP, indicating that it may also contribute to ePPi synthesis. These potential roles of NPP3 in ePPi metabolism have not previously been demonstrated.

Genetic studies have implicated ePPi metabolism in the development of vascular calcification but whether these findings are due to alterations in local or systemic ePPi metabolism is unknown. The cultured aorta model provides a means to test this and demonstrated increased calcification in the absence of NPP1 or ANK, or in the presence of excess TNAP, suggesting that these proteins can act locally to affect extracellular PPi levels in vascular smooth muscle. In particular, the results indicate an important role for TNAP, which is consistent with its critical role in bone mineralization (11) and the upregulation of vascular TNAP in uremia, a condition associated with medial vascular calcification (9).

The results also indicate an important role for extracellular ATP as a source of ePPi in vascular smooth muscle. Nucleotides can be released from cells by vesicular exocytosis or selective transport via ATP channels and nucleotide transporters and can be generated extracellularly by adenylate kinase and nucleoside diphosphokinase. The source of vascular smooth muscle is unknown. In addition to NPPs, ATP can be cleaved by NTPDs. This was the major pathway in rat and mouse aortas comprising ~90% of the ATP clearance rate. This contrasts with cultured smooth muscle cells, wherein ~50% of added ATP is cleaved to PPi (15). This indicates that cultured cells may not accurately reflect extracellular ATP metabolism in arteries and also suggests that changes in the relative abundance of NPP1 and NTPCs could alter ePPi metabolism and vascular calcification.

Unlike cultured cells, there was no measurable release of PPi from cultured aortas in the absence of added ATP. It is unlikely that this is due to the absence of ATP release or PPi release, since calcification was increased in aortas from Enpp1−/− and ank/ank mice. This implies that there is basal production of ePPi, but it is too low to measure. However, some estimates can be made from the data obtained in this study. Based on the reported range of $10^{-10}$ to $10^{-7}$ M for extracellular ATP (22) and the constant maintained in this study, the rate of PPi hydrolysis should be 72–120 pmol·g$^{-1}$·min$^{-1}$, which exceeds the rate of PPi synthesis at the highest extracellular ATP concentration. This is consistent with the lack of PPi release from aortas and the role of ePPi hydrolysis as the major determinant of calcification. The fact that vascular smooth muscle may be a net PPi-consuming tissue suggests that circulating ePPi could be an important determinant of extracellular PPi levels and calcification in vascular smooth muscle in vivo.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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