Acidosis Is a Key Regulator of Osteoblast Ecto-Nucleotidase Pyrophosphatase/Phosphodiesterase 1 (NPP1) Expression and Activity

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Previous work has shown that acidosis prevents bone nodule formation by osteoblasts in vitro by inhibiting mineralisation of the collagenous matrix. The ratio of phosphate (P_i) to pyrophosphate (P_Pi) in the bone microenvironment is a fundamental regulator of bone mineralisation. Both P_i and P_Pi, a potent inhibitor of mineralisation, are generated from extracellular nucleotides by the actions of ecto-nucleotidases. This study investigated the expression and activity of ecto-nucleotidases by osteoblasts under normal and acid conditions. We found that osteoblasts express mRNA for a number of ecto-nucleotidases including NTPdase 1–6 (ecto-nucleoside triphosphate diphosphohydrolase) and NPP1-3 (ecto-nucleotide pyrophosphatase/phosphodiesterase). The rank order of mRNA expression in differentiating rat osteoblasts (day 7) was Enpp1 > NTPdase 4 > NTPdase 6 > NTPdase 5 > alkaline phosphatase > ecto-5-nucleotidase > Enpp3 > NTPdase 1 > NTPdase 3 > Enpp2 > NTPdase 2. Acidosis (pH 6.9) upregulated NPP1 mRNA (2.8-fold) and protein expression at all stages of osteoblast differentiation compared to physiological pH (pH 7.4); expression of other ecto-nucleotidases was unaffected. Furthermore, total NPP activity was increased up to 53% in osteoblasts cultured in acid conditions (P < 0.001). Release of ATP, one of the key substrates for NPP1, from osteoblasts, was unaffected by acidosis. Further studies showed that mineralised bone formation by osteoblasts cultured from NPP1 knockout mice was increased compared with wildtypes (2.5-fold, P < 0.001) and was partially resistant to the inhibitory effect of acidosis. These results indicate that increased NPP1 expression and activity might contribute to the decreased mineralisation observed when osteoblasts are exposed to acid conditions.

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The negative actions of acidosis on the skeleton have long been known (Jaffe et al., 1932). Bone functions as a ‘fail-safe’ store of alkali available to buffer metabolic acid if systemic pH falls below the normal, narrow limits (Arnett, 2010). The solubility of bone mineral is highly pH-sensitive, increasing sharply when pH is decreased below 7.0 (Neuman and Neuman, 1958).

Acidosis also has direct effects on bone cells. Small pH drops within the pathophysiological range result in large increases in the resorptive activity of osteoclasts in vitro (Arnett and Dempster, 1986). Acidosis (pH 6.9) also prevents the formation of mineralised bone nodules in primary cultures of osteoblasts. Part of this inhibition can be attributed to the physico-chemical dissolution of hydroxyapatite (Brandao-Burch et al., 2005); however, expression and activity of tissue non-specific alkaline phosphatase (TNAP) by osteoblasts is also strikingly decreased in acid conditions, suggesting an additional cell-mediated component (Krieger et al., 1992; Brandao-Burch et al., 2005). Collagen formation and deposition are unchanged at pH 6.9 suggesting that the effects of acidosis are restricted to the process of mineralisation (Brandao-Burch et al., 2005).

Bone mineralisation depends on a constant supply of Ca^{2+} and phosphate (P_i) ions for hydroxyapatite crystal formation. Pyrophosphate (P_Pi) is a ubiquitous, potent inhibitor of mineralisation (Fleisch and Bisaz, 1962), and mineralisation in the bone microenvironment ultimately depends on the ratio of P_i to P_Pi concentrations (Felix and Fleisch, 1976; Kornak, 2011). Both P_i and P_Pi, can be generated from extracellular nucleotide triphosphates (NTPs) such as ATP and UTP by the actions of...
ecto-nucleotide pyrophosphatase/phosphodiesterases (NPPs), ecto-nucleoside triphosphate diphosphohydrolases (NTPdases), ecto-5-nucleotidase and TNAP (Stefan et al., 2005; Orriss et al., 2007). Many ecto-nucleotidases have overlapping specificities. For example, NTPdases catalyse the reactions: NTP → nucleotide diphosphate (NDP) + P, and NDP → nucleotide monophosphate (NMP) + P, whereas NPPs primarily hydrolyse NTP → NMP + PP, or NDP → NMP + P (Zimmermann et al., 2012). Osteoblasts have been shown to express three members of the NPP family (NPP1,2,3) (Johnson et al., 2000; Hassel et al., 2002; Orriss et al., 2007).

The important role of NPP1 in bone mineralisation is highlighted by several different knockout models; the naturally occurring NPP1 ‘knockout’ termed the tip-toe walking (ttw/ttw) mouse and the genetically altered NPP1 knockout (Enpp1+/−). The ttw/ttw mouse displays ossification of the spinal ligaments, peripheral joint hyperostosis and calcification of articular cartilage (Okawa et al., 1998). The phenotype of ttw/ttw mice also has similarities to the human disease ‘Ossification of the posterior longitudinal ligament of the spine’ (OPLL). The Enpp1+/− mouse displays a number of defects related to impaired PP; production including calcification of the aorta, spine, joints, cartilage and whizzer vibrissae and increased mineralisation of the osteocyte lacunae (Johnson et al., 2003; Harmeay et al., 2004; Mackenzie et al., 2012b; Hajjawi et al., 2014). Surprisingly, given the reduction in extracellular P/PP, levels, Enpp1+/− mice display reduced trabecular and cortical bone in the long bones and decreased bone strength (Mackenzie et al., 2012b). Recently, an alternative knockout model has also been reported (Enpp1−/−) (Li et al., 2013); these animals, which are on a different genetic background to Enpp1+/− mice, display many of the same phenotypic characteristics such as widespread ectopic calcification (Li et al., 2013).

Our previous work showed that decreased TNAP expression and activity contributes to the inhibitory effects of acidosi on bone mineralisation (Brandao-Burch et al., 2005). Given that NPP1 is also a key regulator of mineralisation, the aim of this study was to determine whether acidosi influences the expression and activity of NPP1 and other P and PP-generating enzymes.

**Materials and Methods**

**Reagents**

All tissue culture reagents were purchased from Life Technologies (Paisley, UK); unless otherwise mentioned, other reagents were obtained from Sigma–Aldrich (Poole, Dorset, UK). Molecular biology reagents were purchased from Invitrogen (Paisley, UK) and Qiagen Ltd (Crawley, UK). Primers for RT-PCR and qPCR were from MWG Biotech (Ebersberg, Germany) and Qiagen, respectively. The NPP1 antibody was obtained from Thermo Fisher Scientific (Loughborough, UK) and the β-actin antibody from Abcam (Cambridge, UK).

**Osteoblast cell culture**

Primary rat osteoblast cells were obtained from 2-day-old neonatal Sprague–Dawley rats as described previously (Orriss et al., 2012b; Taylor et al., 2014). All animal experiments were approved by the University College London Animal Users Committee and the animals were maintained in accordance with the UK Home Office guidelines for the care and use of laboratory animals.

Osteoblasts were cultured at pH 7.4 or 6.9 for up to 14 days, with half medium changes every 2–3 days. Experiments were carried out at 3–4 time points during the osteoblast culture; day 4 (proliferating pre-osteoblasts), day 7 (differentiating osteoblasts), day 10 (mature osteoblasts at the onset of mineralisation) and day 14 (mature, actively mineralising osteoblasts). The pH, pO2 and pCO2 were carefully monitored throughout using a blood gas analyser (ABL705, Radiometer, Crawley, UK). Bone nodule formation by osteoblasts cultured was measured as described previously (Hoebertz et al., 2002; Orriss et al., 2012b; Taylor et al., 2014).

**Osteoblast cell culture from Enpp1−/− mice**

The generation and characterisation of Enpp1−/− mice, which are on a 129sv/TerJ genetic background, have been previously described (Sali et al., 1999). Osteoblasts were isolated from 2-3-day-old wildtype (Enpp1+/+) or homozygote (Enpp1−/−) mice using a 4-step process as previously described (10 mg/ml collagenase II in HBSS for 10 min, collagenase II for 30 min, 4 mM EDTA in PBS for 10 min, collagenase II for 30 min) (Orriss et al., 2014; Taylor et al., 2014). The first digest was discarded and the cells from the other three digests pooled and resuspended in α-minimum essential medium supplemented with 10% foetal calf serum (FCS) and 5% gentamicin (complete mixture abbreviated to α-MEM). Cells were cultured for 2–4 days in a humidified atmosphere of 5% CO2–95% air at 37°C in 25 cm² flasks until confluent. Upon confluence, cells were sub-cultured into 6-well trays in α-MEM at pH 7.4 or 6.9 and supplemented with 2 mM α-glycerophosphate and 50 μg/ml ascorbic acid (mixture abbreviated to ‘supplemented α-MEM’), with half medium changes every 3 days. Medium pH, pCO2 and pO2 were monitored as above.

**Total RNA extraction and complementary DNA strand synthesis**

Osteoblasts were cultured at pH 7.4 or 6.9 in 6-well trays for 7, 10 or 14 days before total RNA was extracted from three wells using TRIZOL® reagent (Life Technologies, Paisley, UK) according to the manufacturer's instructions. Extracted RNA was treated with RNase-free DNase I (35 U/ml) for 30 min at 37°C. The reaction was terminated by heat inactivation at 65°C for 10 min. Total RNA was quantified spectrophotometrically by measuring absorbance at 260 nm. For each sample, 0.5 μg of DNase-treated total RNA was used as a template for first strand cDNA synthesis in a 20 μl reaction also containing 0.5 μg oligo dT, 3 mM MgCl₂, 0.5 mM dNTPs, 20 U recombinant RNasin® ribonuclease inhibitor, ImProm-II® 5x reaction buffer and 200 U ImProm-II reverse transcriptase. The reaction mix was annealed for 5 min at 25°C, followed by extension at 42°C for 60 min and inactivation at 70°C for 15 min. cDNA was stored at −20°C until amplification by RT-PCR or qPCR.

**Quantitative real time polymerase chain reaction (qRT-PCR)**

Osteoblast cDNA was amplified using QuantiTect SYBR Green PCR kit (Qiagen Ltd, Crawley, UK) and specific QuantiTect primers. qRT-PCR (Chromo4, Biorad Laboratories Ltd, Hemel Hempstead, UK) was performed according to manufacturer’s instructions with 40 cycles of denaturation (95°C for 10 sec) and detection (60°C for 30 sec). Data were analysed using the Pfaffl method (Pfaffl, 2001) and is shown as the fold change in gene expression relative to TNAP (Fig. 1C–D) or cells cultured at pH 7.4 (Fig. 1F). All reactions were carried out in triplicate using RNAs derived from four different osteoblast cultures.

**Reverse transcriptase polymerase chain reaction (RT-PCR)**

Rat osteoblast derived cDNA was amplified by RT-PCR in 25 μl reactions containing ~0.5 μg cDNA, 0.2 mM dNTP (10 mM stock), 1.5 mM MgCl₂, 0.2 μM of both sense and antisense primer, 1 U Taq
DNA polymerase in thermophilic DNA polymerase 10x buffer. PCR was performed according to manufacturer's instructions, with cycles of denaturation at 95°C for 30 sec, annealing for 30 sec extension at 72°C for 45 sec and reaction termination at 72°C for 5 min. For analysis, PCR products were loaded on to 1% agarose gels containing 0.3 μg/ml ethidium bromide. Gels were run at 80 mA and the DNA position visualised by exposure to UV light. To account for differences in original cell number and cDNA quality, all samples were normalised against mRNA for β-actin. The expression of TNAP, NPP1-3, NTPdase 1-6 and ANK was investigated in osteoblasts cultured for 7, 10 and 14 days. All reactions were carried out in triplicate using cDNAs derived from three different osteoblast cultures. Primer sequences and annealing temperatures are shown in Table 1.

**Determination of total cellular NPP activity**

The NPP activity of cell lysates was determined colorimetrically (Bio-Tek ELx800 plate reader, Fisher Scientific Loughborough, UK) in cells cultured at pH 7.4 or 6.9 for 7, 10 or 14 days. The assay used to measure total NPP activity was based on the method originally described by Razzell and Khorana (Razzell and Khorana, 1959). Briefly, cells were lysed in a buffer containing 1% TritonX-100 in 0.2 M Tris base with 1.6 mM MgCl₂, pH 8.1. Following centrifugation at 500g, the NPP activity of collected supernatants was measured using 5 mM p-nitrophenyl-thymidine 5'-monophosphate as a substrate. Total protein in cell lysates was determined using the Bradford assay (Sigma–Aldrich, Poole, UK).

**Immunofluorescence**

Rat osteoblasts were seeded onto sterile 1 cm diameter discs, cut from Melinex 75 micron clear polyester film (Du Pont, Dumfries, UK), in 24-well trays at 2.5 × 10⁵ cells/disc, for 14 days at pH 7.4 or 6.9. Upon termination, discs were removed and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min at room temperature, washed 3 × 5 min with PBS and stored at 4°C in PBS.
TABLE 1. Primer sequences for RT-PCR reactions

| Gene   | Primer sequence (S→3') | Product size (bp) | Annealing temperature (°C) |
|--------|------------------------|------------------|---------------------------|
| β-Actin | S - gtc gga cgg tat gca t AS - ttc gct tta ttt cgc gga | 332 | 53 |
| Alpl   | S - cct att tgg gta aga aaa AS - gtt gta ctt cgg aga gag | 238 | 50 |
| Enpp1  | S - gca agc aag gga gac AS - tgg cca cgg gaa tag tgc | 309 | 49 |
| Enpp2  | S - gaa atc gta gta gga gag AS - gtc cct aga gga gac AS - tgg gaa cgg gat tag ggc | 399 | 51 |
| Enpp3  | S - gca tgc aga aga gga gag AS - tgg gag cgg gat tag acc | 396 | 53 |
| NTPdase 1 | S - gaa cct agt gaa gta tct | 332 | 58 |
| NTPdase 2 | S - cca gat gca aag acc aac | 256 | 56 |
| NTPdase 3 | S - cag cca aac ctt cag atg AS - tgc gca aag gca gtt tct | 356 | 53 |
| NTPdase 4 | S - agg cac tgt ggc aga tca AS - cag aag tgg acc agc agg | 362 | 53 |
| NTPdase 5 | S - cag cca aac ctt cag atg AS - tgc gca aag gca gtt tct | 315 | 53 |
| NTPdase 6 | S - gaa cct agt gaa gta tct | 322 | 53 |
| ANK    | S - cata cca cat agc aag AA - aag gga ggc aga tag acc aa | 320 | 55 |

Western blot

Osteoblasts were cultured for 4, 7, 10 or 14 days at pH 7.4 or 6.9 before the monolayers were lysed in ice-cold radio immunoprecipitation (RIPA) lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% SDS 1 mM phenyl methyl sulfonyl fluoride, 1 mg/ml aprotinin, 1 mM Na3VO4 and 2.5 mg/ml deoxycholic acid). Cell homogenates were sonicated for 5 min and stored at −80°C for at least half an hour before use. Protein concentrations from lysates were determined using the Bradford assay. Prior to loading, total protein samples were denatured by incubating at 95°C for 5 min with 5x reducing sample buffer (60 mM Tris–HCl pH 6.8, 25% glycerol, 2% SDS, 10% β-mercaptoethanol and 0.1% bromophenol blue). Protein samples (20 μg/lane) were loaded into SDS–PAGE (8%) gels and transferred onto a polyvinylidene fluoride (PVDF) membrane (Amersham, Buckinghamshire, UK) by the use of a wet tank blotter (Bio-Rad, Hercules, CA) at 150 V for 1 h. The membrane was afterwards blocked with 5% non-fat milk and incubated with rabbit NPP1 (1:200) or β actin (1:250) antibody overnight at room temperature. After washing, blots were incubated in horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoreactivity was visualised using Immobilon™ Western chemiluminescent HRP substrate (Millipore, Watford, UK).

Measurement of extracellular ATP

Prior to measurement of ATP levels, culture medium was removed, cell layers washed and cells incubated with serum-free DMEM at pH 7.4 or 6.9 (1 ml/well) for 1 h. The long-term effects acid on ATP levels were measured in osteoblasts cultured at pH 7.4 or 6.9 for 4, 7, 10 or 14 days. All samples were immediately snap-frozen on dry ice for later ATP quantification. ATP release was measured by luminescence using the luciferin-luciferase assay as described previously (Orriss et al., 2009).

Quinacrine staining

The acridine derivative, quinacrine, is a weak base that binds ATP with a high affinity. When excited by light at 476 nm, it emits fluorescence in the 500–540 nm range and is widely used to visualise ATP-containing subcellular compartments in live cells. Osteoblasts were seeded onto Melinex discs, in 24 well trays at 2.5 x 10^5 cells/disc and cultured for 7 days at pH 7.4 or 6.9. To visualise ATP filled vesicles, Melinex discs were twice washed with PBS before incubation with 30 μM quinacrine for 1 h; discs were washed twice more and mounted onto microscope slides. The cells were immediately observed using fluorescence microscopy.

Statistical analysis

Statistical comparisons were made using both parametric (one-way analysis of variance and adjusted using the Bonferroni method) and non-parametric (Kruskal–Wallis and adjusted using the Dunn method) tests. In all figures where statistical significance is shown, both of these methods gave corresponding results. Unless stated, representative data are presented as means ± SEM for 6–10 replicates. Results presented are for representative experiments that were each repeated at least three times.

Results

Acidosis inhibits bone mineralisation of the collagenous matrix by osteoblasts

Rat osteoblasts cultured in control conditions at pH 7.4 for 14 days formed abundant mineralised bone spicules and nodules, as shown by alizarin red staining for deposited calcium. In acidic medium (pH 6.9), mineralisation of the collagenous matrix was completely prevented as evidenced by the absence of alizarin red staining (Fig. 1A and B).

Osteoblasts express multiple NPPs and NTPdases

Osteoblasts were cultured for up to 14 days at pH 7.4 and expression of NPPs and NTPdases was studied in differentiating osteoblasts (day 7) and mature, bone forming osteoblasts (day 14) using qRT-PCR. Expression of mRNA was detected for Enpp1, Enpp2, Enpp3, NTPdase 1-6 and ALP (Fig. 1C and D).

To determine which of the ecto-nucleotidases were most highly expressed by osteoblasts, we compared the relative expressions of all the ecto-enzymes receptors in differentiating osteoblasts (day 7) and mature, bone forming osteoblasts (day 14) at physiological pH 7.4. Following normalisation of expression using β-actin, TNAP receptor expression was used as the calibrator and expression is presented as fold change relative to TNAP (Alpl) levels. The rank order of mRNA expression in differentiating osteoblasts was Enpp1 > NTPdase 4 > NTPdase 6 > NTPdase 5 > Alpl > Enpp3 > NTPdase 1 > NTPdase 3 > Enpp2 > NTPdase 2 (Fig. 1C). In mature bone forming osteoblasts, the mRNA expression profile was slightly different with the rank order; NTPdase 6 > Enpp1 > NTPdase 4 > Alpl > NTPdase 5 > Enpp3 > NTPdase 3 > Enpp2 > NTPdase 1 > NTPdase 2 (Fig. 1D).

Expression of Enpp1 mRNA is upregulated in osteoblasts cultured at pH 6.9

RT-PCR analysis of mRNA expression at day 7, 10 and 14 demonstrated that Enpp1 mRNA expression was upregulated in
cells cultured in acidotic conditions (pH 6.9), at all stages of differentiation (Fig. 1E) compared to pH 7.4. In contrast, expression of Alpl, Enpp2, Enpp3 and NTPdase 2 was downregulated at pH 6.9 (Fig. 1E). Expression of the other ecto-nucleotidases and the PPi transporter, Ank, was unaffected.

In order to quantify the effects of acidosis on ecto-nucleotidase mRNA expression, qRT-PCR was performed on osteoblasts at the point in culture where the largest effects were seen with RT-PCR (day 10, mature cells at the onset of mineralisation). Compared to pH 7.4, culture at pH 6.9 increased the expression of Enpp1 2.8-fold, whilst expression of Alpl and Enpp3 were decreased 3.2- and 2.5-fold, respectively (*P < 0.05) (Fig. 1F). The mRNA expression of the other ecto-nucleotidases and ANK was not significantly altered by acidosis.

NPP1 protein expression is increased in acidosis

Western blot analysis showed that culture under acid conditions increased NPP1 protein expression in proliferating, differentiating and mature osteoblasts (Fig. 2A). Immunocytochemistry performed on differentiating osteoblasts (7 days at pH 7.4 or 6.9) also demonstrated increased NPP1 protein expression at pH 6.9 (Fig. 2B).

Increased total NPP activity in osteoblasts cultured at pH 6.9

Culture at pH 6.9 also increased total cellular NPP activity; levels were doubled after 7 days at pH 6.9 and increased by ~45% after 10 days (*P < 0.001). Acidosis had no effect on total NPP activity after 14 days of culture (Fig. 2C).

Enpp1−/− osteoblasts display resistance to the inhibitory effects of acidosis on bone mineralisation

Calvarial osteoblasts isolated from Enpp1+/+ and Enpp1−/− mice were cultured for 28 days at pH 7.4 or 6.9. At a normal physiological pH 7.4, Enpp1−/− osteoblasts displayed a 2.5-fold (P < 0.001) increase in bone mineralisation compared to Enpp1+/+ cells (Fig. 3A and B). Reducing the pH to 6.9 resulted in a complete abolition of bone mineralisation in Enpp1−/− cells; in contrast, bone mineralisation showed only partial inhibition in Enpp1−/− osteoblasts (Fig. 3A and B).

Acidosis does not affect ATP release by osteoblasts in vitro

ATP is a key substrate of the NPPs and is released from osteoclasts (Brandao-Burch et al., 2012), osteoblasts (Genetos et al., 2005; Orriss et al., 2009) and osteocytes (Genetos et al., 2007; Hajjawi et al., 2014) in a controlled manner. To determine whether acidosis influenced ATP efflux and, consequently, substrate availability for NPP1, ATP release was measured in normal osteoblasts cultured at pH 7.4 or 6.9 for 4–14 days. ATP release was increased up to eightfold in mature, bone forming osteoblasts compared to pre-osteoblasts. However, acidosis did not result in any significant changes in ATP release at any stage of the culture (Fig. 4A). For the duration of the experiment, osteoblast viability was unaffected by culture at pH 6.9 (not shown). Staining of intracellular ATP-containing vesicles using quinacrine was similar in pH 6.9 and pH 7.4 cultures of normal osteoblasts (Fig. 4B).

Discussion

A number of studies have now shown that acidosis exerts significant negative effects on osteoblast function (Krieger et al., 1992; Ramp et al., 1994; Sprague et al., 1994; Brandao-Burch et al., 2005; Takeuchi et al., 2013). These effects include decreased mineralisation, reduced alkaline phosphatase (TNAP) expression and activity (Krieger et al., 1992; Brandao-Burch et al., 2005) and also increased osteoprotegerin production (Takeuchi et al., 2013).
The present investigation provides the first evidence that the pyrophosphate-generating enzyme, NPP1, also mediates the effects of acid on osteoblasts and bone mineralisation. Mineralisation of collagenous bone matrix is regulated by the ratio of inorganic phosphate (Pi) to the mineralisation inhibitor, pyrophosphate (PPi) within the bone microenvironment. Different members of the ecto-nucleotidase family generate either P, (TNAP, NTPdases) or PP, (NPPs) from extracellular nucleotides (such as ATP) released from cells. This study demonstrated that mRNAs for six members of the NTPdase family (NTPdases 1–6) are expressed by primary rat osteoblasts. In agreement with previous reports (Johnson et al., 2000; Hessle et al., 2002; Orriss et al., 2007), osteoblasts were found to also express three members of the NPP family (NPP1-3). Marked differences in the relative levels of mRNAs for these ecto-nucleotidases were observed in both differentiating and mature osteoblasts. NPP1 has been reported to be the most highly expressed NPP in bone (Vaingankar et al., 2004), an observation which was confirmed in our primary osteoblast cultures. We found that expression of Enpp1 mRNA by osteoblasts was the highest of all the ecto-nucleotidases studied here, including TNAP. In contrast, the expression of NTPdase 1–3 was up to 10-fold lower than TNAP, suggesting that these cell surface enzymes may be less important contributors to extracellular P, levels. NTPdases 4–6 were relatively highly expressed by osteoblasts in our study; however, these enzymes are reported to be localised intracellularly (Yegutkin, 2008), suggesting that they may not contribute significantly to extracellular P, levels.

Our study showed that in culture medium acidified to pH 6.9, so as to completely abolish bone mineralisation (Brandao-Burch et al., 2005), expression of NPP1 by rat osteoblasts at both mRNA and protein levels was strikingly upregulated, as was total NPP activity. The function of Enpp1+/− mouse osteoblasts was similarly pH-dependent, with no bone mineralisation evident in cells cultured at pH 6.9. In contrast, in cultures of Enpp1−/− osteoblasts, mineralisation was >2-fold greater than in wildtypes at pH 7.4, and mineralisation was still evident at pH 6.9, albeit at a significantly reduced level. This partial resistance of Enpp1−/− cells to the effects of acid provides additional evidence for the role of NPP1 in mediating the inhibitory effects of low pH on bone mineralisation. Acid upregulation of NPP1 and total NPP activity in rat osteoblasts occurred in differentiating and maturing cells at days 7–10 of culture. The lack of acid stimulation in the fully mature, bone-forming cells (day 14) may be related in part to the negative feedback inhibition of NPP1 by extracellular ATP and its hydrolysis product, PPi (Orriss et al., 2007); our work has also shown that ATP secretion from osteoblasts is greatest when the cells are fully mature and depositing bone (Orriss et al., 2009).

This study additionally confirms the earlier reports of decreased TNAP expression and activity in acidosis (Krieger et al., 1992; Brandao-Burch et al., 2005). The actions of TNAP are directly antagonistic to those of NPP1, and the opposing actions of these enzymes are important in maintaining mineralisation at a normal level (Johnson et al., 2000). Thus, at pH 6.9, the reduced TNAP activity combined with the increased NPP activity could shift the extracellular P,/PPi ratio in favour of PPi, inhibiting bone mineralisation. Whether low pH affects the expression of other enzymes involved in regulating P, availability for mineralisation, such as PHOSPHO1 (Houston et al., 2004) was not investigated here but presents a worthwhile area for future study.

Despite the decreased extracellular PPi, levels, the Enpp1−/− mouse model displays reduced trabecular and cortical bone in the long bones (Mackenzie et al., 2012b). This contrasts with the in vitro findings that deletion of NPP1 leads to increased levels of bone formation under normal and acid conditions. The number of factors could contribute towards the differences between the in vivo and in vitro data. Firstly, Enpp1−/− mice have a complex phenotype and thus it is possible that alterations in other tissues could indirectly influence bone mass (Mackenzie et al., 2012b), for example, Enpp1−/− animals have...
increased levels of serum sclerostin, a key inhibitor of bone formation (Hajjawi et al., 2014). Secondly, Enpp1⁻/⁻ mice display an unusual walking gate (Sali et al., 1999), mineralisation of the joints and muscle damage (Mackenzie et al., 2012b), which combined are likely to affect the mechanical loading of bones. Finally, earlier work has suggested that the reduced bone in Enpp1⁻/⁻ animals occurs because there is insufficient PPi for TNAP to generate the P for normal bone formation (Mackenzie et al., 2012a). Osteoblasts are typically cultured in vitro with an exogenous source of P (usually β-glycerophosphate), which provides an alternative substrate for TNAP and supply of P, that is not present in vivo.

The current study used calvarial osteoblasts to examine whether acidosis regulates ecto-nucleotidase expression and activity. Previous work demonstrated that the effects of NNPP1 deletion on osteoblast function were influenced by the skeletal origin of the cells (Anderson et al., 2005); calvarial osteoblasts displayed increased mineralisation, as seen in this study, whilst bone marrow osteoblasts showed decreased mineralisation (Anderson et al., 2005). The effect of acidosis on NNPP1 expression and activity in osteoblasts derived from the long bones was not examined in this investigation; however, since NNPP1 expression is reported to be higher in the skull, it is possible that the effects are more pronounced in calvarial cells.

The non-enzymatic, plasma-membrane channel ANK is also considered to act as a PP transporter, contributing to the extracellular pool of P (Johnson et al., 2003). We found that mRNA for ANK was highly expressed by rat osteoblasts at all stages of culture but that its expression was not significantly altered by acidosis, suggesting that this protein may not be involved in mediating the effects of acid on mineralisation.

ATP is a well-documented inhibitor of mineralisation and its actions are thought to be mediated via both the P2 receptors and hydrolysis to produce PPi (Orriss et al., 2007, 2012a). Osteoblasts release ATP constitutively under normal conditions, and this release can be enhanced by fundamental stimuli such as hypoxia (Orriss et al., 2009), fluid shear stress (Genetos et al., 2005) and mechanical loading (Runnem et al., 2012). We found in this study, however, that exposure of osteoblasts to acidic culture medium did not influence ATP release or cell viability at any stage of culture, suggesting that the inhibition of mineralisation at low pH is unlikely to be mediated by increased extracellular ATP. Instead, the decreased mineralisation is likely to involve alterations in TNAP expression (because of differences in NNPP1 and TNAP expression and activity) and, consequently, the amounts of P and PP, produced.

In summary, the results presented in this study provide further evidence for the cell-mediated actions of acid on bone mineralisation, identifying for the first time a role for NNPP1 in this process. This work also adds to the growing body of evidence for the fundamental role of ecto-nucleotidases, extracellular ATP and the P/PP ratio in the regulation of bone mineralisation.

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Literature Cited
Anderson HC, Harmey D, Camacho NP, Garnellia R, Sipe JB, Tague S, Bi X, Johnson K, Terkeltaub R, Millan JL. 2005. Sustained osteomalacia of long bones despite major improvement in other hypophosphatasia-related mineral deficits in tissue nonspecific alkaline phosphatase/nucleotide pyrophosphatase phosphodiesterase 1 double-deficient mice. Am J Pathol 166:1711–1720.
Arnett TR. 2010. Acidosis, hypoxia and bone. Arch Biochem Biophys 503:103–109.
Arnett TR, Dempster DW. 1986. Effect of pH on bone resorption by rat osteoblasts in vitro. Endocrinology 119:19–124.
Brandao-Burch A, Key ML, Patel J, Arnett TR, Orriss IR. 2012. The P2Y receptor is an important regulator of extracellular ATP levels. Front Endocrinol (Lausanne) 3:41.
Brandao-Burch A, Utting J, Orriss IR, Arnett TR. 2005. Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. Calcif Tissue Int 77:167–174.
Felix R, Flesch H. 1976. Role of matrix vesicles in calcification. Fed Proc 35:169–171.
Fleisch H, Bisaz S. 1962. Mechanism of calcification: Inhibitory role of pyrophosphate. Nature 195:91–95.
Genetos DC, Geist DJ, Liu D, Donahue HJ, Duncan RL. 2005. Fluid shear-induced ATP secretion mediates prostaglandin release in MC3T3-E1 osteoblasts. J Bone Miner Res 20:41–49.
Genetos DC, Kephart CJ, Zhang Y, Yellowley CE, Donahue HJ. 2007. Oscillating fluid flow activation of gap junction hemichannels induces ATP release from MLO-Y4 osteocytes. J Cell Physiol 212:207–214.
Hajjawi MO, MacRae VE, Huesa C, Boyde A, Millan JL, Arnett TR, Orriss IR. 2014. Mineralisation of collagen-rich soft tissues and osteocyte lacunae in Enpp1⁻/⁻ mice. Bone 69C:139–147.
Harmey D, Hessle L, Narusawa S, Johnson KA, Terkelba R, Millan JL. 2004. Concentrated regulation of inorganic pyrophosphate and osteopontin by apk2, enpp1, and ank. An integrated model of the pathogenesis of mineralization disorders. Am J Pathol 164:119–1209.
Hessle L, Johnson KA, Anderson HC, Narusawa S, Sali A, Goding JW, Terkelba R, Millan JL. 2002. Tissue-specific alkaline phosphatase and plasma cell membrane glycoprotein-I are central antagonistic regulators of bone mineralization. Proc Natl Acad Sci USA 99:9445–9449.
Hoebertz A, Mahendran S, Burnstock G, Arnett TR. 2002. ATP and UTP at low concentrations strongly inhibit bone formation by osteoblasts: A novel role for the P2Y1 receptor in bone remodeling. J Cell Biochem 86:413–419.
Houston B, Stewart AJ, Farquharson C. 2004. PHOSPHO1-A novel phosphoprotein specifically expressed at sites of mineralisation in bone and cartilage. Bone 34:629–637.
Jaffe HL, Bodanusk A, Chandler JP. 1932. Ammonium chloride decalcification, as modified by calcium intake: The relation between generalized osteoporosis and osteitis fibrosa. J Exp Med 56:823–834.
Johnson K, Goding J, Van Etten D, Sali A, Hu SI, Farley D, Krug H, Hessle L, Millan JL, Terkelba R. 2003. Linked deficiencies in extracellular P and osteopontin mediate pathologic calcification associated with defective PC1- and ANK expression. J Bone Miner Res 18:1094–1104.
Johnson KA, Hessle L, Vangankar S, Weinberg C, Mauro S, Narusawa S, Goding JW, Sano K, Millan JL, Terkelba R. 2000. Osteoblast tissue-nonspecific alkaline phosphatase antagonizes and regulates PCs-1. Am J Physiol Regul Integr Comp Physiol 278:F1365–F1377.
Kernbach U. 2011. Animal models with pathological mineralization phenotypes. Joint Bone Spine 78:561–567.
Krieger NS, Sessler NE, Bushinsky DA. 1992. Acidosis inhibits osteoblastic and stimulates osteoclastic activity in vitro. Am J Physiol 262:F442–F448.
Li Q, Guo H, Chou DW, Berndt A, Sundberg JP, Uitto J. 2013. Mutant Enpp1a/sj mice as a model for generalized arterial calcification of infancy. Dis Model Mech 6:1227–1232.
Mackenzie NC, Huesa C, Rutscher F, MacRae VE 2012a. New insights into NNPP1 function: Lessons from clinical and animal studies. Bone 51:961–968.
Mackenzie NC, Zhu D, Mine EM, van HR, Mannine A, Darryl OL, Millan JL, Farquharson C, MacRae VE 2012b. Altered bone development and an increase in GF-23 expression in Enpp1⁻/⁻ mice. PLoS ONE 7:63217.
Okawa A, Nakamura I, Goto S, Moriya H, Nakamura Y, Ikegawa S. 1998. Mutation in Npgs in a mouse model of ossification of the posterior longitudinal ligament of the spine. Nat Genet 19:271–273.
Orriss IR, Hajjawi MO, Huesa C, MacRae VE, Arnett TR. 2014. Optimisation of the differing conditions required for bone formation in vitro by primary osteoblasts from mice and rats. Int J Mol Med 34:1201–1208.
