Activation of the P2Y<sub>2</sub> receptor regulates bone cell function by enhancing ATP release

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Abstract

Bone cells constitutively release ATP into the extracellular environment where it acts locally via P2 receptors to regulate bone cell function. Whilst P2Y<sub>2</sub> receptor stimulation regulates bone mineralisation, the functional effects of this receptor in osteoclasts remain unknown. This investigation used the P2Y<sub>2</sub> receptor knockout (P2Y<sub>2</sub>R<sup>−/−</sup>) mouse model to investigate the role of this receptor in bone. MicroCT analysis of P2Y<sub>2</sub>R<sup>−/−</sup> mice demonstrated age-related increases in trabecular bone volume (≤48%), number (≤30%) and thickness (≤17%). In vitro P2Y<sub>2</sub>R<sup>−/−</sup> osteoblasts displayed a 3-fold increase in bone formation and alkaline phosphatase activity, whilst P2Y<sub>2</sub>R<sup>−/−</sup> osteoclasts exhibited a 65% reduction in resorptive activity. Serum cross-linked C-telopeptide levels (CTX, resorption marker) were also decreased (≤35%). The resorption defect in P2Y<sub>2</sub>R<sup>−/−</sup> osteoclasts was rescued by the addition of exogenous ATP, suggesting that an ATP deficit could be a key factor in the reduced function of these cells. In agreement, we found that basal ATP release was reduced up to 53% in P2Y<sub>2</sub>R<sup>−/−</sup> osteoclasts. The P2Y<sub>2</sub> receptor agonists, UTP and 2-thioUTP, increased osteoclast activity and ATP release in wild-type but not in P2Y<sub>2</sub>R<sup>−/−</sup> cells. This indicates that the P2Y<sub>2</sub> receptor may regulate osteoclast function indirectly by promoting ATP release. UTP and 2-thioUTP also stimulate ATP release from osteoblasts suggesting that the P2Y<sub>2</sub> receptor exerts a similar function in these cells. Taken together, our findings are consistent with the notion that the primary action of P2Y<sub>2</sub> receptor signalling in bone is to regulate extracellular ATP levels.

Introduction

Adenosine triphosphate (ATP) has long been recognised for its role in intracellular energy metabolism; however, it is also exported to the extracellular environment where it acts as an important signalling molecule (Burnstock 2007a). Outside cells, ATP and related compounds act via purinergic receptors to modulate a range of biological processes. These receptors are classified into two groups: P1 and P2 receptors. There are four P1 receptors (A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub> and A<sub>3</sub>), which are activated by adenosine. The P2 receptors are further subdivided into P2X ligand-gated ion channels and P2Y G-protein-coupled receptors. P2X receptors are activated by ATP, whilst P2Y receptors respond to nucleotides including ATP, adenosine diphosphate (ADP), uridine triphosphate (UTP) and uridine diphosphate (UDP) (Abbracchio & Burnstock 1994, Burnstock 2007b). Currently, seven P2X receptors...
The P2Y receptors display distinct pharmacology with some being activated by adenine-containing nucleotides (P2Y1, P2Y12 and P2Y13), whilst others are stimulated by uridine-containing nucleotides (P2Y2, P2Y4, P2Y6 and P2Y14) (Burnstock 2007a,b). The primary agonist at the P2Y2 receptor is UTP, but it is also activated by ATP. Selective synthetic agonists (e.g. 2-thioUTP) are also available. Receptor stimulation activates phospholipase C and results in Ca2+ release from internal stores. Expression of the P2Y2 receptor has been reported in many tissues including heart, blood vessels, lung, kidney and skeletal muscle (Burnstock 2007a).

Bone cells express multiple P2 receptor subtypes, and knowledge of the functional effects of extracellular nucleotides in bone has increased significantly in recent years (Gartland et al. 2012, Burnstock et al. 2013, Orriss 2015, Noronha-Matos & Correia-de-Sa 2016). P2Y2 receptor expression by osteoclasts has been widely reported (Bowler et al. 1995, Hoebertz et al. 2000, Buckley et al. 2002, Orriss et al. 2011b). Early work using cells from a human osteoclastoma suggested that ATP could act via the P2Y2 receptor to promote bone resorption (Bowler et al. 1995). However, in a follow-up study, UTP failed to stimulate resorption, suggesting this was not the case (Bowler et al. 1998). To date, there are no studies directly describing the functional effects of P2Y2 receptor activation on osteoclasts. In contrast, activation of several other P2Y receptor subtypes (P2Y1, P2Y6, P2Y12 and P2Y14) has been associated with increased osteoclast formation and/or activity (Hoebertz et al. 2001, Orriss et al. 2011b, Su et al. 2012, Syberg et al. 2012b; Lee et al. 2013).

The role of the P2Y2 receptor in osteoblasts has been more extensively investigated. P2Y2 receptor expression by osteoblasts has been extensively reported (Bowler et al. 1995, Maier et al. 1997, Hoebertz et al. 2000), with several studies describing that expression is differentiation dependent with the highest levels seen in mature, bone-forming cells (Orriss et al. 2006, Noronha-Matos & Correia-de-Sa 2012). P2Y2 receptor activation in osteoblast-like cells activates several intracellular signalling pathways including protein kinase C, p38 mitogen-activated protein kinase, c-Jun NH2-terminal protein kinase and RhoA GTPase (Costesi et al. 2005, Pines et al. 2005, Katz et al. 2006, 2008, Gardinier et al. 2014). The P2Y2 receptor has also been shown to mediate the Ca2+ mobilisation induced by oscillatory fluid flow (You et al. 2002).

One of the first functional effects to be attributed to the P2Y2 receptor was the inhibition of bone mineralisation by ATP and UTP (Hoebertz et al. 2002, Orriss et al. 2007, Orriss et al. 2013). Consistent with this, initial skeletal analysis of 8-week-old P2Y2 receptor knockout mice (P2Y2−/−) demonstrated large increases in trabecular and cortical bone parameters in the long bones (Orriss et al. 2007, Orriss et al. 2011a). Furthermore, P2Y2 overexpression leads to decreased bone formation (Syberg et al. 2012a), and polymorphisms in the P2Y2 receptor gene are associated with increased bone mineral density and a decreased risk of osteoporosis (Wessellius et al. 2013). In contrast, a recent study using P2Y2R−/− mice on a different genetic background described small decreases in the trabecular bone in knockout animals (Xing et al. 2014); this work additionally reported that the P2Y2 receptor promotes bone mineralisation.

The P2Y2 receptor may also have a functional role in mediating osteoblast mechanosensitivity. Studies suggest that the P2Y2 receptor promotes mechanotransduction (Xing et al. 2014) and increases cell stiffness and cytoskeletal rearrangement in response to fluid shear stress (Gardinier et al. 2014).

Expression of the P2Y2 receptor has also been reported in MLO-Y4 osteocyte-like cells (Kringlebach et al. 2014). The same study also demonstrated controlled ATP release from these cells and reported that UTP, probably acting via the P2Y2 or P2Y4 receptors, increased this ATP release.

Available evidence thus indicates that the P2Y2 receptor plays significant, although not yet fully defined roles in regulating bone remodelling. This study used the P2Y2R−/− mouse, which was first generated almost 2 decades ago (Cressman et al. 1999), to determine how P2Y2 receptor-mediated signalling influences bone cell function in vitro and in vivo, with a particular focus on its effects in osteoclasts.

**Methods**

**Reagents**

Tissue culture reagents were purchased from Life Technologies (Paisley, UK); unless mentioned, all chemicals were purchased from Sigma-Aldrich (Poole, Dorset, UK). UTP and 2-thioUTP were purchased from Tocris Bioscience (Bristol, UK).

**Animals**

Mice lacking the P2Y2 receptor gene (P2Y2R−/−) were obtained from Jackson Laboratories (Bar Harbor, Maine,
USA). The generation and characterisation of P2Y2R−/− mice (C57BL/6j background) has been previously described (Homolya et al. 1999). All animals were housed under standard conditions with free access to food and water. Animals were bred from homozygote (P2Y2R+/+) and parental strain wild-type (P2Y2R+/−) breeding pairs. All procedures complied with the UK Animals (Scientific Procedures) Act 1986 and were reviewed and approved by the Royal Veterinary College Research Ethics Committee.

Microcomputed x-ray tomographic (µCT) analysis of P2Y2R−/− mice

The tibiae and femora were isolated from male 4-, 8-, 16- and 24-week-old P2Y2R−/− and P2Y2R+/+ mice (n=10), fixed in 10% neutral buffered formalin (NBF) for 24 h and stored in 70% ethanol until scanning. µCT analysis of trabecular and cortical bone parameters was performed on the tibial and femoral metaphysis (SkyScan 1172, Bruker, Belgium). The appearance of the first cartilage bridge was used as a reference point, with an offset of 0.4 mm and 2.5 mm for trabecular and cortical bone, respectively. In all cases, the length of bone analysed was 1 mm. The µCT scanner was set at 50 Kv and 200 µA using a 0.5 mm Al filter and a resolution of 4.3 µm. Analysis of tibial and femoral bone parameters was performed ‘blind’. The images were reconstructed, analysed and visualised using SkyScan NRecon, CTAn and CTVol software, respectively. Bone mineral density (BMD) was calibrated and calculated using hydroxyapatite phantoms with a known density.

Osteoblast formation assay

Osteoblasts were isolated from the calvariae of 3- to 5-day-old P2Y2R+/+ or P2Y2R−/− mice by trypsin/collagenase digestion as previously described (Orriss et al. 2012). Cells were cultured for up to 21 days in alpha-minimum essential medium (α-MEM) supplemented with 2 mM β-glycerophosphate and 50 µg/mL ascorbic acid. Half medium changes were performed every 3 days for the duration of the culture. The total area of bone nodules formed was quantified by image analysis, as described previously (Orriss et al. 2012b).

Primary osteoblasts of bone marrow/stromal cell origin were obtained from the long bones of 6- to 8-week-old male P2Y2R−/− or P2Y2R+/+ mice as described previously (Orriss & Arnett 2012). Cells were plated onto 5 mm diameter ivory discs (10⁶ cells) in 96-multiwell plates in α-MEM supplemented with 10% FCS, 5% gentamicin, 100 nM PGE2, 200 ng/mL M-CSF and 3 ng/mL receptor activator of nuclear factor κB ligand (RANKL, R&D Systems Europe Ltd, Abingdon, UK). After 24 h, discs containing adherent osteoclast precursors were transferred to 6-well trays (4 discs/well in 4 mL medium) for a further 6 days. Culture medium was acidified to pH~7.0 by the addition of 10 meq/l H+ (as HCL) on day 7 to activate resorption (Orriss & Arnett 2012). P2Y2 receptor agonists (10 nM–10 µM UTP or 2-thioUTP) were added from day 3 of culture. Apyrase (a broad spectrum ecto-nucleotidase) was used to determine the effects of endogenous ATP.

Primary osteoblasts were fixed in 2.5% glutaraldehyde and were stained to demonstrate tartrate-resistant acid phosphatase (TRAP). Osteoclasts were defined as TRAP-positive cells with 2 or more nuclei and/or clear evidence of resorption. The total number of osteoclasts and the plan surface area of resorption pits on each disc was assessed ‘blind’ by transmitted light microscopy and reflective light microscopy and dot-counting morphometry, respectively.

Measurement of serum bone markers

Blood was collected from 4-, 8-, 16- and 24-week-old male P2Y2R+/+ and P2Y2R−/− mice by cardiac puncture immediately after termination. Following clotting,
samples were centrifuged at 500 g and the serum was frozen until analysis. Levels of the bone formation marker, N-terminal propeptide of type I collagen (P1NP) and the bone resorption marker, cross-linked C-telopeptide (CTX), were assayed using the P1NP and RatLaps ELISAs, respectively (Immunodiagnostics Systems Ltd, UK).

Histology
Histological analysis was performed on the femur of 8- and 24-week-old male P2Y2R+/+ or P2Y2R−/− mice. Tissues were fixed in 10% NBF, decalcified in 10% EDTA for three weeks and embedded in paraffin wax blocks. Serial sections were cut every 5 µm, and slides were stained with TRAP counterstained with haematoxylin to visualise osteoclasts.

Total RNA extraction and DNase treatment
P2Y2R+/+ and P2Y2R−/− osteoclasts were cultured on dentine discs for 9 days (mature, resorbing cells) before total RNA was extracted using TRIZOL reagent (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. Osteoblasts were cultured for 14 days (mature, bone-forming cells) before RNA collection. 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. RNA was stored at −80°C until amplification by qRT-PCR.

Quantitative real-time polymerase chain reaction (qRT-PCR)
Osteoclast and osteoblast RNA (50 ng) were transcribed and were amplified using the qPCRBIO SyGreen one-step qRT-PCR kit (PCR Biosystems, London, UK), which allows cDNA synthesis and PCR amplification to be carried out sequentially. qRT-PCR was performed according to manufacturer’s instructions with initial cDNA synthesis (45°C for 10 min) and reverse transcriptase inactivation (95°C for 2 min) followed by 40 cycles of denaturation (95°C for 5 sec) and detection (60°C for 30 sec). All reactions were carried out in triplicate using RNAs derived from 4 different cultures. Data were analysed using the Pfaffl method of relative quantification (Pfaffl 2001). Primers were obtained from Qiagen Ltd (Manchester, UK).

Measurement of ATP release
Prior to measurement of ATP release, culture medium was removed, cell layers were washed and cells were incubated with serum-free DMEM (phenol red free). To measure the effects of P2Y2 receptor deletion on basal ATP release, samples were collected after 1 h and immediately measured luminometrically using the luciferin-luciferase assay, as described previously (Orriss et al. 2009). All ATP measurements were normalised to cell number. Cell viability and cell number were determined using the CytoTox 96 colorimetric cytotoxicity assay (Promega UK, Southampton UK).

To examine the effects of acute exposure to UTP or 2-thioUTP (0.1–50 µM), these agonists were added to the serum-free DMEM. Samples were taken for quantification 10, 30, 60 and 90 min after treatment. The luminescence of the DMEM (± UTP/2-thioUTP) was used as a background reading and subtracted from the relevant measurements. Standard curves used to calculate the ATP concentrations in the presence or absence of UTP/2-thioUTP. To investigate the effects of long-term treatment with P2Y2 receptor agonists, osteoclasts and osteoblasts were cultured with UTP or 2-thioUTP (0.1–100 µM) for 7 or 14 days, respectively. Fresh UTP/2-thioUTP was added at each medium exchange. On the day of assay culture, medium was removed and cells were incubated with serum-free DMEM without agonists. Samples were collected after 1 h and were measured immediately.

To determine the effects of P2Y2 deletion on ATP breakdown, cells were swapped to DMEM containing 1 µM ATP, and samples were taken after 2, 5, 10, 30 and 60 min.

Statistical analysis
Data were analysed using GraphPad Prism 6 software (San Diego, CA). Results are expressed as means ± SEM for between 6 and 12 biological replicates. Statistical analyses of bone parameters were performed by two-tailed unpaired Student’s t-test. In vitro data were analysed using an unpaired Student’s t-test, one-way or two-way ANOVA, followed by a Bonferroni post hoc test. For all in vitro work,
Results

P2Y2R−/− mice show age-related increases in trabecular bone

High-resolution μCT analysis revealed that P2Y2R−/− mice display increased levels of trabecular bone compared to age-matched P2Y2R+/+ controls. These differences appear to be age-related with the biggest changes observed in the 24-week animals. Trabecular bone volume (BV/TV) was increased ≤46% in the femur and ≤48% in the tibia of P2Y2R−/− mice (Fig. 1A, B and O). Trabecular number (Tb.N) was increased ≥27% in the femora (Fig. 1C and O) and ≤30% in the tibiae (Fig. 1D and O). Trabecular thickness (Tb.Th) was unchanged up to 8 weeks of age but increased ≤10% and ≤17% at 16 and 24 weeks, respectively (Fig. 1E, F and O). Trabecular bone mineral density (Tb.BMD) was ≤12% higher in P2Y2R−/− mice (Fig. 1G and H). No differences were observed in the cortical bone volume (Fig. 1K, L and O), cortical thickness (Fig. 1K and L), endosteal and periosteal diameter (Fig. 1M and N) and bone length at any age.

Increased bone formation by osteoblasts from P2Y2R−/− mice

The level of mineralised bone nodule formation was increased ~3-fold in P2Y2R−/− calvarial osteoblasts (Fig. 2A and G) and 5-fold in P2Y2R−/− long bone osteoblasts (Fig. 2B). P2Y2 receptor deletion increased basal TNAP activity (≤3-fold) in calvarial and long bone osteoblasts at all stages of differentiation with the largest effects being observed in the mineralising cells (Fig. 2C and D). Serum TNAP activity was up to 60% higher in P2Y2R−/− animals (Fig. 2E); no differences were observed in the serum P1NP levels (Fig. 2F). No differences in total protein content were observed in any TNAP activity experiments.

Osteoclasts from P2Y2R−/− mice exhibit defective resorption

Whilst no differences in osteoclast numbers were observed (Fig. 3A and D), the level of resorption per osteoclast was decreased by 75% in P2Y2R−/− cultures (Fig. 3B and D). Serum CTX levels were reduced up to 35% in P2Y2R−/− mice (Fig. 3C). Qualitative histology suggested that decreased numbers of osteoclasts were evident on the trabecular and endocortical bone surfaces of 24-week-old P2Y2R−/−; however, no differences were observed in 8-week-old animals (Fig. 3E).

Changes in gene expression in P2Y2R−/− osteoclasts and osteoblasts

The effect of P2Y2 receptor deletion on the expression of resorption associated genes and ecto-nucleotidases was investigated in mature, resorbing osteoclasts. mRNA expression of many genes (TRAP, CICN7, RANK and c-fms) showed a downward trend, but only cathepsin K expression was significantly reduced (4.8-fold). Osteoclasts express a range of ecto-nucleotidases that hydrolyse ATP (Hajjawi et al. 2014) and NDPK (nucleoside disphosphokinase), which can regenerate ATP from ADP. P2Y2 receptor deletion did not influence the expression of any of these genes (Table 1).

In osteoblasts, deletion of the P2Y2 receptor increased osteocalcin (Ocn), osteopontin (Opn) and osteoprotegerin (OPG) expression 3.3, 6 and 4.5-fold, respectively. The mRNA expression of Col1α1, Runx2, TNAP, osteonectin, RANKL, MCSF and the ectonucleotidases was unchanged (Table 1).

Activation of the P2Y2 receptor increases bone resorption

Treatment with UTP and 2-thioUTP had no effect on osteoclast formation in P2Y2R+/+ or P2Y2R−/− cells (Fig. 4A and B). However, the area resorbed per osteoclast was dose-dependently increased by up to 80% and 45% in P2Y2R−/− cells treated with UTP and 2-thioUTP (≥100 nM), respectively. No effects on resorption were seen in P2Y2R−/− osteoclasts (Fig. 4C and D).

Reversal of resorption defect in P2Y2R−/− osteoclasts by extracellular ATP

P2Y2R−/− osteoclasts displayed a 53% reduction in ATP release (Fig. 4E) but showed no difference in the rate of ATP breakdown (Fig. 4F). Apyrase (≥1U/mL), a broad spectrum ecto-nucleotidase that rapidly degrades ATP and ADP, inhibited bone resorption by up to 55% (Fig. 4G). To determine if reduced extracellular ATP was the cause of the decreased resorption seen in P2Y2R−/− osteoclasts, cells were cultured with exogenous ATP (1–10 μM). Treatment with ATP (≥1 μM) fully rescued the resorption defect seen in P2Y2R−/− osteoclasts (Fig. 4H).
Figure 1

P2Y$_{R^{-/-}}$ mice display age-related increases in trabecular bone. Trabecular bone volume (BV/TV) was increased by ≤46% and ≤48% in the (A) femur and (B) tibiae of P2Y$_{R^{-/-}}$ mice, respectively. Trabecular number (Tb.N) was increased (C) ≤27% in the femur and (D) ≤30% in the tibia. Trabecular thickness (Tb.Th) was ≤17% and ≤10% higher in the (E) femur and (F) tibia, respectively. (G, H) Trabecular BMD was increased ≤12%. (I, J) Cortical bone volume, (K, L) cortical thickness, (M) periosteal diameter and (N) endosteal diameter were unchanged. Values are means ± SEM ($n=10$), significantly different from controls: * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$. (O) Representative 3D volumetric images of the trabecular and cortical bone of 24-week-old P2Y$_{R^{-/-}}$ and P2Y$_{R^{+/+}}$ mice.
Figure 2
Increased bone formation by osteoblasts from P2Y<sub>2</sub>R<sup>−/−</sup> mice. In cultures of (A) calvarial and (B) long-bone osteoblasts from P2Y<sub>2</sub>R<sup>−/−</sup> mice, the level of mineralised bone nodule formation was increased 3-fold and 5-fold, respectively. Basal TNAP activity was increased by ≤3-fold in P2Y<sub>2</sub>R<sup>−/−</sup> (C) calvarial and (D) long-bone osteoblasts (n=6). (E) Serum P1NP levels were unchanged in P2Y<sub>2</sub>R<sup>−/−</sup> mice (n=10). Values are means ± SEM, significantly different from controls: * = P < 0.05, ** = P < 0.01, *** = P < 0.001. (G) Representative whole well scans (unstained) and phase-contrast microscopy images (alizarin red stained) showing the increased bone formation in cultures of P2Y<sub>2</sub>R<sup>−/−</sup> calvarial osteoblasts. Scale bars: whole well = 0.5 cm, microscopy images = 50 µm.
P2Y₂ receptor agonists increase ATP release from osteoclasts

In P2Y₂⁺/⁺ cells, 10 min after the addition of UTP (≥1 μM), extracellular ATP levels were doubled; the increase in ATP levels was sustained for up to 90 min post treatment (Fig. 5A). No effect of UTP on ATP release was seen in P2Y₂⁻/⁻ osteoclasts at any stage (Fig. 5B, C, and D). Treatment with 2-thioUTP (≥0.1 μM) also dose-dependently increased extracellular ATP levels by ≤50% for up to 90 min in P2Y₂⁺/⁺ osteoclasts (Fig. 5E); 2-thioUTP was without effect in P2Y₂⁻/⁻ cells (Fig. 5F, G, and H).

Figure 3
Osteoclasts from P2Y₂⁻/⁻ mice exhibit defective resorption. P2Y₂ receptor deletion (A) had no effect on osteoclast number but (B) decreased resorption per osteoclast by 75% (n = 8). (C) Serum CTX levels were up to 35% lower in P2Y₂⁻/⁻ mice (n = 10). Values are means ± SEM, significantly different from controls: * = P < 0.05, ** = P < 0.001. (D) Representative transmitted and reflective light microscopy images showing the decreased resorption seen in P2Y₂⁻/⁻ osteoclast cultures. Scale bar = 50 μm. (E) Qualitative histology suggested that the number of TRAP-positive osteoclasts was reduced on the endocortical and trabecular bone surfaces in 24- but not 8-week-old P2Y₂⁻/⁻ mice. Scale bar = 100 μm.
P2Y2 receptor activation induces ATP release in bone

Table 1 The effect of P2Y2 receptor deletion on gene expression in osteoblasts and osteoclasts.

<table>
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Data obtained from qPCR. Values are means ± SEM (n=4). Significantly different from controls * = P<0.05, ** = P<0.01, *** = P<0.001.

RANK = receptor activator of nuclear factor-κB, c-fms = Mcsf receptor, TRAP = tartrate-resistant acid phosphatase, CICN7 = chloride channel, NTPdase = ecto-nucleotide pyrophosphatase/phosphodiesterase 1/3, NDPK = nucleoside diphosphokinase, Ocn = osteocalcin, Opn = osteopontin, TNAP = alkaline phosphatase, On = osteonectin, Col1α1 = collagen 1 alpha 1, Runx2 = runt-related transcription factor 2, RANKL = receptor activator of nuclear factor-κB ligand, Mcsf = macrophage colony stimulating factor, Opg = osteoprotegerin.

The effect of long-term treatment (7 days) with P2Y2 receptor agonists on basal ATP release was also investigated in mature osteoclasts. In P2Y2R+/+ cells, UTP and 2-thioUTP (≥1μM) increased ATP release by up to 70% and 65%, respectively (Fig. 6A). No increase in ATP release was seen in P2Y2R−/− osteoclasts. Standard curves used to calculate ATP levels are shown in Fig. 5K and L. In all experiments, cell viability was unchanged (not shown).

ATP release from osteoblasts is stimulated by UTP and 2-thioUTP

The rate of ATP breakdown was unchanged in P2Y2R−/− osteoblasts (Fig. 6A). ATP release from P2Y2R−/− cells was decreased (≤60%) at all stages of differentiation (Fig. 6B). Long-term treatment (14 days) with UTP and 2-thioUTP increased the levels of ATP release by up to 4-fold and 3-fold, respectively, in P2Y2R+/+osteoblasts (Fig. 6C and D). No effects were seen in P2Y2R−/− osteoblasts.

Acute UTP treatment increased ATP release from P2Y2R+/+ osteoblasts up to 4-fold within 10 min; stimulatory effects were sustained for up to 60 min (Fig. 6E). UTP was without effect in P2Y2R−/− osteoblasts (Fig. 6F, G and H). 2-thioUTP also enhanced ATP release (≤4-fold) from P2Y2R+/+, but not from P2Y2R−/− osteoblasts (Fig. 6I, J, K and L).

Discussion

This study examined the role of P2Y2 receptor-mediated signalling in osteoclasts and osteoblasts. We found that global deletion of the P2Y2 receptor resulted in greater amounts of trabecular bone and increased BMD. Culture of cells derived from P2Y2R−/− mice revealed that osteoclast resorptive activity was decreased whilst bone mineralisation was increased. Mechanistic analysis revealed that P2Y2 receptor activation (acute and prolonged) promotes ATP release from osteoclasts and osteoblasts.

Several P2Y receptors (P2Y1, P2Y6, P2Y12 and P2Y14) and extracellular nucleotides (e.g. ATP, ADP and UDP) have been implicated in the regulation of osteoclast formation and activity (Hoebertz et al. 2001, Orriss et al. 2011b, Su et al. 2012, Syberg et al. 2012b, Lee et al. 2013). However, there are no reports directly describing the functional role of the P2Y2 receptor in osteoclasts. This study found that the P2Y2 agonists, UTP and 2-thioUTP, dose-dependently stimulated bone resorption. Consistent with a pro-resorptive role for UTP and the P2Y2 receptor, we observed that P2Y2R−/− animals had decreased serum CTX levels and that cultured P2Y2R−/− osteoclasts displayed reduced resorptive activity and cathepsin K expression. UDP, the breakdown product of UTP, acts via the P2Y6 receptor to promote osteoclast function (Orriss et al. 2011b). However, since the actions of UTP are lost in P2Y2R−/− osteoclasts, it is unlikely that the effects observed here are due to P2Y6 receptor-mediated signalling.

Earlier studies have reported that P2Y2 receptor activation by ATP and UTP can both inhibit (Hoebertz et al. 2002, Orriss et al. 2007, Orriss et al. 2012a) and promote (Xing et al. 2014) bone mineralisation. Consistent with its role as a negative regulator of bone mineralisation, we observed that P2Y2R−/− osteoblasts exhibited increased levels of bone formation, Ocn expression and TNAP activity. Surprisingly, TNAP mRNA expression was unaffected in P2Y2R−/− osteoblasts. This could indicate that P2Y2 receptor signalling increases enzyme activity by influencing the post-translational modifications of TNAP rather than the overall expression level. We have previously shown that the effects of ATP and UTP are restricted to the mineralisation process through collagen expression and activity being unaffected (Orriss et al. 2007).
The lack of effect of P2Y2 receptor deletion on serum P1NP levels is consistent with these observations.

In agreement with the in vitro findings, our longitudinal µCT study revealed that P2Y2 deletion led to age-related increases in trabecular bone and BMD. These data are also consistent with our earlier description of the bone phenotype of 8-week-old P2Y2R−/− animals (Orriss et al. 2011a) and the observation that P2Y2 receptor overexpression leads to decreased bone formation (Syberg et al. 2012a). However, they are at variance to a recent report of reduced bone levels in P2Y2R−/− mice (Xing et al. 2014). The reasons for these divergent results are unclear but given that parental strain has been shown to affect the phenotype of the P2X7 receptor knockout (Syberg et al. 2012c), the differing genetic background of the animals studied (C57BL/6 compared to SV129 (Xing et al. 2014)) could be a factor. Variations in µCT methodology could also contribute; for example, this study analysed a 1 mm region of the trabecular bone within the metaphyseal portion of the long bones at a resolution of 4.3 µm. In contrast, Xing and coworkers measured the trabecular bone within a narrow region of the diaphysis at a lower resolution (10.5 µm) (Xing et al. 2014).

Unlike the observed effects in the trabecular bone, in both this study and that of Xing and coworkers (Xing et al. 2014), cortical bone parameters were unaffected in P2Y2R−/− mice. This suggests that P2Y2 receptor deletion does not have significant effects on bone growth. Thus, P2Y2 receptor-mediated signalling appears to be more important in bone undergoing rapid turnover. In vivo, osteoblast and ostoclast functions are tightly coupled with osteoclast activation being dependent on osteoblasts. Gene expression analysis revealed a significant increase in osteoblast expression of OPG whilst RANKL expression was unchanged. If reflected in vivo, this would reduce osteoclast formation and activity and could contribute to the decreased bone resorption seen in P2Y2R−/− mice. In agreement, qualitative observations showed that osteoclast numbers on the trabecular and endocortical bone surfaces appeared reduced in these animals. Further,
bone histomorphometric analysis of in vivo parameters such as bone formation rate and osteoclast number would confirm this and build on the findings reported here.

Controlled ATP release has been demonstrated from numerous cell types including bone cells. Several studies have indicated that the primary method of ATP release from osteoblasts is vesicular exocytosis (Romanello et al. 2001, Genetos et al. 2005, Orriss et al. 2009), although the P2X7 receptor may also be involved (Brandao-Burch et al. 2012). In osteoclasts, ATP release involves the P2X7 receptor (Pellegatti et al. 2011, Brandao-Burch et al. 2012). Increasing evidence now suggests that ATP can act to enhance its own release; ATP or UTP-induced ATP release has been demonstrated from MLO-Y4 osteocyte-like cells (Kringelbach et al. 2014), leukocytes (De Ita et al. 2016), urothelial cells (Mansfield and Hughes 2014) and...
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cells from the carotid body (Zhang et al. 2012). The P2Y₂ receptor is thought to mediate this increased ATP release in cells including osteocytes (Kringelbach et al. 2014) and leukocytes (De Ita et al. 2016). Therefore, we investigated whether UTP could exert its functional effects on bone cells indirectly i.e. acting via the P2Y₂ receptor to induce ATP release. We found that P2Y₂R⁻/⁻ osteoblasts and osteoclasts showed reduced levels of basal ATP release. Furthermore, UTP and 2-thioUTP increased ATP release from these cells following both acute (≤90 min) and long-term (≤14 days) treatment. These stimulatory effects were lost in P2Y₂R⁻/⁻ cells suggesting that the increased extracellular ATP levels were mediated via P2Y₂ receptor signalling. For the long-term experiments, UTP and 2-thioUTP were present in the culture medium for 7 or 14 days days prior to testing but not in the medium used for the subsequent ATP release.

Figure 6
The role of the P2Y₂ receptor in ATP release from osteoblasts. (A) No differences were observed in the rate of ATP breakdown between P2Y₂R⁺/⁺ and P2Y₂R⁻/⁻ osteoblasts. (B) Basal ATP release was up to 60% lower from P2Y₂R⁻/⁻ osteoblast. Increased ATP release from P2Y₂R⁺/⁺ but not P2Y₂R⁻/⁻ osteoblasts treated for 14 days with (C) UTP (≤4-fold) and (D) 2-thioUTP (≤3-fold). (E) Acute treatment with UTP (≥10 µM) increased ATP release by ≤4-fold for up to 60 min. (F, G, H) No effect of UTP (10 µM) on ATP release from P2Y₂R⁻/⁻ osteoblasts. (I) ≥1 µM 2-thioUTP also enhanced ATP release (≤4-fold) from P2Y₂R⁺/⁺ osteoblasts but was without effect in P2Y₂R⁻/⁻ cells (J, K, L). Values are means ± sem (n = 12), significantly different from controls: * = P < 0.05, ** = P < 0.01, *** = P < 0.001. Differences between P2Y₂R⁺/⁺ and P2Y₂R⁻/⁻: # = P < 0.05, ## = P < 0.01, ### = P < 0.001.
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![Diagram of purinergic signaling in osteoclast and osteoblast function]

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This suggests that repeated P2Y<sub>2</sub> receptor stimulation could induce changes to the cellular processes, which regulate ATP efflux from bone cells. However, at present, the mechanisms by which this could occur are unknown. Interestingly, P2Y<sub>2</sub> receptor activation in osteoblast-like cells has been shown to induce actin fibre formation in response to fluid shear stress (Gardinier et al. 2014). This ability to regulate cytoskeletal rearrangement could result in alterations in the vesicular release pathway.

Extracellularly, ATP is rapidly broken down by ecto-nucleotidases, restricting its actions to cells close to the release site (Zimmermann et al. 2012). The rate of ATP breakdown and the mRNA expression of ecto-nucleotidases (NPPs, NTPdases) were unchanged in P2Y<sub>2</sub>R<sup>−/−</sup> cells. Thus, our findings suggest that the primary effect of P2Y<sub>2</sub> receptor activation is to stimulate the level of ATP release from bone cells rather than to influence the rate of ATP degradation or regeneration.

Following release, ATP can act on other P2 receptors to influence the function of surrounding cells. In osteoclasts, ATP and its breakdown product ADP act via the P2Y<sub>1</sub> and/or P2Y<sub>12</sub> receptors to promote bone resorption (Hoebertz et al. 2001, Su et al. 2012). Thus, our finding that P2Y<sub>2</sub> receptor activation promotes ATP release suggests indirect actions of UTP on bone resorption (a potential mechanism of action is shown in Fig. 7). Consistent with this idea, we observed that the addition of exogenous ATP rescued the resorption defect in P2Y<sub>2</sub>R<sup>−/−</sup> osteoclasts; although not studied here, ADP would be expected to have a similar effect. Furthermore, apyrase, which breaks down all endogenous ATP, inhibited osteoclast activity. The use of apyrase is likely to cause a rapid accumulation of adenosine. We have shown that adenosine has no effect on osteoclast function (Hajjawi et al. 2016), whilst others report that it promotes resorption (Kara et al. 2010). If the actions of apyrase were a consequence of higher adenosine levels, an increase (or no effect) in resorption would be expected. However, since we observed the opposite, it is more likely that the functional effects of apyrase are due to reduced extracellular ATP levels.

The role of purinergic signalling in osteoblasts has been widely studied, and for some P2 receptors, multiple functional effects have been described (Gartland et al. 2012, Burnstock et al. 2013, Orriss 2015, Noronha-Matos and Correia-de-Sa 2016). The diverse range of experimental models and culture conditions employed in vitro has often resulted in conflicting or confounding results regarding these actions. This is particularly evident for the P2Y<sub>2</sub> and P2X7 receptors, stimulation of which has been shown to both inhibit and promote bone mineralisation (Orriss et al. 2007, Panupinthu et al. 2007, Orriss et al. 2012a, Noronha-Matos et al. 2014, Xing et al. 2014).
The data presented here show that P2Y2 deletion leads to increased levels of bone mineralisation. Based on our findings, one potential mechanism of action is summarised in Fig. 7. We suggest that UTP acts at the P2Y2 receptor to stimulate ATP release, once released ATP can then act via other P2 receptors to block bone mineralisation (Orriss et al. 2012a), as well as exert a direct physiochemical blockade via its breakdown product, pyrophosphate (Orriss et al. 2007, Orriss et al. 2016).

Fluid flow and mechanical stress are well-known stimulators of osteoblast ATP release (Romanello et al. 2001, Genetos et al. 2005, Rumney et al. 2012). This enhanced release of ATP has been implicated in mechanically induced bone formation via increased prostaglandin E2 (PGE2) secretion (Genetos et al. 2005). However, the ATP levels required to induce PGE2 production are 10-fold higher than those needed to inhibit mineralisation and may only occur following mechanical stress. These potentially confounding actions serve to illustrate the highly complex, local effects of purinergic signalling on bone cell function. Thus, how a bone cell responds to these signals is likely to be influenced by factors including local nucleotide concentration, receptor expression profile, ecto-nucleotidase expression and activity, and, for osteoblasts and osteocytes, degree of mechanical stress experienced.

In conclusion, this study describes, for the first time, a role for the P2Y2 receptor in regulating osteoclast function. The in vitro findings also provide further support for the inhibitory actions of P2Y2 receptor signalling on bone mineralisation under normal conditions. Taken together, our findings indicate that the P2Y2 receptor modulates bone homeostasis by regulating extracellular ATP levels and, consequently, local purinergic signalling.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contributions
IRO and TRA helped in experimental design; IRO, DG, KS, MORH and JJP performed experimental work; IRO and TRA wrote and revised manuscript.

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