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The regulation of bone cell function by extracellular nucleotides

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ABSTRACT

Extracellular nucleotides, acting via P2 receptors, modulate bone remodelling by inhibiting osteoblast and stimulating osteoclast activity. The aim of this thesis was to investigate further the effects of extracellular nucleotides on bone cell function under both normal and stress situations. Nucleotide agonists were shown to evoke intracellular Ca\(^{2+}\) responses in rat osteoblasts from \(\geq 0.2 \mu M\). The approximate order of potency was ATP > UTP = ATP\(_\gamma S\) > ADP > UDP > 2-MeSATP = BzATP > \(\alpha\beta\)-meATP, consistent with the expression of functional P\(_{2X}\), P\(_{2X}\), P\(_{2Y}\), P\(_{2Y}\), P\(_{2Y}\) and P\(_{2Y}\) receptors. A dramatic increase in intracellular Ca\(^{2+}\) responses to ATP or UTP was observed in osteoblasts cultured for 8-10 days compared to 4 days, indicating that osteoblast responsiveness to nucleotides increases with cell differentiation. P2 receptor mRNA and protein expression in osteoblasts was shown to be differentiation dependent and was characterised by a shift from P\(_{2X}\) to P\(_{2Y}\) expression, with mature osteoblasts strongly expressing P\(_{2Y}\), P\(_{2Y}\) and P\(_{2Y}\) receptors. Closer investigation revealed ATP and UTP decrease alkaline phosphatase expression and activity, indicating that extracellular nucleotides primarily inhibit mineralisation rather than organic matrix synthesis. Taken together these data suggest the P\(_{2Y}\) receptor, and possibly the P\(_{2Y}\) receptor could function as "off-switches" for mineralised bone formation. Constitutive ATP release from primary rat osteoblasts was found to occur via vesicular exocytosis in a differentiation dependent manner. Moreover, transient exposure to hypoxia (2% O\(_2\)) or hyperthermia (40\(^\circ\)C) induced a rapid, significant increase in ATP release. In contrast, continuous culture at 2% O\(_2\), 34\(^\circ\)C or 40\(^\circ\)C caused marked impairment of osteoblast function and ATP release. Given the negative effects of extracellular nucleotides on bone cell function, locally increased concentrations following stress situations may contribute to the bone loss associated with hypoxia or inflammation \textit{in vivo}. A bone densitometer was used to screen mice deficient in selected P2 receptors. Bone mineral content and density were increased up to 18% in P\(_{2X}\) mice, decreased up to 10% in P\(_{2Y}\) mice and P\(_{2X}\) mice but unaffected in P\(_{2X}\) mice. In summary, the work described here provides further evidence for the role of extracellular nucleotides and their receptors in the regulation of bone cell function in health and disease.
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PREFACE

The primary aim of this research was to investigate the effects of extracellular nucleotides on bone cell function during normal and stress situations. Previous work has demonstrated the important role of extracellular nucleotides and their receptors in the modulation of bone remodelling and the work carried out here builds on this pre-existing knowledge. Chapter 1, the general introduction, provides an overview of (1) the differentiation and function of bone cells; (2) extracellular nucleotides and purinergic signalling, and (3) purinergic signalling and bone cell function.

Bone cells express a number of P2 receptors, activation of which causes increased intracellular calcium (Ca$^{2+}$). In Chapter 2, several techniques allowed the detailed study of P2 receptors expression in osteoblasts as they differentiate with time in culture. Using the high-throughput fluorescence imaging plate reader (or FLIPR), it is possible to measure intracellular responses to agonist stimulation; this system utilises a 96-well format and allows the simultaneous addition of test compounds to cell layers. Using osteoblasts derived from primary rat calvaria, the FLIPR was employed to screen multiple purine agonists, including ATP, UTP and ADP, for activity. In agreement with expression of functional P2 receptors, nucleotides stimulated increased intracellular Ca$^{2+}$ in osteoblasts with the following order of potency ATP > UTP = ATPγS > ADP > UDP > 2-MeSATP = Bz-ATP > αβ-meATP. Using the FLIPR, responses to nucleotide agonists were tested in osteoblasts cultured for 4, 8 and 10 days; the amplitude of response increased dramatically with time in culture whilst cell number only doubled. These data indicated osteoblast responses to ATP and UTP increased with differentiation and time in culture. Semi-quantitative reverse transcriptase PCR (RT-PCR) and immunofluorescence were employed to investigate mRNA and protein levels, respectively. RT-PCR demonstrated P2X$_2$ and P2X$_3$ receptor expression to be highest in proliferating osteoblasts and less prominent in mature cells. P2X$_7$ and P2Y$_1$ receptor expression remained relatively constant, whilst P2Y$_2$ receptor expression increased dramatically with differentiation. P2Y$_4$ and P2Y$_6$ receptor levels appeared highest at intermediate periods. Immunofluorescence confirmed these trends. Study of the inhibitors suramin and reactive blue 2 suggested that nucleotides exhibit their inhibitory effects on bone formation via the P2Y$_2$.
receptor subtype, although the involvement of the P2Y4 receptor subtype cannot be discounted. Furthermore, correlating with the increased P2Y2 receptor expression, bone nodule formation assays demonstrated that addition of ATP or UTP to mature osteoblasts can effectively "switch off" bone formation.

In Chapter 3, the nucleotide-mediated inhibition of bone nodule formation was studied in more detail. Using light microscopy it became apparent that deposition of organic matrix is generally unaffected in nucleotide treated cultures. As alkaline phosphatase (ALP) is important in mineralisation the effect of nucleotides on enzyme activity were quantified; both ATP and UTP but not ADP and UDP, significantly inhibited ALP activity in vitro. RT-PCR analysis demonstrated a marked down regulation of ALP and osteocalcin mRNA in nucleotide treated cultures, whilst expression of other bone matrix proteins was unaffected. These findings suggest ATP and UTP can act via the P2Y2 receptor and possibly the P2Y4 receptor to down regulate ALP expression, and by primarily inhibiting mineralisation disrupt bone formation.

Chapter 4 investigates ATP release from osteoblasts under normal and stress situations. Using our established primary cell culture system, basal ATP release was measured from bone forming rat osteoblasts via the luciferin-luciferase assay. Levels of ATP release increased with differentiation with more mature osteoblasts releasing up to 7-fold more ATP. The mechanism of ATP release from osteoblasts was investigated using specific inhibitors of vesicular exocytosis, and mechanosensitive ion channels; data produced suggested that basal ATP release is mediated, at least in part via vesicular mechanisms.

Many pathological situations causing bone loss are characterised by a localised decrease in oxygen tension (hypoxia) and inflammation (increased temperature). As extracellular nucleotides have striking negative effects on bone remodelling, the effect of hypoxia (2% O2), hyperthermia (40°C) and hypothermia (34°C) on osteoblast function and ATP release were investigated. Low oxygen significantly inhibited osteoblast proliferation and function as well reducing cell viability as determined by MTT, bone nodule formation and LDH cytotoxicity assays, respectively. A transient exposure to hypoxia causes a rapid, significant 2–3-fold increase in ATP release from
osteoblasts, an affect not caused by increased cell lysis. In contrast, the level of ATP release from cells continually cultured at 2% O₂ appeared slightly decreased.

Prolonged culture at 34°C or 40°C also significantly impaired mineralised bone nodule formation and cell proliferation. Transient hypothermia did not influence ATP release, however, transient hyperthermia increased ATP release 2-fold. Unlike hypoxia-induced release, hyperthermia-induced ATP release appeared more sustained and lasted hours as opposed to minutes. Long-term culture at hypothermic or hyperthermic temperatures also significantly impairs ATP release. These data suggest long-term culture under stressful conditions will markedly affect osteoblast function, whilst transient exposures stimulate ATP release increasing the extracellular concentration and potentially initiating local purinergic signalling.

Knockout animals have yielded a lot of information regarding the physiological roles of many gene products, including the P2 receptors. Chapter 5 investigates the effect of P2X₂, P2X₃, P2X₂/₃ and P2Y₁ receptor removal on bone phenotype, using a PIXImus bone densitometer. The single P2X receptor knockouts were scanned aged 2, 4, 6 and 9 months, and the P2X₂-null animals displayed a significantly increased bone mineral content, bone mineral density and weight, however the effects appeared age related. Conversely, P2X₃-receptor deficient animals exhibited no differences in bone phenotype. P2X₂/₃ double knockouts were scanned aged 6 and 8 months; they displayed a decreased bone mineral density but not content. The P2Y₁ receptor knockouts were scanned at 2 months only and found to exhibit small but significant decreases in bone mass.

Chapter 6 is a general discussion in which these results are summarised and discussed in a broader context. Potential future experiments to further knowledge about extracellular nucleotides and bone cell function are also suggested.

Appendix I contains a list of all the abbreviations used and Appendix II provides details of all the publications and abstracts arising from this research, together with one paper containing work performed but not included in this thesis “Hypoxia is a major stimulator of osteoclast formation and activity” by Arnett et al, 2003.
CHAPTER 1

GENERAL INTRODUCTION

OVERVIEW OF BONE STRUCTURE AND FUNCTION

The skeletal system is a dynamic living tissue comprised of bone and cartilage. Bone is a specialised connective tissue with three main functions: firstly, by providing support and site for muscle attachment the skeleton has a mechanical role. Secondly, it protects the vital organs and bone marrow from damage and thirdly, as bone comprises of approximately 65% inorganic mineral, it functions as a large reservoir of ions to help maintain calcium (Ca$^{2+}$) and phosphate (PO$_4^{3-}$ or Pi) homeostasis (Baron, 2003). The extracellular matrix of bone comprises of inorganic mineral salts deposited within an organic collagen matrix and three main cell types: osteoblasts, osteoclasts and osteocytes, all of which are distinct in their function, activity and appearance. The combined action of these cells ensures constant skeletal remodelling and enables growth, adaptation and repair.

Individual bones are often characterised according to their shape. Long bones such as the femur are longer than they are wide and usually found in the limbs. Short bones, including those in the wrists and ankles, are generally equal in breadth and length. Flattened bones are relatively thin and often curved and include the bones found in the skull (such as the calvariae) and the mandible (Recker, 1992).

Anatomically, each bone is subdivided into distinct regions, best illustrated by the long bones. The central cavity, termed the medullary canal, is filled with red and yellow marrow; red marrow being the site of blood cell formation whereas yellow marrow is mainly adipose tissue. Encompassing the medullary canal is the shaft or diaphysis; a region primarily composed of cortical bone. The metaphysis is the cone shaped region separating the diaphysis from the bone ends or epiphysis. Primarily formed of trabecular bone, the epiphysis is delineated from the metaphysis by the
epiphyseal line; this region is a remnant of the broad cartilaginous growth plate (the epiphyseal plate) that became ossified upon cessation of bone growth. Encasing the entire skeleton is a layer of connective tissue termed the periosteum. The outer periosteal layer is a sheath of fibrous connective tissue containing blood vessels and nerves, which surrounds an inner cell layer (cambrian layer) chiefly containing undifferentiated progenitor cells (Baron, 2003).

Each bone shares the same basic architecture comprising of an outer, protective layer of cortical (or compact) bone surrounding the inner trabecular (or cancellous) bone and the bone marrow. Cortical and trabecular bone contain the same cell types and matrix elements but display structural and functional differences. By volume, cortical bone is 80-90% calcified bone tissue, whereas trabecular bone is only 15-25% bone (the remainder being occupied by bone marrow, blood vessels and connective tissue) (Baron, 2003). The lower volume of calcification in trabecular bone means it is less dense than cortical bone. Structurally, cortical bone consists of thin sheets (3–7 μm) called lamellae arranged in concentric layers around a central Haversian canal, each canal being lined with a layer of endosteum and housing a blood vessel. These cylindrical structures are referred to as osteons or Haversian systems and located in-between them are interstitial lamellae; layers of bone that are remnants of previously remodelled osteons. Surrounding these central Haversian systems are the outer and inner circumferential lamellae, the latter giving way to the inner cavities containing the trabeculae and marrow. In combination, these structural properties give cortical bone a mainly mechanical and protective function (Recker, 1992; Baron 2003).

Trabecular bone is found at the ends of long bones, the inner parts of flat bones and in the vertebrae. The microstructure of cancellous bone appears “honeycombed”, consisting of thin (50–400 μm) interconnecting rods called trabeculae, each trabecular element comprises of several lamellae embedded with osteocytes. Covering the bone surface is a single cell layer of osteoblasts and some osteoclasts and this interface between the marrow and trabecular bone, often termed the endosteal or trabecular envelope, is a key site of bone metabolism. Since remodelling only occurs at exposed locations, the large surface area of trabecular bone means it fulfils a mainly metabolic function (Baron, 2003; Recker, 1992).
Microscopically, bone can also be classified into two types: woven or lamellar. Woven bone contains collagen fibres arranged in randomly orientated bundles and irregularly distributed large osteocytes. It is produced when osteoblasts synthesize osteoid rapidly, such as in embryonic life and childhood, but also in adults with pathological conditions characterized by rapid new bone formation for example in Paget’s disease and fracture repair (Baron, 2003). In comparison, lamellar bone comprises of regular parallel bands of collagen fibres arranged in sheets. Remodelling of the skeleton gradually replaces the immature woven bone with mature lamellar bone and therefore, virtually all bone in a healthy adult is lamellar.

OSTEOBLASTS

Origin & phenotype

Bone formation or osteogenesis is performed by osteoblasts, mononuclear cells derived from mesenchymal stem cells, a lineage that also gives rise to adipocytes, chondrocytes, myoblasts and fibroblasts (Grigoriadis et al, 1988). Once committed to the osteoblastic lineage, these stem cells are referred to as osteoprogenitor cells. Morphologically, osteoblasts are most akin to fibroblasts but can be distinguished by the presence of mineralised extracellular matrix (ECM). Active osteoid secreting osteoblasts are large, polarised and cuboidal in shape, with a prominent rough endoplasmic reticulum, whereas quiescent osteoblasts, often referred to as “bone lining cells”, have a flat morphology.

The differentiation and activity of osteoblasts can be spilt into three distinct phases: (1) proliferation of osteoprogenitors, (2) ECM development and maturation, characterised by the upregulation of genes associated with the mature osteoblast phenotype (type I collagen, osteocalcin (OCN) and alkaline phosphatase (ALP)), and (3) mineralisation of the organic matrix. The progression of osteoblast differentiation is associated with a gradual decrease in cellular proliferation.
Osteoblast differentiation

The use of multipotent mesenchymal progenitors in vitro has provided abundant information regarding osteoblast differentiation and although knowledge has increased over recent years, many questions remain unanswered especially relating to the interactions between different signalling pathways. Osteoblast differentiation or osteoblastogenesis is a complex process and requires the sequential activation and suppression of phenotype specific genes (Figure 1.1). This section discusses the main transcription factors and local regulators involved in differentiation, most being involved in both skeletogenesis and post-natal osteoblast development.

**Figure 1.1. A schematic diagram illustrating the transcription factors, cytokines and hormones important in osteoblast differentiation**

This diagram shows the principal phases of osteoblast differentiation and the major factors (local and systemic) implicated at each stage (see text for description).
Transcription factors

Osteoblast differentiation is mediated by a number of transcription factors, the earliest and most specific being runt related transcription factor 2 (Runx2), also called core binding factor α1 (Cbfα1). The heterodimeric Runx2 belongs to the Runx family of transcription factors. In mammals, the Runx family has three members (Runxl, -2, -3); each contains a characteristic runt domain responsible for DNA binding and regulates a distinct developmental pathway (haematopoiesis, osteogenesis and neurogenesis, respectively). All mesenchymal condensations during early embryonic development express Runx2, making it a common factor in both chondrocyte and osteoblast differentiation. Runx2 activates transcription by binding to osteoblast-specific cis-acting elements (OSE) located in the promoter regions of specific genes, for example two OSEs, OSE1 and OSE2, are located upstream of the OCN gene (Ducy et al., 1996). Work using transgenic mouse models demonstrated the fundamental role of Runx2 in osteoblast differentiation; for example, overexpression of Runx2 in vitro leads to osteoblast-specific gene expression in fibroblasts and myoblasts and in vivo causes ectopic endochondral bone formation (Ducy et al., 1997). Homozygous Runx2-deficient mice lack osteoblasts and osteoclasts and have skeletons exclusively made of cartilage (Komori et al., 1997), whereas heterozygous Runx2 knockouts exhibit delayed ossification in certain cranial bones (Otto et al., 1997). In mature osteoblasts, Runx2 influences osteogenesis by controlling the expression of numerous genes required for bone formation including ALP (Ducy et al., 1996), osteopontin (Sato et al., 1998), OCN (Ducy et al., 1996) and bone sialoprotein (Benson et al., 1999).

Other osteogenic transcription factors include Osterix (Osx), Muscle segment homologue 2 (Msx2), homeobox containing transcription factor Distal-less gene 5 (Dlx5), HLH proteins (Id, Twist, Dermo) and transcription factors of the activating protein-1 (AP-1) complex. Osx is a zinc finger transcription factor believed to function downstream of Runx2, an idea supported by the lack of Osx expression in Runx2 knockout mice and the complete lack of bone formation in Osx-deficient mice (Nakashima et al., 2002). Both Msx2 and Dlx5 are important during development and appear antagonistic; Msx2 acts to prevent terminal osteoblastic differentiation, whereas Dlx5 expression coincides with the expression of bone marker genes and seems to exert positive regulatory effects (Bendall et al., 2000). Removal of either factor produces animals with multiple defects including abnormal osteogenesis (Acampora et
Members of the AP-1 family of transcription factors are important regulators of bone cell proliferation and differentiation. Suggestive of a role stimulating osteogenesis, overexpression of ΔFosb, a fosB splice variant, leads to increased bone formation and impaired adipogenesis (McCabe et al, 1996; Sabatakos et al, 2000). Moreover, enhanced expression of Fra-1, another member of the AP-1 family, also causes increased bone mass. Fra-1 is abundant in mature osteoblasts and by increasing the expression of bone specific genes it can stimulate bone formation (Jochum et al, 2000). The early response gene c-fos, which mediates cell growth and development, is expressed in osteoprogenitor cells but not mature osteoblasts indicating a role for this transcription factor in osteoblast differentiation (Machwate et al, 1995). By repressing genes of the mature osteoblast phenotype, HLH proteins negatively regulate differentiation and allow osteoprogenitor expansion; these proteins have to be downregulated to allow differentiation to proceed (Ogata et al, 1991).

Regulators of osteoblast differentiation

By controlling the expression and/or activity of these and other transcription factors, many local signalling factors act to regulate osteoblast differentiation and osteogenesis.

The hedgehog family of signalling molecules contains Indian, Sonic and Desert (Ihh, Shh and Dhh, respectively), all of which are essential in different developmental pathways. Although primarily expressed in chondrocytes, Ihh appears to be a key regulator of the developing endochondral skeleton: as illustrated by Ihh-deficient mice, which display reduced chondrocyte proliferation and lack osteoblasts in endochondral bone (St Jacques et al, 1999). Exactly how Ihh signals to promote osteoblast differentiation and osteogenesis remains unclear but two important components in mediating its effects are the cell surface receptor Patched and the transmembrane protein, Smo. Activation of Patched induces ALP activity and mineralisation in vitro (Nakamura et al, 1997), conversely osteoprogenitors devoid of Smo fail to respond to Ihh, inhibiting osteoblast differentiation (Long et al, 2004).

The bone morphogenetic proteins (BMPs) are potent regulators of both osteoblast differentiation and bone formation. With the exception of BMP-1, all belong to the transforming growth factor β (TGF-β) superfamily, members of which regulate proliferation, differentiation and cell death in many tissues (Massague et al, 2000;
In osteoprogenitors, BMP-induced signalling induces the expression and/or activity of Runx2, Osx and Msx-2 and of the ≥15 BMPs implicated in osteoblast differentiation, BMP-2, -4, -5, -6 and -7 are thought to be particularly important. For example, BMP-2 stimulates precursors into the osteogenic lineage and enhances ALP activity, parathyroid hormone (PTH) responsiveness and OCN production in vitro (Yamaguchi et al, 2000). Moreover, reports suggest that BMPs regulate osteoblast cell survival during the induction of differentiation (Chen, 2001). Knockout animals have provided limited information regarding the involvement of these factors in skeletal development as BMP-2, BMP-4 and BMP-7 null mice all die shortly before or after birth (Yamaguchi et al, 2000).

The FGFs are a large family of polypeptides that function to control proliferation and differentiation in many cell types. Initiation of FGF signalling in osteoprogenitors, via binding to specific FGF receptors (FGFR), stimulates proliferation and differentiation: a role emphasized by transgenic mouse models such as FGF-18 null mice, which display delayed ossification and reduced expression of osteogenic markers (Liu et al, 2002). Moreover, conditional inactivation of FGFR2 produces animals with skeletal dwarfism, decreased bone density and impaired osteoprogenitor proliferation and mature osteoblast function (Yu et al, 2003). FGFs function by enhancing the activity of osteogenic transcription factors; for example, FGF-2 facilitates the binding of Runx2 to its OSE consensus sequence thereby providing a mechanism enabling FGF signalling to directly stimulate the expression of Runx2-dependent osteoblast genes (Debiais et al, 2004). FGFs also influence bone remodelling by regulating the expression of other growth factors that affect bone cell function including TGF-β (Noda & Vogel, 1989) and prostaglandins (Kawaguchi et al, 1995).

Wnts are secreted, lipid-modified glycoproteins that regulate cellular activities including proliferation, migration and gene expression (Moon et al, 2002). By signalling through the Frizzled family of receptors, Wnts activate at least three intracellular pathways including the Wnt/β-catenin or canonical pathway. Activation of this pathway stabilises β-catenin, allowing it to interact with a number of different transcriptional regulators and control the expression of target genes. The involvement of Wnt/β-catenin signalling in osteoblast differentiation is an area of active research and recently Wnt10b was reported to induce Runx2, Dlx5 and Osx expression but
suppress the adipogenic transcription factors, PPARγ and C/EBPα. Additionally, Wnt10b knockout mice display decreased trabecular bone (Bennett et al, 2005). The recent observation that β-catenin localisation does not occur in Ihh-null mice suggested Wnt signalling occurs downstream of Ihh during osteogenesis; this indicates cross talk exists between these pathways, however, at present the exact nature of this interaction remains unclear (Hu et al, 2005).

Cellular Interactions

Cell-to-cell and cell-to-matrix interactions also play an important role in osteoblast differentiation and function and if lost can lead to apoptosis (Jilka et al, 1998). Cadherins, which are Ca²⁺ dependent transmembrane glycoproteins, mediate cell-to-cell interactions. In osteoblasts, expression of N-cadherin (Marie, 2002) and cadherin-11 (Kii et al, 2004) appears to influence differentiation as partial disruption of their activity indirectly favours progenitor cell commitment to the adipogenic lineage (Castro et al, 2004). Furthermore, although N-cadherin null mutations are lethal (Radice et al, 1997), cadherin-11 deficient mice exhibit a reduced bone density in the calvaria and long bone metaphysis (Kawaguchi et al, 2001).

Integrins mediate cell-to-matrix interactions by coupling proteins of the extracellular matrix to structural proteins of the cytoskeleton. These interactions enable propagation of modulatory signals that influence cell differentiation, adhesion, and cytoskeletal organisation. Important in osteoblast differentiation and bone development are the β1 integrins (α1β1, α1β2, α3β1, α2β1); for example interactions between type I collagen and α1β1/α2β1 integrins accelerate osteoblast differentiation by activating the mitogen activated protein kinase (MAPK) signalling pathway and increasing Runx2 activity (Xiao et al, 1997; Xiao et al, 1998).

BONE FORMATION

Bone is formed by the action of mature osteoblasts in a two-step process, with the first stage being synthesis and deposition of the organic matrix. Osteoblasts synthesize type I collagen and many non-collagenous bone matrix proteins (NCPs), which are packaged into vesicles and then released via exocytosis. In the extracellular environment, thin layers of these non-mineralised proteins become embedded in the
ground substance to form a matrix known as osteoid; subsequent mineralisation of which produces the calcified bone tissue. The ground substance is primarily composed of proteoglycans and glycoproteins. Also synthesised by osteoblasts, proteoglycans are macromolecules consisting of a central core protein with covalently attached acidic glycosaminoglycan side chains; important proteoglycans in bone include decorin, biglycan and fibromodulin.

**Collagen**

The collagens are important structural extracellular matrix proteins accounting for around 25% of total body protein. To date some 27 types of collagen have been identified, with the main forms found in connective tissue being type I, II, III, V and XI. Type I collagen accounts for approximately 90% of the total protein in bone and plays a fundamental role in the development and proper function of the skeleton. It is a heterotrimeric molecule comprising of two identical α1 (I) chains and one α2 (I) chain, which combine to form a triple helix structure. Collagen biosynthesis is a multi-step process involving a number of post-translational modifications. Initially the α chains are synthesized with a short N-terminal signal sequence plus propeptides at both N- and C-terminals, and following translation are injected into the endoplasmic reticulum (ER) lumen. All α-chains are characterised by a repeating Gly-X-Y motif, where X is usually proline and Y is hydroxyproline. Within the ER, individual α-chains undergo a series of reactions that facilitate triple helix formation, including the hydroxylation of proline by prolyl-4-hydroxylase to produce 4-hydroxyproline, a reaction that enables the formation of stabilising intrastrand hydrogen bonds. Prolyl-4-hydroxylase is oxygen-dependent and, following catalysis requires ascorbate (vitamin C) for regeneration; in its absence, the inhibition of proline hydroxylation produces collagen that denatures (or “melts”) at a lower temperature. A select number of lysine residue are also hydroxylated, a modification necessary for the glycosylation and cross-linking of the collagen molecule. These reactions are catalysed by one of three lysyl hydroxylases (LH), each encoded by an individual gene termed PLOD-1, -2 or -3 (Valtavaara et al, 1997, Valtavaara et al, 1998). Differential expression of the PLOD genes causes the formation of tissue specific cross-links and may explain why the type I collagen in skin exhibits a different bonding pattern to the type I collagen in bone. PLOD-1 expression is widespread, thus LH1 produces cross-links which are relatively
ubiquitous between tissues (Valtavaara et al, 1997). In contrast, expressions of PLOD-2 and -3 are more tissue specific and the observed upregulation of PLOD-2 in differentiated osteoblasts means the LH2 isoform is likely to confer a bone specificity to type I collagen (Uzawa et al, 1999).

Once formed, the stable triple helix is secreted into the extracellular matrix via exocytosis, where proteolytic cleavage converts procollagen into collagen via removal of the propeptides. Individual collagen triple helices then combine to form fibrils, ranging in diameter from 10–300 nm and up to hundreds of micrometers in length. These fibrils are stabilised by the oxidative deamination of ε-amino groups of specific lysine and hydroxylysine residues, a reaction catalysed by lysyl oxidase. This forms highly reactive aldehyde moieties that rapidly form the covalent cross-links necessary for the development of functional mature collagen. Inhibition of this reaction drastically reduces collagens tensile strength, leading to defective bone and cartilage formation. Finally, collagen fibrils condense to form structures known as collagen fibres.

**Bone matrix proteins**

NCPs constitute the remaining 10-15% of the organic matrix, and are either exogenously derived (~25%) or synthesised by osteoblasts (~75%), however their roles in the bone microenvironment are by no means fully characterised. Exogenous NCPs include the serum-derived proteins, albumin and α2HS-glycoprotein. Many of the NCPs synthesised by osteoblasts undergo post-translational modifications such as phosphorylation and/or glycosylation. Important NCPs involved in bone formation including OCN, osteonectin, osteopontin, bone sialoprotein (BSP) and matrix gla protein (MGP). OCN is a widely used marker of osteoblast differentiation, primarily because of its restricted expression profile (mineralising osteoblasts and hypertrophic chondrocytes). It is the most abundant NCP in bone and is post-translationally modified by vitamin K dependent γ-carboxylases to produce residues with a high affinity for Ca$^{2+}$. OCN-deficient mice exhibit increased bone mineral density but show no defects in resorption indicating a role in the regulation of mineralisation (Ducy et al, 1996). MGP is expressed in vascular smooth muscle cells, chondrocytes and osteoblasts where it acts to inhibit mineralisation; a role illustrated by MGP-knockout mice, which develop calcification in extraskeletal sites including the arteries and
growth plate (Luo et al., 1997). The glycoprotein osteonectin is transiently expressed in tissues undergoing rapid proliferation, remodelling or changes in architecture. The functions of osteonectin in bone remain unclear although osteonectin-null mice display decreased bone remodelling because of reduced osteoblast and osteoclast number. As the defect in bone formation is greater, knockouts animals exhibit a progressive age-related reduction in bone mass (Delany et al., 2000). Osteonectin may also play a role in cell attachment (Young et al., 1992).

Some NCPs belong to a family called the SIBLINGS (Small Integrin Binding Ligand N-linked Glycoproteins) members of which contain the integrin-binding consensus sequence RGD (Arg–Gly–Asp). Both osteopontin and BSP belong to this family and are important in mediating cell-to-cell and cell-to-matrix interactions, anchorage of osteoclasts to bone and can bind Ca\(^{2+}\) with high affinity (Reinholt et al., 1990; Asou et al., 2001). BSP, a virtually bone specific attachment protein, is a potent nucleator of hydroxyapatite crystals, an effect dependent on the poly (glutamic) acid sequences present in the protein. Conversely osteopontin, which is structurally similar to BSP but lacks the glutamic acid structural motif, potently inhibits hydroxyapatite crystal deposition and propagation (Boskey et al., 1993; Hunter & Goldberg, 1993). Further illustrating this inhibitory role, osteopontin knockout mice display a small increase in mineralisation (Boskey et al., 2002). Osteoblasts synthesize many other cell attachment proteins, each containing the RGD sequence, including: fibronectin, vitronectin and osteoadherin.

Following osteoid secretion, there is a lag period prior to calcification termed the osteoid maturation period, which, in humans, lasts about 10 days. Subsequent mineralisation involves the deposition of inorganic ions into the osteoid matrix and produces mature bone. The mineralisation process, which is regulated by the activities of phosphatases, NCPs and cell-to-cell/matrix interactions, will be discussed in detail in Chapter 4.
BONE GROWTH AND DEVELOPMENT

During embryonic development, osteoblast differentiation and bone formation occur via two distinct pathways: endochondral ossification or intramembranous ossification. Intramembranous ossification occurs in the clavicle and some cranial bones. In these regions, mesenchymal stem cells under the influence of local growth factors proliferate and differentiate into pre-osteoblasts and then osteoblasts. At ossification centres, osteoblasts form immature woven bone that is later remodelled into the mature lamellar bone characteristic of the adult skeleton.

Endochondral ossification is associated with foetal bone development and day-to-day bone growth. This process involves the formation of a cartilage template that is later replaced by bone. The initial site of ossification is in the bone diaphysis and is termed the primary ossification centre. It is here where the activity of local growth factors (BMPs, FGF, parathyroid hormone-related peptide (PTHrP), Ihh) and specific transcription factors (Sox9, Sox5 & Sox6) cause mesenchymal stem cells to condense, differentiate into chondrocytes and secrete cartilaginous matrix. At any one time there are four distinct subpopulations of chondrocytes in growth plates: resting, proliferating, prehypertrophic and hypertrophic; each is arranged into distinct zones and surrounded by undifferentiated mesenchymal cells forming the perichondrium. Hypertrophic chondrocytes are responsible for mineralising the previously deposited cartilaginous matrix and display a dramatic increase in expression of ALP, type X collagen and matrix metalloproteinase 9 (MMP-9). The gradual development of a calcified extracellular matrix around the hypertrophic chondrocytes causes them to secrete vascular endothelial growth factor (VEGF), a factor that along with MMP-9 facilitates the invasion of blood vessels from the perichondrium. Vascular invasion promotes resorption of the calcified cartilage, and osteoblast differentiation from perichondrial mesenchymal cells; the osteoblasts then replace the cartilage template with bone. Later osteoclast activity breaks down this bone to form the medullary cavity. Subsequently, chondrocytes become localised to the epiphysis of the growing bone, where the process continues away from the primary ossification centre to facilitate longitudinal growth. The interface between the midshaft and the epiphysis is termed the growth or epiphyseal plate and lengthening of the bone diaphyses pushes the two growth plates apart. Longitudinal growth continues until maturity, when
hormonal changes inhibit further cartilage production and calcify the epiphyseal cartilage in a process termed secondary ossification.

The various steps of chondrogenesis and endochondral ossification are tightly controlled to prevent abnormal growth or ossification. Important regulatory factors include Runx2, Sox9, Ihh, PTHrP, TGF-β and FGFs. Runx2 is highly expressed in non-hypertrophic chondrocytes and in a role distinct from that in osteoblastogenesis, acts to promote differentiation to the prehypertrophic stage; a role identified using knockout animals which display defects in this process (Inada et al, 1999). Sox9 is a transcription factor fundamental in the conversion of condensed mesenchymal cells to chondrocytes and its expression is necessary for induction of the other Sox members (Sox5, -6) involved in differentiation (Akiyama et al, 2002). Hypertrophic chondrocytes are characterised by the expression of Ihh and the PTHrP receptor, which work in combination to regulate chondrocytic differentiation. Following secretion, Ihh binds to cells in the perichondrium to influence PTHrP production and osteoblast differentiation. PTHrP and Ihh are thought to interact in a negative feedback, where PTHrP induced by Ihh binds to its receptor on prehypertrophic chondrocytes and suppresses differentiation (Vortkamp et al, 1996). FGFs exert an inhibitory effect on Ihh production to ensure a balance exists between the stimulation and inhibition of chondrocyte differentiation (Wagner & Karsenty, 2001).

OSTEOCYTES

Actively mineralising osteoblasts can become trapped within the bone matrix, where they may undergo a terminal differentiation step to form an osteocyte. These cells are characterised by an extensive network of cytoplasmic processes that permeate through the canaliculi and contact adjacent cells; interactions that are essential for cell maturation, activity and survival as they enable osteocytes to communicate with and obtain nutrients from surface osteoblasts. Gap junctions, formed by the membrane protein connexin 43, facilitate intercellular communication (Civitelli et al, 1993). Mice lacking connexin 43 display skeletal malformations, delayed mineralisation and osteoblast dysfunction (Furlan et al, 2001). Their widespread distribution and ability to respond to strain with biochemical signals makes osteocytes the most likely candidates for sensing mechanical load and influencing the appropriate bone
remodelling in vivo (Lanyon, 1993). Although not fully elucidated, signalling pathways mediating these effects are thought to involve prostaglandin E$_2$ (PGE$_2$) and nitric oxide, both of which are important in mechanically induced bone formation. Osteocyte apoptosis, a process influenced by mechanical loading, may also influence cell signalling and thus bone remodelling (Noble et al, 1997; Noble et al, 2003)

**OSTEOCLASTS**

**Phenotype**

Osteoclasts are large, multinucleate cells responsible for bone resorption. Actively resorbing osteoclasts are highly polarised with four distinct membrane domains: (1) the sealing or clear zone, (2) the ruffled border, (3) the functional secretory domain and (4) the basolateral upper domain. Morphologically, osteoclasts resemble macrophages and bone resorption employs some of the same cellular machinery as phagocytosis.

**Osteoclast differentiation**

Osteoclast formation (or osteoclastogenesis) requires the fusion of mononuclear precursors derived from haematopoietic stem cells of the monocyte/macrophage lineage; promyeloid precursors that give rise to many cell types including macrophages and dendritic cells. Naturally occurring mutations and targeted knockout mutations have resulted in the identification of at least 24 genes that positively or negatively regulate osteoclastogenesis. Two cytokines are essential for osteoclast formation: macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL); together they induce the expression of genes that typify the osteoclast lineage including cathepsin K and tartrate-resistant acid phosphatase (TRACP). This section discusses the principal factors involved in osteoclastogenesis; for a more comprehensive review, the reader is referred to Quinn et al, 2005 and Blair et al, 2005. Figure 1.2 shows a schematic diagram of the osteoclast differentiation pathway and the principal factors involved.

*In vivo* osteoclastogenesis is tightly regulated, however, the inhibition of osteoclast production and/or function leads to defective bone resorption and increased levels of
mineralised tissue. For example, osteopetrosis, a rare hereditary condition characterised by the failure of osteoclast-mediated resorption, leads to trabecular and cortical thickening, loss of the bone marrow cavity and dense but fragile bones. Conversely uncontrolled osteoclast formation and/or activity will result in excessive bone resorption, a decrease in bone mass and an increased fracture risk, commonly termed osteoporosis.

**Important factors involved in osteoclast differentiation and activity**

This diagram shows the different stages of osteoclast differentiation and the main transcription factors, cytokines and hormones involved (see text for description).
**Important regulators of osteoclastogenesis**

The myeloid and B-cell-specific transcription factor PU.1 regulates the initial differentiation from haematopoietic stem cell and is currently the earliest known marker of osteoclast differentiation. Disruption of the PU.1 gene produces an osteopetrotic phenotype characterised by arrested osteoclast and macrophage development (Tondravi *et al*, 1997).

The homozygous osteopetrotic (*op/op*) mouse first demonstrated the important role of M-CSF in osteoclastogenesis; these animals possess a mutated M-CSF gene, lack osteoclasts and macrophages and their stromal cells fail to release active M-CSF (Yoshida *et al*, 1990). However, exogenous application of M-CSF partially restores the impaired bone resorption (Takahashi *et al*, 1991). Both osteoblasts and T-lymphocytes secrete M-CSF, which binds to the *c-fms* receptor on osteoclast precursors. Levels of *c-fms* depend on PU.1 expression, and receptor activation enhances the proliferation, differentiation and survival of both haematopoietic precursors and mature osteoclasts (Lagasse & Weissman, 1997).

RANKL, the fundamental osteoclast differentiation factor, was initially identified as being important in T-lymphocyte and dendritic cell function, however, its crucial role in osteoclastogenesis was revealed during investigations into the osteoclast inhibitory factor, osteoprotegerin (OPG) (Yasuda *et al*, 1998a; Yasuda *et al*, 1998b). This cytokine, also known as TNF-related activation induced cytokine (TRANCE), osteoclast differentiation factor (ODF), and OPGL, belongs to the tumour necrosis factor (TNF) superfamily. Both osteoblasts and activated T-lymphocytes secrete RANKL and by upregulating osteoblastic expression, many factors indirectly stimulate osteoclastogenesis. RANKL exists in two forms, surface bound and soluble, with proteolytic cleavage of the former directly producing the latter. The essential role in osteoclast production is further illustrated by RANKL-deficient mice, which although possessing osteoclast progenitors, lack mature osteoclasts and exhibit severe osteopetrosis (Kong *et al*, 1999). Additionally, RANKL can act on pre-existing mature cells to increase bone resorption (Burgess *et al*, 1999).

RANKL binds to a member of the TNF receptor (TNFR) superfamily termed RANK, a transmembranous receptor widely expressed on cells of the monocyte/macrophage lineage including pre- and mature osteoclasts. RANK-deficient
mice also lack osteoclasts and exhibit a severe osteopetrosis, although this phenotype can be rescued by transplantation of normal bone cells (Dougall et al., 1999).

OPG is a secreted TNFR-related protein that functions to regulate bone mass; this soluble decoy receptor, produced by osteoblasts, acts to inhibit osteoclastogenesis by preventing the binding of RANKL to RANK on osteoclast precursors (Lacey, 1998). OPG-deficient mice display severe osteoporosis accompanied by calcification of the aorta and renal arteries, whilst OPG overexpression blocks osteoclastogenesis leading to osteopetrosis (Bucay et al., 1998). Levels of OPG and RANKL are therefore coordinated to regulate bone resorption and any modification to this equilibrium will directly influence bone remodelling.

**Intracellular signalling pathways in osteoclast formation and activity**

Binding of M-CSF, RANKL and other osteoclastogenic cytokines to their appropriate receptors activates numerous intracellular signalling cascades, which result in the expression of specific genes involved in osteoclast differentiation, activation and survival. Activation of c-fms by M-CSF controls the expression of intermediate early transcription factors such as c-fos, an important regulator of osteoclast differentiation. Homozygous c-fos-deficient mice are osteopetrotic, have deficits in bone remodelling and tooth eruption and display high levels of macrophages because differentiation follows this pathway (Wang et al., 1992).

RANK activation by RANKL induces at least five distinct signalling pathways: inhibitor of NFκB (IKK), c-Jun N-terminal kinase (JNK), p38, extracellular signal regulated kinase (ERK) and src pathways (Boyle et al., 2003). The cytoplasmic domain of RANK contains specific motifs that enable the binding of TNFR-associated factors or TRAFs (including TRAF1, -2, -3, -5 and -6) (Wong et al., 1998; Kim et al., 1999). TRAF6 is one of the key adaptor proteins involved in osteoclastogenesis as it can signal via several pathways to activate nuclear factorκB (NFκB). Following activation, NFκB translocates to the nucleus where it acts to enhance the transcription of genes associated with the osteoclast phenotype. Mice deficient in TRAF6 (Lomaga et al., 1999) or both p50 and p52 subunits of NFκB (Franzoso et al., 1997) exhibit severe osteopetrosis due to a complete inhibition of osteoclast formation. Stimulation of other TNFR family members can also influence NFκB activity, therefore allowing
other cytokines to replace, augment or prevent RANKL-mediated signalling. For example, interferon-γ (IFN-γ) causes the rapid degradation of TRAF6 and strongly inhibits activation of the JNK pathway and NF-κB. RANKL-mediated signalling also stimulates nuclear factor of activated T cells 1 (NFATc1), and accumulating evidence now suggests this transcription factor also plays an important role in osteoclastogenesis (Ikeda et al., 2004; Matsuo et al., 2004). In contrast to the IKK1/2 and JNK pathways, activation of the ERK cascade is thought to negatively regulate osteoclastogenesis (Boyle et al., 2003).

The c-src and p38 pathways are more important in mature osteoclasts. The signalling induced by c-src acts to induce cell survival, cytoskeletal rearrangement and osteoclast cell motility (Wong et al., 1999). Mice deficient in c-src display an osteopetrotic phenotype because the osteoclasts present are incapable of resorption (Boyce et al., 1992). Activation of the p38 pathway induces the transcriptional activator mi/Mitf, which controls the expression of genes characterising the mature osteoclast phenotype such as TRACP and cathepsin K.

**BONE RESORPTION**

The sequence of cellular events required for bone resorption involves 5 main steps: (1) formation and migration of the osteoclast to the resorption site, (2) attachment of the osteoclast to bone, (3) polarisation, (4) resorption and (5) osteoclast apoptosis or return to the non-resorbing stage. Figure 1.3 shows an actively resorbing osteoclast and the principal components involved.

The initial step of resorption is osteoclast recruitment, a process probably mediated by cytokines including M-CSF, RANKL and VEGF. Once in position, to enable them to function, osteoclasts must attach to the bone surface. Interactions between the osteoclast and bone matrix occur at the clear or sealing zone, a region that contains no organelles and is rich in filamentous actin (f-actin). These microfilaments are organised into a ring (actin ring) separating the basolateral membrane from the ruffled boarder (a highly fenestrated region of membrane orientated towards the bone matrix). This structure isolates the resorption pit (Howship’s lacunae) and prevents leakage of bone degradation products. Matrix recognition at the site of attachment is thought to involve integrins, and osteoclasts express at least four types: αvβ3, αvβ5, α2β1 and
αvβ1 (Nesbitt et al., 1993). These integrins are thought to facilitate attachment via interactions with bone matrix proteins containing the RGD consensus sequence, such as osteopontin (Miyauchi et al., 1991).

Following attachment, osteoclasts secrete protons (H\textsuperscript{+}), Cl\textsuperscript{-} and proteolytic enzymes (including TRACP, cathepsin K, aryl sulphatases) across the ruffled boarder into the resorption pit. Within the osteoclast cytoplasm, a vacuolar-type V-ATPase proton pump packages H\textsuperscript{+} into acidic vesicles, which following diffusion to the ruffled border, are released via exocytosis. This action decreases the pH of the resorption pit to ~ pH 4.0 and enables the dissolution of the strongly basic hydroxyapatite. Following the removal of the inorganic components, proteases can degrade the now exposed organic matrix. One of the most important enzymes involved in bone resorption is cathepsin K, which cleaves collagen at multiple sites within the triple helix facilitating its breakdown (Garnero et al., 2003). Transcytotic vesicles remove the organic and inorganic degradation products from the lacunae, diffuse across the cell and release their contents via the functional secretory domain into the extracellular environment (Salo et al., 1997). On completion of bone resorption, osteoclasts either return to a quiescent non-resorbing stage or die by apoptosis.

![Figure 1.3. An actively resorbing osteoclast](image)
BONE REMODELLING

The adult skeleton is in a dynamic state being continually broken down and replaced by the coordinated action of osteoblasts and osteoclasts in a process termed bone remodelling. These tightly coupled processes allow bone growth, repair and shape changes, $\text{Ca}^{2+}$ regulation and the adaptation of bone to mechanical loading. Bone remodelling proceeds in one direction and takes place at discrete sites throughout the skeleton; these regions, first described by Frost in the 1960’s, are termed bone multicellular units (BMUs) (Frost, 1962).

At a new BMU site, remodelling begins with recruitment of osteoclasts, which over the period of approximately 10 days degrade the bone matrix; resorption proceeds until the lacunae is $\sim 100 \mu \text{m}$ in diameter and $50 \mu \text{m}$ deep. Subsequent recruitment of osteoblasts to the BMU permits the deposition of osteoid, beginning at the bottom of the lacunae. After $\sim 80$ days this process is complete and the osteoid is mineralised with hydroxyapatite giving the BMU its tensile strength. Finally, the remodelled area enters a quiescent phase to complete the $\sim 120$ day process. At any one time, 5-20% of the skeleton is being remodelled by $\sim 2$ million BMUs (Mundy, 1999).

There is a net increase in bone mass until the 3rd-4th decade of life, followed by a period where the bone mass stays relatively constant, thereafter, at the level of the BMU a gradual net decrease in bone mass occurs. In men, this decrease is osteoblast-mediated, as the cells do not entirely fill the lacunae, resulting in a small deficit at each BMU. Women generally experience a rapid decrease in bone density during the postmenopausal years, this occurs for several reasons; firstly, in addition to the osteoblast-mediated bone loss observed in men, the lacunae produced by osteoclasts are deeper than the standard $50 \mu \text{m}$ causing a greater deficit at each BMU. Secondly, oestrogen deficiency after the menopause further enhances bone loss by causing an increased number of BMUs (Mundy, 1999).
CONTROL OF BONE REMODELLING

Bone remodelling is tightly controlled to avoid excessive bone formation or resorption; many hormones, growth factors and cytokines act, both locally and systemically, to control bone cell function via direct or indirect mechanisms. This section discusses some of the important regulators of bone remodelling including systemic hormones, prostaglandins, nitric oxide, insulin growth factors (IGFs), protons, mechanical stimuli, hypoxia and temperature.

Non-steroid hormones

There are two broad groups of hormones important in bone cell function, steroid and non-steroid hormones.

Parathyroid hormone (PTH), the major systemic regulator of Ca\(^{2+}\) homeostasis, is synthesised by the chief cells of the parathyroid gland. It is secreted during periods of relative Ca\(^{2+}\) deficiency, and acts to maintain the extracellular concentration. The skeletal effects of PTH are complex, dosage and cellular differentiation dependent, involve osteoblasts and osteoclasts and are both catabolic and anabolic. During skeletogenesis, PTH in conjunction with \(Ihh\) is essential in controlling differentiation into the chondrogenic or osteogenic lineages. Intermittent dosing of PTH appears to stimulate the proliferation/differentiation of osteoprogenitor cells and increase bone formation, whereas continuous application appears to enhance early development but suppress the later stages of osteogenic development (Hollnagel et al, 1997). PTH also stimulates osteoclastic bone resorption, an effect initially thought to be mediated indirectly via osteoblasts; however, recent findings have provided evidence for a direct stimulatory action of PTH on human osteoclasts (Dempster et al, 2005).

PTHrP, despite being encoded by a different gene, binds to PTH receptors and has similar actions. It is expressed on both osteoblasts and chondrocytes and acts locally to regulate cell proliferation and differentiation (Suda et al, 1996; Karaplis & Goltzman, 2000). The role of PTHrP in skeletal development is highlighted by knockout mice, which exhibit widespread abnormalities in endochondral bone formation (Karaplis et al, 1994).
Calcitonin is synthesised by the thyroid glands and functions to lower circulating Ca\(^{2+}\) and Pi by acting on the kidneys and bone. Since committed osteoclast precursors express calcitonin receptors they are often used as an osteoclastic marker (Hattersley & Chambers, 1989; Lee et al, 1995). Calcitonin is a powerful inhibitor of osteoclast activity \textit{in vitro} (Chambers et al, 1982; Arnett & Dempster, 1987) and \textit{in vivo} can prevent bone loss and reduce risk of fracture in postmenopausal women (Rico et al, 1995). Although calcitonin can inhibit osteoblast and osteocyte apoptosis, the exact actions on these cells requires further investigation (Plotkin et al, 1999).

Thyroid hormones act systemically to increase the basal rate of oxygen consumption, metabolism and heat production. Moreover, by stimulating bone resorption, regulating osteoblastic gene expression and controlling the production of several growth factors (IGF-1) and cytokines (interleukin 6 (IL-6)), thyroid hormone can also regulate bone remodelling (Mosekilde et al, 1990). Both excess and reduced levels of thyroid hormone have profound consequences on body homeostasis including marked skeletal effects. In overt hyperthyroidism (Graves' disease), metabolic rate and heat production are strikingly increased, as is osteoclast activity and bone turnover; thus, prolonged exposure to excess thyroid hormone leads to reduced bone mass, increased fracture risk and osteoporosis. In contrast, hypothyroidism is characterised by a lower metabolic rate and body temperature as well as reduced bone turnover (Lakatos, 2003).

Recent work has identified the peptide hormone leptin as an important mediator of bone mass, although its effects remain unclear. Osteoblasts express both leptin and its receptor and \textit{in vitro} leptin promotes osteoblast proliferation, differentiation and survival, collagen synthesis and mineralisation (Gordeladze et al, 2002). Conversely, both leptin and leptin-receptor deficient mice are obese with an increased bone mass, suggesting this hormone inhibits bone formation possibly indirectly via the sympathetic nervous system (Ducy et al, 2000; Takeda et al, 2002).

\textit{Steroid hormones}

All steroid hormones function in a similar manner; following ligand binding the hormone-receptor complex translocates to the nucleus, where acting as a transcription factor it can activate or repress expression by binding, as homo- or heterodimer, to
hormone response elements (HREs) in the promoter region of target genes (Mangelsdorf et al, 1995).

Glucocorticoids, such as cortisol, are derived from cholesterol and secreted by the adrenal cortex. They have wide-ranging, albeit species dependent, effects on osteoblast differentiation and bone remodelling. In rats, glucocorticoids increase the number of osteoprogenitors, stimulate osteoblast differentiation and enhance expression of osteoblast markers including ALP (Bellows et al, 1998; Bland, 2000). In humans, the effects are less clear as in vitro data suggest glucocorticoids are required for osteoblast differentiation however, in vivo administration decreases bone formation, increases bone resorption and leads to a decrease in bone mass. These in vivo effects are probably mediated indirectly because glucocorticoids inhibit Ca$^{2+}$ uptake from the gut, thus stimulating PTH release and increasing bone resorption.

Given the dramatic bone loss that occurs in postmenopausal women, the sex steroids, particularly oestrogens, are fundamental in the control of bone remodelling. There are many naturally occurring oestrogens, including 17β-oestradiol, and they act via two distinct intracellular receptors, ERα and ERβ, to reduce bone resorption by inhibiting either osteoclastogenesis or osteoclast function (Robinson et al, 1997). Additionally, oestrogens act on osteoblasts to stimulate proliferation (Robinson et al, 1997) and the expression of several osteoblastic marker genes including type I collagen, OCN and ALP (Gray et al, 1987; Qu et al, 1998). Oestrogens can also influence bone remodelling indirectly by modulating the production of cytokines and growth factors from bone cells (Jilka, 1998).

The actions of androgens (including testosterone) on bone cell function are less well-investigated, nevertheless earlier work demonstrated expression of androgen receptors on osteoblasts and osteoclasts (Bellido et al, 1995; Pederson et al, 1999). These hormones influence bone metabolism by stimulating osteoblast proliferation, decreasing bone resorption and regulating IL-6 and TGF-β expression and secretion (Bellido et al, 1995; Kasperk et al, 1997).

Vitamin D$_3$ (1,25(OH)$_2$D$_3$) is a steroid-related hormone important in Ca$^{2+}$ homeostasis. The action of UV light forms inactive vitamin D$_3$ (cholecalciferol) in the skin; subsequent hydroxylations in the liver and kidneys produce the active form, 1,25-
dihydroxyvitamin D$_3$. The major biological function of vitamin D$_3$ is to maintain normal blood levels of Ca$^{2+}$ and Pi: when the plasma concentration of Ca$^{2+}$ decreases, PTH stimulates the production of vitamin D$_3$, which acts to restore Ca$^{2+}$ levels to normal by increasing transport in the intestines, bone and kidney. Vitamin D$_3$ deficiency profoundly impairs bone and cartilage mineralisation, leading to osteomalacia in adults and rickets in children, conditions characterised by the formation of soft bones with an increased tendency to fracture. Vitamin D$_3$ regulates osteoblast differentiation by activating or repressing the transcription of genes such as ALP, type I collagen and OCN (van Leeuwen et al, 2001). However, like PTH, the effects appear complex, with marked differences between species. In human and some rat osteoblasts, vitamin D$_3$ regulates the early stages of osteoblast differentiation and favours the osteogenic over the adipogenic pathway (Beresford et al, 1992; Beresford et al, 1994). In contrast, in primary rat calvarial cells vitamin D$_3$ promotes differentiation into adipocytes (Bellows et al, 1994). Furthermore, there are conflicting reports regarding the effect of vitamin D$_3$ on Runx2 activity, with some saying its is stimulated and others saying it is inhibited (Drissi et al, 2002; Viereck et al, 2002).

Local regulators

Tumour necrosis factor-α (TNFα), a major inflammatory mediator active in diseases such as rheumatoid arthritis, is synthesised by activated macrophages and T-lymphocytes. This cytokine can potently enhance osteoclastogenesis indirectly via actions on osteoblasts or directly by stimulating NFκB activity. Recent evidence suggests TNFα can function either independently or synergistically with RANKL, however, in combination these cytokines have a greater effect on osteoclast formation (Kudo et al, 2002). The effects of TNFα on osteoclast activity are less clear but are probably mediated via interactions with osteoblasts, as TNFα failed to stimulate resorption in isolated osteoclast cultures (Thomson et al, 1987; Azuma et al, 2000). The pro-resorptive effects of TNFα are compounded by its ability to suppress osteoblast differentiation and function.

The pleiotrophic cytokine interleukin-1 (IL-1) has two isoforms with identical activities (IL-1α, IL-1β) and is produced by stromal and haematopoietic cells. In
mouse and human marrow cultures as well as stromal-cell free cultures, IL-1 in the presence of RANKL and M-CSF, acts to stimulate osteoclastogenesis (Lader & Flanagan, 1998; Ma et al, 2004). IL-1 promotes mature osteoclast survival and can stimulate resorption either directly or indirectly by enhancing RANKL expression in osteoblasts (Jimi et al, 1998; Xing et al, 2003). Furthermore, IL-1 can promote the secretion of other proinflammatory cytokines including IL-6 and TNFα. IL-1 acts to inhibit bone formation by decreasing osteoblastic expression of ALP and type I collagen (Tanabe et al, 2004). These catabolic effects mean IL-1 has been implicated in the bone loss associated with inflammatory disease (Kwan et al, 2004).

Many cell types, including osteoblasts, synthesise IL-6, which is a member of the gp130 cytokine family. Although osteoclastogenic, IL-6 mediates most of its effects indirectly; it stimulates osteoblasts to produce downstream effectors including RANKL, which can either act on osteoclasts directly or in a paracrine manner to further stimulate their manufacture by osteoblasts (Kotake et al, 1996).

The potent multifunctional cytokine TGFβ has three isoforms (TGFβ1, -2, -3) with TGFβ1 being the most abundant in the bone matrix. Once synthesised by osteoblasts TGFβ is stored in a latent form within the bone matrix, until release and activation following bone resorption. TGFβ is a potent mitogen for osteoprogenitor cells and therefore can increase the number of cells expressing the osteoblast phenotype. In vitro TGFβ exerts different effects depending on culture conditions; in co-cultures of osteoblasts and haematopoietic cells TGFβ suppresses osteoclastogenesis by increasing OPG secretion and/or decreasing RANKL expression (Takai et al, 1998; Quinn et al, 2001). Conversely, in mouse marrow cultures, TGFβ suppresses apoptosis and enhances osteoclast formation in cells stimulated with RANKL and M-CSF, an effect abolished by soluble TGFβ receptor II (Fuller et al, 1998; Sells Galvin et al, 1999).

Prostaglandins such as PGE2 are synthesized from membrane phospholipids by the actions of phospholipase A2 and cyclooxygenases; they are potent multifunctional regulators of bone formation and resorption that function by mediating the actions of other growth factors, cytokines and mechanical stimulation. PGE2 has anabolic actions and promotes the recruitment of osteoblast precursors and stimulates
proliferation, collagen synthesis and ALP activity (Hakeda et al., 1985; Raisz et al., 1999). In some cases the actions of prostaglandins and extracellular nucleotides are interrelated, for example, osteoblasts stimulated by fluid flow release enhanced levels of ATP, which then acts in a paracrine way to stimulate PGE$_2$ production and release (Genetos et al., 2005). The effects of prostaglandins on osteoclast function appear to be species dependent as they stimulate resorption in whole organ cultures (Hoebertz et al., 2001) and isolated human osteoclasts (Lader & Flanagan, 1998) but inhibit resorption in isolated chick osteoclasts (Arnett & Dempster, 1987).

Nitric oxide (NO) mediates many physiological processes including vascular relaxation, neurotransmission and mechanically induced bone formation. This highly reactive free radical is generated by nitric oxide synthase (NOS), an enzyme with three differentially expressed isoforms: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). NO has been implicated in the local regulation of bone metabolism and exerts potent biphasic effects on bone cell function. Osteoblasts constitutively produce NO at a low level and once released it may act in an autocrine manner to stimulate proliferation and cytokine production (van't Hof & Ralston, 2001). The eNOS isoform appears to be particularly important in this process as eNOS knockouts display defective bone formation and reduced osteoblast number (Aguirre et al., 2001). Low concentrations of NO may also be necessary for osteoclast motility and activity. High NO concentrations, which commonly occur after stimulation with proinflammatory cytokines, are associated with osteoblast cell death (Damoulis & Hauschka, 1997) and impaired osteoclast formation and activity (van't Hof & Ralston, 1997).

Both insulin-like growth factors (IGF-1, IGF-2) are present in the systemic circulation, synthesized in numerous tissues including bone and are potent modulators of growth in vivo. In bone, many systemic hormones regulate synthesis of IGF; for example, glucocorticoids inhibit IGF-1 expression in osteoblasts, an event thought to contribute towards the decreased bone formation observed following glucocorticoid treatment (Delany & Canalis, 1995). IGFs are weakly mitogenic for osteoblasts, enhance mature cell function and prevent apoptosis (Hill et al., 1997). They promote bone formation by increasing levels of type I collagen and decreasing the expression of collagen degrading enzymes, matrix metalloproteinase (MMP)-13 and collagenase 3 (Canalis et al., 1995). These effects appear to be important for maintenance of bone
mass as IGF-1 null mice display decreased bone formation (Bikle et al, 2002). Addition of IGF-2 to murine mouse marrow cultures enhanced osteoclast formation in a dose dependent manner, suggesting IGFS may also influence osteoclastogenesis in vivo (Fukuoka et al, 2005).

**Acidosis**

Acid is a by-product of metabolism and given the negative effects of systemic acidosis, it is necessary for multicellular organisms to maintain the acid-base balance within narrow limits. The large inorganic component of the skeleton provides a massive reservoir of base to buffer excess protons (H+) if the kidneys and lungs alone cannot maintain the acid-base balance. Initially, the negative skeletal effects of acidosis were thought to result from simple physico-chemical dissolution of bone mineral to buffer a decreased pH, however, this is now known to be untrue (Green & Kleeman, 1991). Acidosis directly stimulates bone resorption, with osteoclasts exhibiting a clear acid activation curve; around pH 7.1-7.2 small decreases of only a few hundredths of a unit will cause a doubling of resorption pit formation by rat osteoclasts in vitro (Arnett & Dempster, 1986; Arnett & Dempster, 1987; Arnett & Spowage, 1996). Acidification is a key requirement for resorption, and once activated, osteoclasts can be further stimulated by many agents including vitamin D₃ (Meghji et al, 2001), ATP, ADP (Morrison et al, 1998), RANK and RANKL (Arnett, 2003). Contrastingly, acidosis inhibits the production of many matrix components (Kaysinger & Ramp, 1998; Frick & Bushinsky, 1999) and progressively blocks the formation of mineralised bone nodules by rat osteoblasts in vitro (Brandao-Burch et al, 2005). The solubility of Ca²⁺ and Pi is increased 2-fold and 4-fold, respectively when the pH is reduced and thus the primary action of low pH is to block mineralisation (Larsen & Jensen, 1989).

**Mechanical stimulation**

Mechanical loading (resulting in strain) is necessary for the maintenance of bone mass and adaptation of the skeleton. Periods of unloading such as during cast immobilisation are characterised by significant bone loss (Duncan & Turner, 1995; Ehrlich & Lanyon, 2002). The effects of mechanical loading on bone remodelling depend on several factors including the frequency, duration and magnitude of the strain but in general, short periods of intermittent loading are most effective at inducing an
adaptive response (Turner, 1998). The osteogenic effects of bone loading also depend on the extent to which the strain is different from normal and appear to decrease with ageing. Mechanotransduction is the process by which cells, most likely osteocytes and osteoblasts, perceive physical stimuli and respond with biochemical signals. Although the exact mechanisms involved remain unclear, it is thought that physiological levels of strain distort the bone tissue and cause movement of extracellular fluid through the canaliculi. Fluid flow can stimulate cells via direct membrane deformation or by inducing an increase in intracellular Ca\textsuperscript{2+} (Pavalko et al, 1998), the subsequent signalling events cause cells to increase expression of Runx2 (Ziros et al, 2002) and release anabolic growth factors particularly PGE\textsubscript{2} and NO (Jiang et al, 2001). Following release, these chemical mediators can act to regulate bone remodelling by inhibiting resorption and stimulating bone formation in vivo.

**Hypoxia**

Bone is highly vascular receiving a blood flow of 200-400 ml/min, approximately 8% of the total cardiac output in a resting adult human. Long bones are fed by multiple arterial supplies that diverge into arterioles and control the blood supply into the cortical capillaries and bone marrow (De Marneffe, 1951). Disruption of this blood supply causes profound ischemia followed by vascular congestion, osteocyte death and finally osteoporosis, illustrating the importance of microvasculature in bone remodelling and repair.

Oxygen tension (pO\textsubscript{2}) in arterial blood is about 95 mmHg (~ 12%) and in venous and capillary blood is about 40 mmHg (~ 5%), approximately a quarter of that in atmospheric air. In normal tissues the interstitial pO\textsubscript{2} is usually between 5-12% and has been measured in the bone marrow of healthy volunteers at ~ 6.5% (Harrison et al, 2002) and in the synovial fluid of normal joints at 6-7% (Lund-Olesen, 1970). Localised hypoxia occurs when blood supply to a tissue is reduced or disrupted, for example following fracture or tissue damage. It can also arise because of natural processes including aging and the menopause, which influence the delivery of O\textsubscript{2} to the tissues. Following vascular disruption, the ensuing hypoxic gradient will lead to a very low pO\textsubscript{2} in the centre of the affected area; in diseased tissues such as osteoarthritic joints the pO\textsubscript{2} has been reported to fall to < 1% (Lewis et al, 1999).
In order to function cells must be able to sense and respond to changes in oxygen tension. Acute exposure to hypoxia activates both fast and delayed cellular responses; fast responses include a rapid increase in intracellular Ca$^{2+}$ and a switch from aerobic mitochondrial-based metabolism to anaerobic glycolysis-based metabolism. The delayed responses involve altered gene expression primarily caused by the upregulation/stabilisation of specific transcription factors, the most important being hypoxia-inducible factor (HIF). Under normal conditions, cells constitutively produce the heterodimeric HIF, which consists of two subunits (HIF-α and HIF-β) each containing an amino-terminal basic-helix-loop-helix-PAS domain required for dimerisation and DNA binding (Wang et al., 1995). The HIF-β subunit or arylhydrocarbon nuclear translocator (ARNT) is a constitutive nuclear protein. In contrast, oxygen tightly regulates HIF-α expression and levels increase exponentially as oxygen concentrations decrease. There are three closely related forms of the HIFα subunit (HIF-1α, -2α, -3α), each encoded by a distinct gene locus. In terms of structure and regulation, HIF-1α and -2α are analogous, however less closely related is the HIF-3α subunit, which lacks DNA binding activity. Expression of HIF-1α is universal, whereas HIF-2α expression is restricted to certain cell types including osteoblasts (Akeno et al., 2001). HIF-3α is a negative regulator of the other HIFα subunits and by binding HIF-β functions to inhibit hypoxia induced, HIF-mediated gene expression (Hara et al., 2001).

Normally, oxygen and iron sensitive enzymes hydroxylate a proline residue (pro564) on HIF-1α, targeting it for intracellular destruction via the ubiquitin-proteosome pathway (Pugh & Ratcliffe, 2003). These enzymes, belonging to the prolyl hydroxylase family, have an absolute requirement for molecular oxygen and therefore provide a direct link between oxygen availability and the regulation of HIF (Bruick & McKnight, 2001). During hypoxia, suppression of enzyme catalysis enables HIF-1α to accumulate, translocate to the nucleus and dimerise with HIF-β. The active HIF complex acts to enhance transcription by binding to the hypoxia response element (HRE), via the consensus sequence 5′-RCGTG-3′, situated in the promoter region of target genes (Semenza, 1996). There are three broad groups of hypoxia-inducible genes: (1) factors related to oxygen delivery such as erythropoietin and VEGF, (2) enzymes involved with glucose metabolism especially those of the glycolytic pathway,
and (3) factors involved in cell proliferation and survival including IGF-2 (Wenger et al., 2002). Conserved across most mammalian cells, this oxygen sensing system allows employment of the appropriate cellular responses to hypoxia.

Despite the high level of skeletal vasculature, both chondrocytes and osteocytes occupy avascular matrix bound sites and receive nutrients by passive diffusion. In vivo, HIF-1α mRNA has been detected in articular cartilage (Stokes et al., 2002), and a critical role in developing bone was illustrated when HIF-1α deletion caused the death of chondrocytes in the cartilaginous growth plate (Aigner et al., 2001; Schipani et al., 2001). Hypoxia may also represent a novel mechanotransduction pathway as mechanical unloading (24 hours) leads to rapid increase in hypoxic and HIF-1α expressing osteocytes; however, this effect is transient and a subsequent short period of loading restores oxygen homeostasis (Dodd et al., 1999; Gross et al., 2001).

Earlier work on cultured osteoblasts demonstrated diverse responses to hypoxia including the induction of many genes involved in fracture repair such as VEGF (Steinbrech et al., 1999; Steinbrech et al., 2000), IGF₂ (Akeno et al., 2001), TGF-β and type I and III collagen (Warren et al., 2001). In contrast, expression of the fundamental osteoblast differentiation factor Runx2 is decreased (Park et al., 2002). The effect of hypoxia on osteoblast function will be discussed further in Chapter 4. It is well documented that low oxygen stimulates the formation and activation of cells derived from marrow precursors, including those of the monocyte/macrophage lineage (Bradley et al., 1978; Broxmeyer et al., 1990; Lewis et al., 1999). Pathological bone loss commonly occurs during ischemia, fractures, tumours, inflammation and/or infection where the pO₂ is reduced and these conditions have striking effects on other cells of the monocyte/macrophage lineage. During the course of this research, low oxygen (2%) was shown to dramatically increase osteoclast formation and activity (please see paper by Arnett et al., 2003 in Appendix II).

**Temperature**

Homeothermic animals regulate their body temperature and possess mechanisms to detect changes in both internal and external temperatures and compensate accordingly. When the temperature decreases, the body adjusts by reducing heat loss and increasing heat production. Conversely, when the temperature rises, the body increases its heat
loss and reduces heat production. These regulatory responses involve a number of autonomic, somatic and endocrine responses and are necessary because even small deviations from the 37°C norm markedly affects cellular metabolism. Extreme changes in temperature can lead to organ failure and even death. Changes in body temperature can occur locally or systemically for example; the increased temperature seen during inflammation or infection is part of the body’s innate defence mechanisms and is caused by the activities of proinflammatory cytokines such as IL-1 and IL-6 (Netea et al, 2000). Some pathological conditions are also associated with changes in core body temperature such as in hyperthyroidism or hypothyroidism.

All cells must be able to sense and respond to changes in ambient temperature. At present the mechanisms involved remain unclear, however, considerably more information exists regarding cellular responses to heat shock as opposed to cold shock. The heat shock response is ubiquitous, highly conserved and essentially acts as a defence mechanism to protect cells from a range of harmful conditions including heat stress, hypoxia and inflammation (Feder & Hofmann, 1999). This response involves the induction of proteases and heat shock proteins (Hsps); Hsps were initially identified following heat shock and are a diverse family of molecular chaperones that play an essential role in protein folding and translocation (Ellis et al, 1991). Hsps are classified, according to size, into six major families (Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and the small heat shock proteins). Within each Hsp family, there are members that are either constitutively expressed or inducible. It is now accepted that certain Hsps function under normal conditions to aid in protein folding, however, heat stress acts to further increase protein levels. Induction of Hsp expression requires a heat shock element (HSE) in the promoter region; these HSEs are bound by heat shock factors (HSFs), of which there are three in human cells (Morimoto et al, 1998). The ubiquitously expressed HSF-1 has a principal role in stress-induced expression of Hsp genes. Normally, Hsps particularly Hsp70, bind and inactivate HSF-1; however, increased temperature causes Hsp70 to assume its chaperoning function freeing HSF-1 to bind, as a trimer, to the HSE in target genes and enhancing transcription (Zhong et al, 1998). Prokaryotic cells express cold shock proteins (Csps), which act as RNA chaperones to prevent the formation of inappropriate secondary structures. To date Csps have not been identified in eukaryotic cells and are not thought to be involved in their cold shock response (Graumann & Marahiel, 1998).
The mechanisms via which cells sense changes in external temperature remain undefined but may involve members of the transient receptor potential (TRP) superfamily. Individual TRP channel subunits contain six transmembranous domains and assemble as tetramers to form cation-permeable (such as Ca$^{2+}$, Na$^{+}$) pores. Subdivided into seven subfamilies, including TRPC, TRPV, TRPM and TRPA, the TRP channels are important in sensory physiology with roles in vision, taste, smell, mechanosensation and thermosensation. Of these, the TRPV channels are thought to be most important in temperature sensing. TRPV1, TRPV2 and TRPV3 are activated by temperatures of 43°C, 52°C and 39°C, respectively and therefore could function as heat sensors (Xu et al, 2002; Patapoutian et al, 2003; Bender et al, 2005). Whereas TRPV4, TRPM8 and TRPA1 channels are stimulated by 27°C, 23-28°C and 17°C respectively thus making them cold sensors (Peier et al, 2002; Story et al, 2003). Cells expressing these receptors possess the ability to respond to a wide range of temperatures from noxious heat ($\geq 43^\circ$C) to uncomfortable cold ($\geq 17^\circ$C). At present, an involvement of TRP channels in the responses of bone cells to temperature and other noxious stimuli remains unproven. Nevertheless, as osteoblasts express the non-thermosensitive TRPV6 channel, expression of other thermosensitive TRP channels on bone cells is possible (Weber et al, 2001). The effects of temperature on osteoblast function are discussed further in Chapter 4.
EXTRACELLULAR NUCLEOTIDES AND PURINERGIC SIGNALLING

Overview

All cells require energy in order to carry out the biochemical reactions necessary for survival; the free energy donor in most processes is adenosine triphosphate (ATP). ATP belongs to a large family of molecules essential in cellular function called the nucleotides, all of which are synthesised de novo from simple organic building blocks (amino acids and sugars). Each nucleotide consists of a purine (adenine or guanine) or pyrimidine (thymine, uracil or cytosine) base, a sugar (ribose or deoxyribose) and a phosphate (mono-, di- or tri-) group joined to the sugar by an ester linkage. Nucleotide biosynthesis is a complex process involving multiple enzymatic reactions and feedback inhibition to prevent overproduction. Once formed nucleotides are readily interconvertible for example, removal of one, two or three phosphate ions from ATP generates adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine, respectively. Nucleotides are ubiquitous across all cell types, are involved in numerous intracellular biochemical processes and metabolic pathways and are the substrates for a wide range of enzymes.

In 1929, following the observation that adenosine and AMP could elicit biological effects on the mammalian heart, Drury and Szent-Gyorygi first proposed a role for purines as extracellular signalling molecules; however, it was 1972 before the concept of purinergic neurotransmission was proposed. In his hypothesis, Burnstock suggested ATP was a neurotransmitter involved in non-adrenergic and non-cholinergic responses of smooth muscle in the gastrointestinal tract and bladder (Burnstock et al, 1970; Burnstock, 1972). This concept took time to gain acceptance since ATP was first recognised for its role in intracellular biochemical processes and because such a ubiquitous and simple molecule seemed an unlikely extracellular messenger. In 1976, the finding that ATP was a co-transmitter released during nerve transmission (Burnstock, 1976a) had a significant influence on the purinergic field and led to the first report of purinergic receptors (Burnstock, 1976b). This was followed, two years later, by the proposal that there were specific purinoreceptors for nucleosides (P1 receptors) and nucleotides (P2 receptors) (Burnstock, 1978). The P2 receptors are
further subdivided into the ionotrophic P2X and the metabotropic P2Y (first proposed by Burnstock & Kennedy, 1985), on the basis of their structure, pharmacological profile and tissue distribution (Abbracchio & Burnstock, 1994). Currently, seven P2X receptors and eight P2Y receptors have been characterised (Ralevic & Burnstock, 1998; Burnstock 2004). The most potent agonists at purinergic receptors are the adenine and uridine-containing nucleotides; adenosine potently activates P1 receptors, whilst P2 receptors respond to ATP, ADP, uridine triphosphate (UTP) and uridine diphosphate (UDP).

**P1 receptors**

The endogenous purine nucleoside adenosine, is formed either by the sequential breakdown of ATP to ADP, AMP and finally adenosine or by the hydrolysis of S-adenosyl-L-homocysteine. It potently activates four different P1 receptor subtypes: $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$ (Ralevic & Burnstock, 1998; Fredholm *et al*, 2001). Each P1 receptor is G-protein linked and activation modulates adenylate cyclase (AC) activity. $A_1$ and $A_3$ receptors are $G_i$ coupled and decrease cAMP (3'-5' cyclic adenosine monophosphate) production by inhibiting AC, whereas $A_{2A}$ and $A_{2B}$ receptors are $G_s$ coupled and stimulate AC leading to increased cAMP production. Individual P1 receptors display distinct pharmacological profiles and tissue distributions, being expressed in amongst others the brain, heart, lungs, liver, bladder, large intestine and spinal cord (Ralevic & Burnstock, 1998). Adenosine signalling through the P1 receptors mediates diverse biological effects including vasodilatation in the heart and neuroprotection during hypoxia. There are selective agonists and antagonists for each P1 receptor facilitating mechanistic investigation and potentially providing therapeutic targets. Although P1 receptor-mediated signalling is important in chondrocytes and cartilage, bone cells do not express P1 receptors and therefore they were not the focus of this research.

**P2X receptors**

All seven members of the P2X receptor family ($P2X_{1-7}$) are ligand gated ion channels. Each receptor subunit has two transmembranous domains (TM1 and TM2), separated by an extensively N-glycosylated extracellular loop that constitutes a large portion of the protein and always contains 10 cysteine residues (North, 2002). These conserved
cysteines are thought to contribute towards the protein tertiary structure by disulphide bond formation. TM1 is involved in channel gating, TM2 lines the ion pore, and a hydrophobic H5 region close to the pore vestibule is the ATP binding site. Each P2X subunit possesses a short intracellular N-terminus and C-terminus, the only exception being the P2X7 receptor. The P2X subunits share a 35-48% sequence identity and vary in size from 379 to 595 amino acids. The stoichiometry of P2X receptors involves at least three subunits combining to form homo or heteromultimers. Currently there are four known heteromultimers: P2X2/3, P2X1/5, P2X2/6 and P2X4/6, although how individual P2X subunits influence receptor phenotype remains unclear. ATP is the main natural ligand at P2X receptors, although many synthetic ATP analogues also activate specific P2X receptor subtypes. Conversely, all uridine-containing nucleotides are inactive. All P2X receptors mediate fast signalling and activation induces a rapid, non-selective cation current permeable to Na+, K+ and Ca2+ ions.

The P2X1 receptor

Homomeric P2X1 receptors are potently activated by ATP and its synthetic analogue α,β-methylene ATP (α,β-meATP), but can be inhibited by cyclic pyridoxine-α4,5-monophosphate-6-azophenyl-2',5'-disulphonate (MRS2220) or 2',3'-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) (North, 2002). Agonist stimulation causes rapid receptor desensitisation (decline in the current elicited during the continued presence of ATP) with a slow recovery. P2X1 receptors are permeable to Ca2+ and Na+ ions, with expression detected in smooth muscle, platelets and the cerebellum (Ralevic & Burnstock, 1998). P2X1 receptor-deficient mice demonstrated an important role for this receptor in the vasoconstriction of renal vasculature (Inscho et al, 2004).

The P2X2 receptor

At present, there are no selective agonists or antagonists for P2X2 receptors; however, one unique property of this receptor subtype is its sensitivity to protons and low concentrations of zinc and copper ions. A pH of ~ 7.0 causes a significant potentiation in ATP-evoked currents, a response considerably effected by removal of a specific amino acid residue (His-319) (Nakazawa et al, 2003). Following agonist stimulation, the homomeric P2X2 receptors become particularly permeable to Ca2+ but unlike other P2X receptors exhibit little or no desensitisation. Expression of the P2X2 receptor is
widespread and includes nervous tissue, the bladder and intestine. P2X₂ knockout mice display reduced ventilatory responses to hypoxia (Rong et al, 2003) plus deficits in pain related behaviours and normal sensory processes suggesting this receptor mediates some of the sensory effects of ATP (Cockayne et al, 2005).

The P2X₃ receptor

The synthetic analogue 2-methylthio-ATP (2-MeSATP) and to lesser extent α,β-meATP potently activate homomeric P2X₃ receptors, which like P2X₁ receptors, experience rapid desensitisation following ligand binding. These receptors are predominantly expressed in sensory nerves but are also present on endothelial and epithelial cells. Involvement of this receptor in mediating peripheral pain responses and urinary bladder volume reflexes was demonstrated in vivo using receptor knockout mice (Cockayne et al, 2000; Zhong et al, 2001).

The P2X₂/₃ receptor

The heteromeric P2X₂/₃ receptor is expressed in certain sensory neurons and shares some of the properties of both homomeric P2X₂ and P2X₃ receptors including potentiation by low pH, slow desensitisation and activation by α,β-meATP (North, 2002). Moreover, P2X₂/₃-null mice display the same deficits as both single P2X₂ and P2X₃ knockouts such as impaired ventilatory responses to hypoxia (Rong et al, 2003) and urinary bladder reflexes (Cockayne et al, 2005).

The P2X₄ receptor

Homomeric P2X₄ receptors are potently activated by ATP, however they have no selective antagonists and are relatively insensitive to the more general inhibitors, suramin and pridoxal-5-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS). Upon activation, P2X₄ receptors become particularly permeable to Ca²⁺ and desensitisation is intermediate between that observed at P2X₁ and P2X₂ receptors (North, 2002). Receptor expression is widespread being especially prominent on cells from the CNS.
The P2X5 receptor

Compared to other P2X receptors, homomeric P2X5 receptors elicit only small currents in response to agonist (ATP) stimulation (North, 2002). This receptor is inhibited by suramin and PPADS and, following activation, experiences slow desensitisation. P2X5 receptors are widely expressed in proliferating cells of the skin (Greig et al., 2003), gut, bladder (Groschel-Stewart et al., 1999), thymus (Glass et al., 2000), skeletal muscle (Meyer et al., 1999; Ryten et al., 2002) and spinal cord, where receptor activation acts to stimulate cell division. It is also prominent in several cell types during embryonic development (Ryten et al., 2001).

The P2X1/5 receptor

Cells expressing heteromeric P2X1/5 receptors provide responses to ATP that have several distinct features; firstly, they are more sensitive to ATP than their homomeric counterparts and, secondly, α,β-meATP evokes a sustained current which is not seen in either P2X1 or P2X5 homomultimers (North, 2002). Expression of P2X1/5 receptors has been reported in some blood vessels (Burnstock & Knight, 2004).

The P2X6 receptor

Unlike the other members of the P2X receptor family, P2X6 subunits do not form functional homomultimers (Ralevic & Burnstock, 1998). P2X6 subunit expression is widely distributed throughout the CNS where it forms heteromultimers with P2X4 or P2X2 receptors.

The P2X2/6 and P2X4/6 receptor

Expression of the P2X2/6 and P2X4/6 receptors has been reported in the brain stem and central nervous system neurons, respectively (Burnstock & Knight, 2004). However, for both heteromeric channels information regarding receptor pharmacology is still limited (North, 2002).

The P2X7 receptor

The P2X7 (formerly P2Z) receptor is distinct from other family members in several ways; firstly, it has a significantly longer C-terminus and secondly, it is the only P2X subunit not to form functional heteromultimers. This receptor has a relatively low
sensitivity for ATP (> 100 μM) and preferentially binds to 2',3'-O-(benzoyl-4-benzoyl)-ATP (Bz-ATP). P2X7 receptors can be inhibited by several different antagonists including Brilliant Blue G and the large organic cation, KN-62 (North, 2002). Prolonged or repeated exposure to high agonist concentrations, mediates the formation of cytolytic pores (Murgia et al, 1992), whereas, transient receptor stimulation causes the formation of nonselective membrane pores permeable to molecules up to 900 Kda in size (Di Virgilio, 1995). The P2X7 receptor is predominantly expressed on immune cells including macrophages, microglia and certain lymphocytes. This receptor has been implicated in the inflammatory response as leukocytes derived from P2X7 knockout mice failed to release IL-1β in response to ATP (Labasi et al, 2002). Moreover, these animals were less susceptible to monoclonal antibody induced arthritis.

**P2Y receptors**

In keeping with other G-protein coupled receptors (GPCRs), all P2Y receptors possess the characteristic seven transmembrane domain structure; an extracellular N-terminus and an intracellular C-terminus that gives rise to the structural diversity between subtypes and contains protein kinase binding motifs. The P2Y receptors display a 19-55% sequence homology and there are eight family members: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14 (Ralevic & Burnstock, 1998; Burnstock, 2004). The missing numbers in the series represent receptors cloned from non-mammalian vertebrates or receptors currently undergoing functional characterisation.

Each P2Y receptor couples to a heterotrimeric G protein and can be further subdivided according to pharmacology, structure and downstream signalling. The P2Y1, P2Y2, P2Y4 and P2Y6 receptors couple to Gq/11, thus receptor stimulation activates phospholipase C, which hydrolyses phosphatidyl inositol 4,5-bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG) and results in Ca2+ release from internal stores. In contrast the P2Y12, P2Y13 and P2Y14 receptors couple to Gi, inhibit AC and alter cAMP levels. The P2Y11 receptor subtype is distinct and couples to both Gq/11 and Gi (King & Burnstock, 2002; Burnstock, 2004).

Classification in terms of pharmacology splits the P2Y receptors into the adenosine nucleotide-preferring receptors (P2Y1, P2Y12, P2Y13 (human and rodent) and human
P2Y\textsubscript{11}), the uridine nucleotide-preferring receptors (human P2Y\textsubscript{4} and P2Y\textsubscript{6}) and finally the receptors of mixed selectivity (human and rodent P2Y\textsubscript{2} and rodent P2Y\textsubscript{4}). Structurally P2Y receptors can be subdivided into two groups, the first of which contains P2Y\textsubscript{1}, P2Y\textsubscript{2}, P2Y\textsubscript{4}, P2Y\textsubscript{6} and P2Y\textsubscript{11} and the second P2Y\textsubscript{12}, P2Y\textsubscript{13} and P2Y\textsubscript{14}. For the first subgroup, a Y-Q/K-X-X-R motif in TM7 participates in ligand binding; this is additional to the fundamental H-X-X-R/K motif in TM6 that all P2Y receptors possess. In the second group, the additional ligand-binding motif is K-E-X-X-L, this alternative sequence suggesting a different mode of agonist binding (Abbracchio \textit{et al}, 2003).

\textit{The P2Y\textsubscript{1} receptor}

The P2Y\textsubscript{1} receptor is activated by adenine containing nucleotides, particularly ADP and, to a lesser extent, ATP; conversely, UTP and UDP are inactive. This is the only P2Y receptor subtype with specific, high-affinity, synthetic agonists (2-MethylthioADP (2-meSADP)) and antagonists (\textit{N}^{\delta}-methyl-2'-deoxyadenosine-3',5'-bisphosphate (MRS2179) and 2-chloro-\textit{N}^{\delta}-methyl-(\textit{N})-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate (MRS2279)). The P2Y\textsubscript{1} receptor has a widespread distribution with expression reported in tissues such as brain, heart, muscle, intestine, platelets and astrocytes (Burnstock & Knight, 2004). P2Y\textsubscript{1}-deficient mice exhibit decreased platelet aggregation and increased bleeding indicating an important role for this receptor in induction of platelet aggregation (Fabre \textit{et al}, 1999; Leon \textit{et al}, 2001).

\textit{The P2Y\textsubscript{2} receptor}

The P2Y\textsubscript{2} receptor (formerly P2U) displays a mixed selectivity being potently activated by both ATP and UTP but insensitive to nucleotide diphosphates. Receptor expression is widespread and has been identified in amongst others the heart, blood vessels, lung, kidney, astrocytes and skeletal muscle (Burnstock & Knight, 2004). Currently assigned roles for the P2Y\textsubscript{2} receptor include astrogliosis, transmitter release, mucus escalation and airway hydration. Knockout studies revealed a critical role for P2Y\textsubscript{2} receptors in the regulation of ion transport in airway epithelial cells (Cressman \textit{et al}, 1999; Homolya \textit{et al}, 1999).
The P2Y₄ receptor

Both ATP and UTP activate the P2Y₄ receptor and in many species, UTP is the more potent with the exception of rat where these agonists are equipotent. Compared to other family members, P2Y₄ receptor expression is less widespread although is found in brain, epithelial cells, macrophages, monocytes, and smooth muscle. Knockout mice have no overt phenotype but lack UTP- and ATP-induced chloride secretory responses in jejunum epithelial cells, indicating a role for P2Y₄ receptors in this process (Robaye et al, 2003).

The P2Y₆ receptor

The P2Y₆ receptor is selective for uridine-containing nucleotides, particularly UDP. This receptor displays widespread expression being present in tissues as diverse as kidney, lung, spleen, thymus, smooth muscle, heart and epithelium. The P2Y₆ receptor has a proposed role in lymphocytic maturation (King & Burnstock, 2002); however, as no knockout model is available, further functions remain undefined.

The P2Y₁₁ receptor

ATP, ADP and the synthetic nucleotides 2-MeSATP and Bz-ATP all activate this receptor, whilst it is weakly antagonized by suramin and reactive blue 2. Expression is relatively widespread having been detected in the spleen, brain, placenta, intestine and granulocytes. At present no P2Y₁₁ knockout model is available therefore functional effects of receptor activation remain undefined, although this receptor has been implicated in the differentiation of granulocytes into neutrophils (Communi et al, 2000).

The P2Y₁₂ receptor

The P2Y₁₂ receptor is another member of the P2Y family primarily activated by ADP and is structurally related to the UDP-glucose receptor (49% identity). Expression is more restricted having only been identified in the brain and on platelets, where the receptor plays a role in haemostasis. A truncated form of the P2Y₁₂ receptor is non-functional and in one patient was associated with a bleeding disorder (Hollopeter et al, 2001).
The P2Y\textsubscript{13} receptor

The recently identified P2Y\textsubscript{13} receptor is activated by ADP and has a high sequence homology with the P2Y\textsubscript{12} receptor (Marteau \textit{et al}, 2003). Expression of this receptor has been reported in the spleen, liver, brain and erythrocytes (Fumagalli \textit{et al}, 2004). Recently, P2Y\textsubscript{13} receptors were implicated in a negative feedback that leads to the inhibition of ATP release from erythrocytes (Wang \textit{et al}, 2005).

The P2Y\textsubscript{14} receptor

There is some debate over whether this is a true P2Y receptor or not because it specifically responds to UDP-glucose and UDP-galactose but not UTP or UDP. The P2Y\textsubscript{14} receptor, also known as the UDP-glucose receptor, has no defined physiological role although transcripts are widely expressed throughout the neuroaxis (Abbracchio \textit{et al}, 2003).

Sources and fates of extracellular nucleotides

The mechanisms of ATP release and breakdown are discussed in more detail in Chapters 4 and 6.

Physiological roles of purinergic signalling

It is now widely recognised that purinergic signalling is involved in both neuronal and non-neuronal tissues, where extracellular nucleotides can mediate short-term, fast actions or long-term, slower, trophic actions. Evidence now implicates extracellular purines and pyrimidines in biological processes as diverse as smooth muscle contraction and relaxation, inflammation, platelet aggregation and endocrine and exocrine secretions [see review by Burnstock & Knight, 2004].

ATP mediates short-term signalling by acting as a cotransmitter in both the central and peripheral nervous system. For example, sympathetic co-transmission of noradrenaline and ATP has been demonstrated in the smooth muscle of blood vessels, whereas ATP and acetylcholine are co-transmitters from peripheral or central nerve terminals including the parasympathetic nerves supplying the urinary bladder. Other short-term actions include the regulation of ion transport in epithelial cells and the
stimulation of insulin from the β cells of the pancreas (Abbracchio & Burnstock, 1998).

Long-term purinergic signalling plays a role in the regulation of embryonic development, cell proliferation, differentiation and apoptosis, however the exact consequences are dependent on many factors including concentration, target cell and receptor expression. For example, low micromolar concentrations of nucleotides can stimulate DNA synthesis and proliferation in a number of cell types including fibroblasts, haematopoietic cells and keratinocytes; however, millimolar concentrations of ATP can induce apoptosis, probably via the P2X7 receptor (Abbracchio & Burnstock, 1998).
PURINERGIC SIGNALLING AND BONE

There is now growing evidence that extracellular nucleotides, signalling through P2 receptors play an important role in the regulation of bone remodelling. This section will provide a brief overview of current knowledge regarding purinergic signalling and bone cell function.

**Extracellular nucleotides and osteoblasts**

Osteoblasts express at least seven P2 receptor subtypes (see Table 1), however, only four have been assigned functional effects based on in vitro data. In many cell types, the P2X5 receptor plays a role in stimulating proliferation (Burnstock & Knight, 2004); following the observation that 10-100 µM ATP stimulates DNA synthesis in human osteoblast-like cells via activation of the mitogen activated protein (MAP) kinase pathway (Nakamura et al., 2000) a similar role was proposed in osteoblasts. The role of the P2X7 receptor in osteoblast function is less clear, however, it has been implicated in osteoblast apoptosis (Gartland et al., 2001).

*In vitro* evidence suggests that purinergic signalling via the P2Y1 receptor, can act to modulate osteoblast responses to systemic factors. In rat UMR-106 osteoblast-like cells, compared to treatment with PTH alone, co-stimulation with ADP and PTH caused increased CREB phosphorylation and, by binding to the Ca/CRE region in the promoter region, they synergistically increased expression of the transcription factor c-fos (Buckley et al., 2001). Similar effects were also observed in human osteoblast-like SaOS-2 cells, albeit through a different pathway; in these cells activation of both the serum response element (SRE) and the Ca/CRE in the promoter region were required for increased c-fos expression (Bowler et al., 1999). Consequently, locally released extracellular nucleotides may cooperate with systemic factors to activate signalling in osteoblasts above a key threshold, which alone neither stimulus could achieve; allowing the integration of local and systemic factors in the control of bone remodelling (Bowler et al., 2001).
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Receptor agonists</th>
<th>Species and cell type</th>
<th>Evidence</th>
<th>Reference</th>
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<tr>
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<tr>
<td>P2X3</td>
<td>ATP (H⁺ sensitive)</td>
<td>Rat (primary)</td>
<td>Immunocytochemistry, in situ hybridisation</td>
<td>Hoebertz et al, 2000</td>
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<td>Immunocytochemistry</td>
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<td>Human (primary, SaOS-2)</td>
<td>Immunocytochemistry</td>
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<td>Mouse (primary)</td>
<td>RT-PCR</td>
<td>Ke et al, 2003</td>
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<td>2-MeSADP &gt; ADP &gt; ATP</td>
<td>Rat (primary)</td>
<td>In situ hybridisation</td>
<td>Hoebertz et al, 2000</td>
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<td>Human (MG-63)</td>
<td>RT-PCR</td>
<td>Maier et al, 1997</td>
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<td>UTP &gt; ATP</td>
<td>Rat (primary)</td>
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<td>RT-PCR</td>
<td>Maier et al, 1997</td>
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<td>RT-PCR</td>
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<tr>
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<td>α,β(1meATP = ATP</td>
<td>Human (primary)</td>
<td>RT-PCR</td>
<td>Buckley et al, 2002</td>
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<td>Rat (primary)</td>
<td>Immunocytochemistry, in situ hybridisation</td>
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<tr>
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<td>Hoebertz et al, 2000</td>
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<td>Human (primary)</td>
<td>RT-PCR</td>
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<td>RT-PCR, Immunocytochemistry</td>
<td>Buckley et al, 2002 &amp; Gartland et al, 2003</td>
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<td>Rat (primary)</td>
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<td>Buckley et al, 2002</td>
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<td>Human (ostoclastoma)</td>
<td>RT-PCR</td>
<td>Bowler et al, 1995</td>
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<td>Human (primary)</td>
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<td>Human (primary)</td>
<td>RT-PCR</td>
<td>Buckley et al, 2002</td>
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<td>RT-PCR</td>
<td>Korcock et al, 2005</td>
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<td>Human (primary)</td>
<td>RT-PCR</td>
<td>Buckley et al, 2002</td>
</tr>
<tr>
<td>P2Y11</td>
<td>BzATP &gt; ATP &gt; ADP</td>
<td>Human (primary)</td>
<td>RT-PCR</td>
<td>Buckley et al, 2002</td>
</tr>
</tbody>
</table>

Table 1. Expression of P2 receptors by osteoblasts and osteoclasts
Both UTP and ATP, at concentrations as low as 1–10 μM cause strong inhibition of mineralised bone nodule formation by cultured rat osteoblasts, whereas adenosine and ADP were without effect (Hoebertz et al, 2002). These potent actions of ATP and UTP point to the involvement of either P2Y2 or P2Y4 receptors. Further evidence for P2Y2-mediated inhibition comes from the observation that adenosine 5'-O-(3-thiotriphosphate (ATPγS), a potent P2Y2 agonist, also inhibits bone formation in vitro (Jones et al, 1997). Additionally, P2Y2 receptors have been shown to mediate the propagation of intercellular Ca2+ waves (Jorgensen et al, 2000) and oscillatory fluid flow-induced Ca2+ mobilization (You et al, 2002) in osteoblast-like cell lines. Recently, the P2Y2 receptor has also been implicated in the ATP/UTP mediated stimulation of Erg-1 (Pines et al, 2003) and Runx2 (Costessi et al, 2005), via the protein kinase C (PKC) and extracellular related kinase (ERK) pathways.

At present, a role for the P2X2, P2Y4 or P2Y6 receptors in osteoblast development and function remains to be defined.

Activation of P2 receptors on osteoblasts also influences the production of other local factors important in bone cell function. For example, in osteoblast-like cells, ATP was shown to increase the expression of IL-6 mRNA (Ihara et al, 2005) and stimulate the release of arachidonic acid (Wanatanbe-Tomita et al, 1997). Moreover, several groups have reported ATP release from osteoblast-like cells; this will be discussed further in Chapter 4.

**Extracellular nucleotides and osteoclast function**

Osteoclasts also express multiple P2 receptors (see Table 1). Involvement of P2 receptors in the control of osteoclast function was first suggested by Bowler et al (1995), after ATP was found to stimulate resorption by cells derived from an osteoclastoma. Initially, this effect was thought to be mediated via the P2Y2 receptor, however, in a follow-up study UTP failed to stimulate bone resorption (Bowler et al, 1998), suggesting this was not the case. Subsequently, ATP was found to stimulate the formation and activity of rodent osteoclasts; the resorptive activity being further increased when osteoclasts were first activated by culture in acidified medium (Morrison et al, 1998). These pro-resorptive effects were suggested to involve the P2X2 receptor since it is the only P2 receptor sensitive to protons. Moreover, a recent
study found low micromolar concentrations of ATP, ADP and 2-MeSADP to be potent stimulators of both the formation and resorptive activity of rodent osteoclasts, and resorption in bone organ cultures (Hoebertz et al., 2001). These findings, together with cytochemical evidence, suggest the involvement of the P2Y1 receptor in mediating the osteolytic effects of ATP and ADP (Hoebertz et al., 2000; Hoebertz et al., 2001; Hoebertz et al., 2003). Thus, activation of both the P2X2 and the P2Y1 receptors can potentially influence osteoclast activity. Conversely, a study on human osteoclasts suggested that ATP functions indirectly, via the upregulation of RANKL on osteoblasts, to stimulate resorption (Buckley et al., 2002).

The role of the P2X7 receptor in osteoclast activity has received considerable attention in recent years and in vitro evidence suggests this receptor could be involved in a number of different processes. Experiments using cells derived from human peripheral blood demonstrated P2X7 receptor antagonism inhibited osteoclast formation (Gartland et al., 2003a) suggesting as in macrophages (Chiozzi et al., 1997), a potential role in cell fusion. Conversely, P2X7-deficient mice possessed functional osteoclasts in vivo and using knockout precursor cells osteoclasts could be generated in vitro, indicating the P2X7 receptor is not required for cell fusion (Ke et al., 2003). Additionally, the P2X7 receptor may also play a role in intercellular communication between bone cells (Jorgensen et al., 2002).

Activation of both the P2X7 receptor by Bz-ATP (300 μM) (Korcok et al., 2004) and the P2Y6 receptor by UDP (10 μM) (Korcok et al., 2005) induced translocation and activation of NFκB in osteoclasts and their precursors. Moreover, the activation of P2Y6 receptor prevented the apoptosis induced by TNFα, indicating a role for this receptor in osteoclast survival (Korcok et al., 2005).

At present, how the P2X1, P2X4, P2Y2, P2Y4 and P2Y11 receptors signal to modulate osteoclast activity remains unclear.

**Purinergic signalling and cartilage**

Cartilage is a fundamental component of the skeletal system being necessary for bone formation via endochondral ossification and essential for the smooth movement of joints. The expression of purinergic receptors in cartilage was first suggested
following the observation that ATP, ADP and to a lesser extent UTP stimulated PGE$_2$ production from cultured human chondrocytes (Caswell et al, 1991). Recent characterisation, demonstrated expression of P2X$_2$, P2X$_5$, P2Y$_1$ and P2Y$_2$ receptors along with the P1 receptors, A$_{2A}$ and A$_{2B}$ in chondrocytes (Koolpe et al, 1999; Hoebertz et al, 2000).

Extracellular nucleotides act to stimulate cartilage resorption, an effect enhanced by IL-1β and IL-1α, and intercellular communication through the extracellular matrix (Leong et al, 1994; D'Andrea & Vittur, 1996). Chondrocytes release ATP, which, by acting in an autocrine/paracrine manner, can induce PGE$_2$ release (Graff et al, 2000). Nucleotide-induced PGE$_2$ release is further enhanced by pro-inflammatory cytokines including IL-1β, IL-1α and TNF-α; this suggests nucleotides may function synergistically with these factors and potentially contribute indirectly towards the pathophysiology of arthritis (Caswell et al, 1992; Koolpe et al, 1999). Conversely, other data indicates a constructive role for extracellular nucleotides as they can stimulate proteoglycan synthesis, inhibit their breakdown and induce cartilage repair (Kumahashi et al, 2004).

Although an area of active research, the effects of extracellular nucleotides on chondrocyte function and cartilage were not the focus of this thesis and will not be discussed further.

**GENERAL AIM OF THESIS**

The aim of this thesis was to investigate further the involvement of purinergic receptors and extracellular nucleotides in bone cell function under normal and stress situations.
CHAPTER 2

FUNCTIONAL EXPRESSION OF PURINERGIC RECEPTORS BY OSTEOBLASTS

INTRODUCTION

Growing evidence suggests extracellular nucleotides, signalling through P2 receptors, might play a significant role in bone biology modulating both osteoblast and osteoclast function (Hoebertz et al, 2003). The P2 receptors are grouped into the P2X ligand-gated ion channels and P2Y G-protein linked receptors (Abbracchio & Burnstock, 1994). Currently there are seven known P2X subtypes (P2X_1-7) and eight P2Y subtypes P2Y_1, P2Y_2, P2Y_4, P2Y_6, P2Y_11, P2Y_12, P2Y_13 and P2Y_14; each of these receptors has been cloned, characterised and displays distinct tissue expression and pharmacology (Burnstock & Knight, 2004).

Studies in the early 1990s showed that extracellular nucleotides act on osteoblast-like cells to induce formation of inositol triphosphate (IP_3) and to elevate intracellular calcium (Ca^{2+}) transiently; pharmacological profiles were suggestive of the expression of P2Y_1- and P2Y_2-like receptors [reviewed by Hoebertz et al, 2003]. Subsequent studies on primary human osteoblasts indicated heterogeneity of P2 receptor expression, since all cells exhibited intracellular Ca^{2+} responses to ATP and UTP (suggestive of P2Y_2 and/or P2Y_4 receptors) but only a sub-population responded to ADP (which is selective for the P2Y_1, P2Y_12 and P2Y_13 receptors) (Dixon et al, 1997). This was taken to indicate that expression of P2 receptors changes during the osteoblast life cycle, depending on the differentiation state. A summary of the P2 receptor subtypes expressed on osteoblasts is provided in Table 1.

Several of the P2 receptor subtypes expressed on bone cells have assigned functional effects (see Chapter 1 for more detailed information). For example, in vitro data has implicated the P2X_5 receptor in osteoblast proliferation as 10-100 μM ATP, but not UTP, stimulate DNA synthesis in osteoblast-like MG-63 (Nakamura et al,
2000) and MC3T3-E1 cells (Shimegi, 1996). The involvement of the P2X2 receptor in the modulation of osteoblast activity remains unclear, whereas P2X7 receptors may mediate apoptosis in response to high agonist concentrations (Gartland et al, 2001).

Earlier in vitro evidence indicated that relatively low concentrations of extracellular nucleotides exert P2 receptor-mediated effects that would shift the bone remodelling balance strongly in the negative direction (Hoebertz et al, 2003). Low micromolar concentrations of ATP and ADP, signalling through the P2Y1 receptor, stimulate the formation and resorptive activity of rodent osteoclasts, and resorption in bone organ cultures (Morrison et al, 1998; Hoebertz et al, 2001). In contrast, both UTP and ATP cause strong inhibition of mineralised bone nodule formation by cultured rat osteoblasts (Hoebertz et al, 2002). These potent actions of ATP and UTP point to the involvement of either P2Y2 or P2Y4 receptors. Additionally, P2Y2 receptors have been shown to mediate the propagation of intercellular Ca2+ waves (Jorgensen et al, 2000) and can stimulate the activity of transcription factors including Erg-1 (Pines et al, 2003) and Runx2 (Costessi et al, 2005). Nucleotide signalling in bone via P2Y1 and P2Y2 receptors can also modulate local remodelling responses to key osteotropic agents such as PTH (Buckley et al, 2001; Bowler et al, 2001).

Despite abundant evidence indicating P2 receptor expression on osteoblasts, how levels change with osteoblast proliferation and differentiation remained poorly investigated. In other cell types including haematopoietic cells, purinergic receptor expression is differentiation dependent (Martin et al, 1997). The aim of the work presented in this chapter was document changes in P2 receptor expression (P2X2, 5, 7 and P2Y1, 2, 4, 6) in normal populations of bone-nodule-forming rat osteoblasts as differentiation and maturation proceed. Changes in mRNA and protein levels were investigated using reverse transcriptase PCR (RT-PCR) and immunofluorescence, respectively. Nucleotide-agonist induced changes in intracellular Ca2+ were studied using the high-throughput fluorescence imaging plate reader (FLIPR).
MATERIALS AND METHODS

Cell culture

Primary rat osteoblast cells were obtained by sequential enzyme digestion of excised calvarial bones from 2-day-old neonatal Sprague-Daley rats using a 3-step process (1% trypsin in PBS for 10 minutes; 0.2% collagenase type II in Hanks balanced salt solution (HBSS) for 30 minutes; 0.2% collagenase type II in HBSS for 60 minutes). The first two digests were discarded and the cells resuspended in Dulbecco’s Modified Essential Medium (Gibco, Paisley, UK) supplemented with 10% Foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin (complete mixture abbreviated to DMEM). Cells were cultured for 2-4 days in a humidified atmosphere of 5% CO₂-95% air at 37°C in 75cm² flasks until confluent. Upon confluence cells were seeded into 6, 24 or 96-well trays (10⁵ and 5 x 10⁴ cells/well, respectively) in DMEM supplemented with β-glycerophosphate (2 mM), ascorbic acid (50 µg/ml) and dexamethasone (10 nM). Osteoblasts were cultured for 4-21 days with half medium changes every 2-3 days. Throughout the culture, medium pH, pCO₂ and pO₂ were monitored using a blood gas analyser (ABL-700, Radiometer, Copenhagen).

To investigate the ability of ATP and UTP to prevent bone formation and the P2 receptor subtypes involved, fresh nucleotides and/or receptor antagonists were added to the medium every 2-3 days. As concentrations of ATP/UTP of 1-100 µM (Chapter 3; Hoebertz et al, 2002) significantly inhibit bone nodule formation, an intermediate concentration of 10 µM was employed for all the experiments performed here. The purinergic receptor antagonists suramin and reactive blue 2 were tested at 0.1-100 µM.

Measurement of intracellular Ca²⁺

Osteoblastic cells were seeded into poly-D lysine-coated black walled, clear-bottomed 96-well trays (BD Biosciences, Oxford UK) at a density of 5 x 10⁴ cells/well and cultured for 4, 8 or 10 days with half medium changes every other day. Cells were twice washed with PBS and loaded for 30 minutes with the cell-permeant Fluo-4 AM (2 µM) in PBS containing 2.5% pluronic acid in 100 µl dimethyl sulphoxide (DMSO). After removal of the fluorophore loading solution, cell layers were washed twice more
and 150 µl of PBS added per well. The cell plates were loaded into a fluorescence imaging plate reader (FLIPR, Molecular Devices, Wokingham, UK) together with a separate 96-well tray containing nucleotide agonists, an ionomycin positive control (1 µM) and a PBS negative control. The agonists were distributed in a randomised pattern to minimise any cell plating effects due to well position. The FLIPR was programmed to transfer the agonists simultaneously to all 96-wells, 30 seconds after commencement of recording; fluorescence was excited at 488 nm and emission measured at 510-560 nm. Duration of recording was typically 3 minutes; at the end of experiments, cell layers were fixed with 2.5% glutaraldehyde for 5 minutes and stained with 1% alizarin red (w/v) to demonstrate the presence of bone nodules. Due to technical constraints (acidification of medium, monolayer peeling) it was not possible to culture osteoblasts in the 96-well trays for periods exceeding 10 days.

Cell proliferation assay

Osteoblast proliferation was measured using the MTT assay. The assay exploits the ability of mitochondrial dehydrogenases, in metabolically active cells, to reduce the yellow tetrazolium 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) generating a purple formazan product. Rat calvarial osteoblasts were cultured in standard 96-well trays as described above. MTT reagent stock solution (5 mg/ml in PBS) was added to each cell layer (final concentration 0.5 mg/ml) and plates incubated in the dark at 37°C for five hours; culture medium was then carefully removed and formazan crystals solubilised by the addition of concentrated DMSO. Absorbance was read in a plate reader at 550 and 650 nm (ELx800, Bio-Tek International, Fisher Scientific UK, Loughborough): a standard curve for determination of cell numbers was constructed using cells seeded at $10^2$ to $10^6$/well.

Total RNA extraction and complementary DNA strand synthesis

Osteoblasts were cultured in 6-well trays for 6-18 days in supplemented DMEM; every 2-3 days total RNA was extracted from two wells using TRIZOL® reagent (Invitrogen, Paisley, UK) according to the manufacturers instructions. Before first strand complementary DNA (cDNA) synthesis, extracted RNA was DNase treated with RNase free DNase I (35 U/ml) for 30 minutes at 37°C. The reaction was terminated by heat inactivation at 65°C for 10 minutes. Total RNA was quantified
spectrophometrically 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 minutes at 25°C, followed by extension at 42°C for 60 minutes and inactivation at 70°C for 15 minutes. CDNA was stored at -20°C until amplification by RT-PCR.

**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 manufacturers’ instructions, with cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds (see Table 2 for annealing temperatures), extension at 72°C for 45 seconds and reaction termination by 72°C for 5 minutes. For analysis, PCR products were loaded onto a 1% agarose gel containing 0.3 μg/ml ethidium bromide. Gels were run at 80 mA for 30 minutes and the DNA position visualised by exposure to UV light. Product size was determined by comparison to a DNA ladder run concurrently. To account for differences in original cell number and cDNA quality all samples were normalised with β actin prior to further reactions. The expression of P₂X₂, P₂X₅, P₂X₇, P₂Y₁, P₂Y₂, P₂Y₄ and P₂Y₆ receptors was investigated in osteoblasts cultured for 6, 8, 11, 13 and 15 days. Expression of the bone matrix protein, osteocalcin (OCN), was employed as a marker of differentiation. All reactions were carried out in triplicate using cDNAs derived from three different osteoblast cultures. Primer sequences and annealing temperatures are shown in Table 2. The identity of PCR products generated by these primers has previously been confirmed by sequencing (Bailey *et al*, 2000; Bailey *et al*, 2001; Shibuya *et al*, 1999)

**Immunofluorescence**

Rat osteoblastic cells were seeded onto sterile 1 cm discs, cut from Melinex clear polyester films (Du Pont, Dumfries UK), in 24-well trays at 5 x 10⁴ cells/disc and
cultured in supplemented DMEM for 4-18 days. Every three days discs were removed \((n = 5)\) and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 minutes at room temperature, washed 3 x 5 minutes with PBS and stored at 4°C in PBS-methiolate (0.05%) until staining. Each disc was incubated with a blocking solution of 10% normal horse serum (NHS) in PBS-methiolate containing 0.1% Triton x 100 for 1 hour. Primary antibodies raised in rabbit were diluted in 10% NHS in PBS-methiolate at a ratio of 1:200 (P2X receptors) or 1:100 (P2Y receptors). Discs were incubated overnight in the primary antibody solution with gentle agitation; negative controls were incubated overnight in 10% NHS in PBS-methiolate, containing no primary antibody. Following removal of the primary antibody solution, cells were subjected to three further 5 minute washes with PBS before incubation for 1 hour with the donkey anti-rabbit Cy3-labelled secondary antibody solution (1:400) and a DAPI counter stain (1:3500), diluted in PBS with 1% NHS and 0.05% methiolate. After three further 5 minute PBS washes discs were mounted onto microscope slides using Citifluor AF2 solution and either stored at 4°C or viewed under a fluorescence microscope (Cy3 absorbance and emission at 550 nm and 570 nm, respectively). All images were acquired using identical camera settings to allow comparison of staining intensity.

**Quantification of bone nodule formation**

Osteoblast cultures were examined daily using an Olympus IMT-2 inverted microscope with phase contrast optics (Olympus Optical Company Ltd, London, UK) and after 7 and 10 days plates were stopped to indicate the progression of nodule formation. After 15-24 days the experiment was terminated and cell layers were washed with PBS, fixed with 2.5% glutaraldehyde for 5 minutes, washed again with PBS and following three further washes with 70% ethanol, plates were left to air dry. Mineralised bone nodules were visualised by staining with alizarin red (1% solution w/v in water) for 5 minutes, rinsed with 50% ethanol to remove excess stain, then air-dried. The plates were imaged at 800 dpi using a high-resolution flat-bed scanner (Epson Perfection Photo 3200). Using ‘Adobe Photoshop’ (version 5, Adobe Systems Inc.) images of individual wells were first changed to greyscale before conversion to binary images using Scion Image software (Scion Corporation; [http://www.scioncorp.com](http://www.scioncorp.com)). These images were then subjected to automated analysis,
using constant 'threshold' and 'minimum particle' levels, to determine the number and plan surface area of mineralised bone nodules.

All images of osteoblast monolayers were taken using a Leica digital camera, mounted on an Olympus IMT-2 inverted microscope with phase contrast optics.

**Reagents**

All chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise mentioned. Fluo-4 AM was obtained from Molecular Probes Inc (Invitrogen, Paisley UK). Reverse Transcriptase, Taq DNA polymerase and other molecular biology reagents were purchased from Promega UK (Southampton, UK) and all primers from MWG Biotech (Ebersburg, Germany). P2Y primary antibodies were obtained from Alomone (Jerusalem, Israel), P2X antibodies from Roche Bioscience (Palo Alto, CA, USA), donkey anti-rabbit Cy3 labelled secondary antibodies from Jackson Immunoresearch (Philadelphia, USA) and Citifluor AF2 solution from Citifluor (London, UK).

**Statistical analysis**

Statistical comparisons were made by one-way analysis of variance (ANOVA) and adjusted using the Bonferroni method. Representative data are presented as means ± SEM for five to ten replicates. Results are representative for experiments that were each repeated at least three times.
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<th>Product size (bp)</th>
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Table 2. Primer sequences and annealing temperatures used in Chapter 2 RT-PCR reactions

P2X primers taken from sequences published by Shibuya et al, 1999, P2Y1, P2Y2, P2Y4 primers taken from Bailey et al, 2000 and P2Y6 primers taken from Bailey et al, 2001. Previous sequencing of PCR products ensured amplification of the correct gene. β actin and osteocalcin (OCN) primers were designed in our laboratory.
RESULTS

*Nucleotide-induced increases in intracellular Ca\(^{2+}\) in primary rat osteoblasts*

Concentrations of ATP, UTP and ADP over the range 0.002 \(\mu\)M to 1000 \(\mu\)M were tested in osteoblasts cultured for 8 days. All three agonists triggered increases in intracellular Ca\(^{2+}\), measured using the intracellular fluorophore, Fluo-4 AM; responses typically peaked 10 seconds after addition, and decayed back to baseline within 2-3 minutes. Cellular responses were illustrated by expressing baseline fluorescence prior to agonist addition as 100% and all subsequent readings as a percentage of this value (Figure 2.1A). Immediately after agonist addition, a small artefactual decrease in fluorescence was normally observed.

Of the nucleotide agonists tested, ATP was the most potent; eliciting increased intracellular Ca\(^{2+}\) from concentrations of 0.2 \(\mu\)M, with peak responses occurring at ~20 \(\mu\)M (Figure 2.1B). A decrease in response occurred with concentrations exceeding 100 \(\mu\)M. UTP was somewhat less potent than ATP with a maximal response between 20-100 \(\mu\)M, although this varied between cultures. Comparatively speaking ADP was clearly the least potent of the three with concentrations of 2-200 \(\mu\)M eliciting significantly smaller responses.

At day 8 of culture, other P2 agonists, including (in decreasing order of potency) ATP\(_{\gamma}\)S, UDP, 2-MeSATP, Bz-ATP and \(\alpha,\beta\)-meATP also increased intracellular Ca\(^{2+}\) in osteoblasts; however, with the exception of ATP\(_{\gamma}\)S none were as potent as ATP. UDP is an agonist at P2Y\(_4\) and P2Y\(_6\) receptors, Bz-ATP acts at P2X\(_7\) and \(\alpha,\beta\)-meATP can activate P2X\(_3\) and P2X\(_3\) receptors, indicating these receptor subtypes may be present on rat osteoblasts. In contrast, no intracellular response was observed to the P1 agonist’s adenosine and AMP (Figure 2.1C).
Figure 2.1. Purinergic agonists stimulate increased intracellular Ca\(^{2+}\) in rat osteoblasts.

(A) Cellular responses to agonist stimulation were very transient, returning to basal within ~30 seconds. The arrow represents agonist addition at t = 0 and the slight drop in fluorescence observed is a common artefact seen with this technique. (B) ATP, UTP and ADP all caused an increase in intracellular Ca\(^{2+}\) (n = 10). Responses are expressed as percentage increase in fluorescence following agonist addition. (C) Other P2 agonists elicited an increase in intracellular Ca\(^{2+}\) (n = 10), but with the exception of ATPyS, none were as potent as ATP. Adenosine and AMP, which are inactive at P2 receptors, had no effect on intracellular Ca\(^{2+}\) (*** = p<0.001, ** = p<0.01). Each FLIPR experiment was performed at least three times.
**Bisphosphonates do not induce increased intracellular Ca\(^{2+}\) in osteoblasts**

The bisphosphonates are a class of drugs clinically used to treat conditions characterised by bone loss, such as osteoporosis. Through direct actions on osteoclasts, they inhibit bone resorption, although the exact mechanisms involved depend on the class of bisphosphonate. First generation bisphosphonates do not contain any nitrogen atoms and function via cellular uptake, followed by enzymatic conversion to a non-hydrolysable ATP analogue, accumulation of which is toxic to the cell. Second or third generation bisphosphonates usually contain nitrogen in at least one of their side chains. They disrupt cholesterol synthesis by inhibiting farnesyl diphosphate synthase, the enzyme that converts mevalonate to cholesterol (Green, 2004). Several bisphosphonates share structural similarities with nucleotides and so the first and third generation bisphosphonates, Clodronate and Zolendronate were tested (1-500 μM) for effects on intracellular Ca\(^{2+}\) in osteoblasts, using the FLIPR. Even at the highest concentration, neither drug elicited an increase in intracellular Ca\(^{2+}\) in primary rat osteoblasts (Figure 2.2).

![Figure 2.2. Bisphosphonates do not increase intracellular Ca\(^{2+}\) in rat osteoblasts.](image)

The bisphosphonates Zolendronate and Clodronate (1-500 μM) did not cause an increase in intracellular Ca\(^{2+}\) in rat osteoblasts (n = 5).
Intracellular Ca\textsuperscript{2+} responses of osteoblasts to nucleotides: dependence on time in culture

Primary rat osteoblasts cultured for 4, 8 or 10 days were challenged with ATP, UTP and ADP over the concentration range 2-200 \(\mu\)M. Intracellular Ca\textsuperscript{2+} responses increased strikingly with time in culture; all concentrations of ATP induced significantly higher responses at 10 days compared to 4 days (up to 6-fold), whilst at 8 days compared to 4 days, \(\geq 20 \mu\)M ATP caused significantly increased responses (up to 3-fold) (Figure 2.3A). Following a similar trend, 2-200 \(\mu\)M UTP also induced significantly enhanced responses at both day 8 and day 10 compared to day 4 (Figure 2.3B). ADP induced less pronounced alterations in response with only 100-200 \(\mu\)M causing significantly increased Ca\textsuperscript{2+} release at 8 and 10 days (Figure 2.3C).

To determine whether increased cell number accounted for these results, cell proliferation assays were performed in parallel with the FLIPR experiments (Figure 2.3D). Between 4 and 10 days, cell number only increased by 2.4-fold whilst intracellular Ca\textsuperscript{2+} responses increased up to 6-fold, 3.6-fold and \(\sim 2\)-fold following treatment with ATP, UTP and ADP respectively. These data indicate cell proliferation is a major cause of the enhanced responses to ADP, but cannot entirely explain the increased cellular responses to ATP and UTP.

Experimentation on osteoblasts cultured for greater than 10 days was not possible due to a marked reduction in medium pH (pH 7.10 instead of 7.40), caused by the small volume of fluid bathing the cells, and monolayer peeling. Both of these factors detrimentally affect osteoblast activity and functional responses, leading to inaccurate readings from the FLIPR
Figure 2.3. Osteoblast responses to ATP, UTP and ADP change with time in culture.

(A) 2-200 μM ATP caused increased intracellular responses at day 10 compared to day 4 (up to 6-fold). Stimulation with 20-200 μM ATP induced larger intracellular responses at day 8 of culture compared to day 4 (up to 3-fold) \((n = 10)\). (B) UTP at 2-200 μM caused significantly higher increases in intracellular Ca\(^{2+}\) at both 8 and 10 days compared to 4 days (up to 3.6-fold) \((n = 10)\). (C) Treatment with 100-200 μM ADP induced significantly increased intracellular responses at 8 and 10 days, compared to 4 days \((n = 10)\). (D) Cell number increased 2.4 fold between 4 and 10 days of culture \((n = 10)\) \((*=p<0.05, **=p<0.01, ***=p<0.001)\). All FLIPR time course experiments were performed in triplicate.
Expression of P2 receptor mRNA in osteoblasts

Primary rat osteoblasts were cultured for up to 15 days in 6-well plates, and total cellular RNA was extracted at day 6, 8, 11, 13 and 15 to enable the levels of selected mRNAs to be determined by PCR amplification. For each of the seven P2 receptors investigated, mRNA was detected and representative results are shown in Figure 2.4. In all experiments, osteoblast monolayers formed mineralised nodules indicating the presence of mature functional osteoblasts.

RNA levels in individual samples were normalised using the housekeeping gene β-actin. OCN mRNA expression, used as a marker of osteoblast differentiation, was barely detectable at 6 days but increased to high levels at 11-15 days indicating the presence of mature bone forming osteoblasts. The individual P2 receptors showed considerable variation in mRNA levels throughout the culture period confirming the idea of differentiation dependent receptor expression (Figure 2.4). Expression of P2X2 and P2X5 receptor mRNAs peaked at 8 days but then declined to undetectable or near-undetectable levels by 15 days. In contrast, P2X7 receptor mRNA was detected at all time points between 6 and 15 days. Expression of P2Y1 receptor mRNA remained relatively constant level until 15 days, when levels appeared to decline. In contrast, P2Y2 receptor mRNA showed a striking, progressive upregulation with time in culture suggesting this receptor plays an important role in mature osteoblasts. P2Y4 and P2Y6 receptor mRNAs were most highly expressed between 8 and 13 days of culture.
Figure 2.4. *Expression of P2 receptor mRNA in primary rat osteoblasts.*

Total RNA was extracted after 6, 8, 11, 13 and 15 days of culture. Samples were normalised with mRNA for β actin, and osteocalcin (OCN) mRNA was assessed as a marker of osteoblast differentiation. Marked differences in the expression of some P2 receptor mRNAs were evident over time. P2X2 and P2X5 receptor mRNAs were expressed in early cultures, whereas expression of mRNA for the UTP-selective P2Y2 receptor increased strongly in later cultures. Expression of P2X7 and P2Y1 receptor mRNA remained relatively constant until 15 days when levels decreased. P2Y4 and P2Y6 receptor mRNAs tended to be greatest between days 8 and 13. Each PCR reaction was carried out in triplicate using cDNA from three separate experiments. The product sizes (in base pairs) for each gene amplified are: P2X2 (357), P2X5 (418), P2X7 (354), P2Y1 (289), P2Y2 (311), P2Y4 (294), P2Y6 (331), β actin (332) and OCN (418).
Detection of P2 receptor subtypes in osteoblasts by immunofluorescence

Specific primary antibodies directed against individual P2 receptors and a Cy3 labelled secondary antibody enabled the determination of receptor protein by fluorescence microscopy. The development and specificity of the P2X polyclonal antibodies has been reported previously (Oglesby et al, 1999; Xiang et al, 1998). The P2Y antibodies are commercially available and are directed against specific epitopes located in the 3rd intracellular loop between TM5 and TM6 (P2Y1, P2Y2) or in the C-terminus (P2Y4, P2Y6); they have been employed previously by Ryten et al (2004a) and Cheung et al (2003).

In all cases, cell size appears to decrease with culture progression; this reflects the compaction of osteoblasts at the later time points. In all cases, no immunostaining was observed in the negative controls (no primary antibody). Protein and mRNA expression generally followed similar trends, however in some cases protein expression was detected when mRNA was absent. Marked changes in immunostaining for P2 receptors with time in culture were observed; representative images of are shown in Figure 2.5 and 2.6, for P2X and P2Y receptors, respectively. Staining for individual receptors was not always uniform across cell layers, reflecting variations in differentiation and proliferation.

In agreement with the RT-PCR results, both P2X2 (Figure 2.5A & 2.5B) and P2X5 (Figure 2.5C & 2.5D) receptor staining was strongest at earlier time points (4-8 days), and was increasingly confined to sub-populations of cells as cultures progressed. This restricted expression was particularly noticeable in the cells stained for P2X5 receptors and may well reflect uneven differentiation across the cell monolayer resulting in regions of less differentiated still proliferating osteoblasts. For both P2X2 and P2X5 receptors, mRNA and protein expression demonstrated slight variance, with receptor protein being detected at later time points when mRNA was absent. P2X7 receptor protein expression appeared widely distributed throughout the monolayer and remained at a relatively constant, albeit low level throughout the culture period (Figure 2.5E & 2.5F). Similar to the other P2X receptors, P2X7 receptor protein expression appeared to persist for longer than the mRNA expression.
In accordance with the RT-PCR data, P2Y₁ receptor protein expression remained relatively widespread and constant throughout the culture period (Figure 2.6A & 2.6B). RT-PCR demonstrated a large increase in P2Y₂ receptor mRNA expression between 6 and 15 days of culture and immunofluorescence confirmed protein expression also increased strongly with time (Figure 2.6C & 2.6D). Both P2Y₄ and P2Y₆ receptors had only previously been identified on human osteoblast-like cell lines (Maier et al, 1997), however these data provide clear evidence for the widespread expression of receptor protein on rat osteoblasts. Expression of both P2Y₄ (Figure 2.6E & 2.6F) and P2Y₆ (Figure 2.7G & 2.6H) receptors tended to be greatest at intermediate time points.
Figure 2.5. *Expression of P2X receptors in rat osteoblasts*

Changes in P2X receptor expression over time in culture were studied by immunofluorescence using specific primary polyclonal antibodies, Cy3-labelled anti-rabbit secondary antibody (red) and DAPI nuclear stain (blue). Photomicroscopy values were the same for each image. Each picture is a representative of the staining that occurred in three separate experiments. Image (A) illustrates the strong P2X$_2$ receptor expression at 8 days of culture, whilst (B) demonstrates the lack of P2X$_2$ receptor expression at 14 days. Image (C) represents the high level of P2X$_5$ receptor expression at early stages of culture; in contrast, (D) illustrates the very low level of expression in mature osteoblasts. Images (E) and (F) illustrate the constant, low level of P2X$_7$ receptor expression seen in these cultures. In all images the scale bar = 10 μm.
Changes in P2Y receptor expression over time in culture were studied by immunofluorescence using specific primary polyclonal antibodies, Cy3-labelled anti-rabbit secondary antibody (red) and DAPI nuclear stain (blue). Photomicroscopy values were the same for each image. Each picture is a representative of the staining that occurred in three separate experiments. Images (A) and (B) represent the relatively constant P2Y_1 receptor expression in rat osteoblasts. P2Y_2 receptor expression increased dramatically from (C) day 4 to (D) day 14. Image (E) shows P2Y_4 receptor expression at day 8 and image (F) how it increased at 11 days. Image (G) illustrates the peak expression of P2Y_6 receptors at intermediate time points, whilst image (H) illustrates the lower expression at day 14. In all images scale bar = 10 μm.
Reactive blue 2 does not prevent UTP-mediated inhibition of bone nodule formation

ATP and UTP both potently activate rat P2Y2 and P2Y4 receptors. The widespread expression of the P2Y4 receptor observed here questions the findings of Hoebertz et al (2001), which implicated the P2Y2 receptor subtype in the inhibition of bone formation by nucleotides. The purinergic receptor antagonist, reactive blue 2, inhibits P2Y4 receptors but is inactive at P2Y2 receptors; in an attempt to clarify the P2 receptor subtype mediating the inhibition of bone formation, this agent was tested in vitro at a range of concentrations (0.1-100 μM). UTP-treated osteoblasts demonstrated an approximate 85% decrease in bone formation and at 0.1-10 μM, reactive blue 2 failed to prevent this inhibitory effect (Figure 2.7A). Reactive blue 2 alone at 0.1-10 μM did not adversely affect osteoblast function (Figure 2.7B). Osteoblasts cultured with 100 μM reactive blue 2 (with or without UTP) failed to form bone nodules, suggesting that at high concentrations this agent is toxic.

Suramin inhibits bone nodule formation in rat osteoblasts

Currently there are no antagonists specific for the P2Y2 receptor subtype. Suramin is a non-specific inhibitor acting at multiple P2 receptor subtypes including the P2Y2 receptor, however it is inactive at the P2Y4 receptor. Osteoblasts were treated with 0.1-100 μM suramin (with or without 10 μM ATP/UTP) and cultured for up to 21 days. Consistently, treatment with suramin impaired proliferation and completely prevented bone nodule formation, indicating that prolonged exposure to this agent was toxic to osteoblasts. Thus, this inhibitor could not be employed to further clarify the P2 receptor subtype involved in mediating the anti-osteogenic effects of nucleotides on osteoblast function.
Figure 2.7. **Reactive blue 2 does not prevent the inhibitory actions of UTP**

(A) Treatment with UTP (10 μM) inhibited bone nodule formation by rat osteoblasts by up to 85%; reactive blue 2 at 0.1-10 μM did not prevent the anti-osteogenic effect of UTP in vitro \( (n = 6) \). (B) Application of reactive blue 2 (0.1-10 μM) alone did not affect bone nodule formation by rat osteoblasts in vitro \( (n = 6) \). These results are representative of experiments performed at least three times.
**Late-acting inhibition of bone nodule formation by nucleotides**

The images in *Figure 2.8* are low power scans of osteoblast monolayers cultured for 17 days without (*Figure 2.8A*) and with (*Figure 2.8B*) 10 μM ATP. These pictures were acquired using a high-resolution scanner (Epson Perfection Photo 3200) and illustrate how long-term culture in the presence of extracellular nucleotides exerts a striking inhibitory effect on mineralised nodule formation *in vitro*.

Rat calvarial osteoblasts were cultured for 17 days and from about day 10 onwards, mineralised bone nodules with characteristic ‘trabecular’ morphology began to form. ATP or UTP (10 μM) was added to osteoblast cultures from day 1, 7, 10 or 14 and for 1-7 and 1-10 days. Incubation with ATP or UTP from day 1, 7 or 10 caused a striking 18-fold reduction in mineralised nodule formation (*Figure 2.9A* & *2.9B*). In contrast, nucleotide addition for the first 7 days of culture had no effect on mineralised nodule formation at day 17, however, application for the first 10 days elicited a small ~20% decrease (*Figure 2.9C* & *2.9D*). When ATP was present from 14-17 days only, further bone nodule formation by osteoblasts was prevented (although bone already formed was unaffected) (*Figure 2.9E*).

The observation that P2 receptor expression changes with osteoblast differentiation, combined with these functional observations led to a more detailed investigation regarding the inhibitory effects of nucleotides on bone formation (see Chapter 3).
Figure 2.8. Culture with 10 \( \mu M \) ATP inhibits bone nodule formation by rat osteoblasts.

These are low power images of osteoblast monolayers cultured for 17 days (A) without or (B) with 10 \( \mu M \) ATP. Unlike the control cultures, ATP-treated cultures displayed an almost complete inhibition of bone nodule formation \textit{in vitro} (\( n = 6 \)). In both images the scale bar = 0.5 cm.
Figure 2.9. Late-acting inhibition of bone nodule formation by ATP or UTP

(A & B) Addition of ATP or UTP from day 7 or 10 inhibited nodule formation to the same extent (up to 18-fold) as from day 1. (C & D) Nucleotide addition for 1–7 days did not impair nodule formation, whilst addition for 1–10 days caused a slight decrease (~ 20%). (E) ATP addition to mature bone forming osteoblasts inhibited further nodule formation, however, preformed bone was unaffected (***=p<0.001, **=p<0.01, *=p<0.05). All experiments were performed in triplicate (n = 6).
DISCUSSION

Purinergic signalling appears to have a major role controlling cellular function in a wide array of tissues and cells (Burnstock & Knight, 2004). The work shown here demonstrates that functional responses of normal osteoblasts to the nucleotides ATP and UTP increase during differentiation with time in culture; these changes were notably accompanied by an increase in the expression of the P2Y receptors particularly the UTP-sensitive P2Y2 receptor and the P2Y4 and P2Y6 receptors.

The fluorescence imaging plate reader (FLIPR) system enabled efficient, high throughput, quantitative screening of intracellular Ca2+ responses of cell populations to agonists. Previous studies have used the FLIPR to demonstrate the presence of functional P2 receptors in epithelial cells (Coutinho-Silva et al, 2005); however, this study investigated the acute actions of ATP and other nucleotides on primary osteoblasts maintained in long-term cultures. Several nucleotide agonists elicited rapid increases in intracellular Ca2+; the most potent of these being ATP, the “universal agonist” at P2 receptor subtypes, followed by UTP, which is selective for P2Y2 and P2Y4 receptors. Peak intracellular Ca2+ responses to ATP and UTP occurred in the range 2–200 μM, consistent with the blockade of bone nodule formation caused by the nucleotides at the same concentrations (Chapter 3; Hoebertz et al, 2002). Smaller intracellular Ca2+ responses were also evoked by several other nucleotide agonists, consistent with the expression of functional P2X2, P2Y4, P2Y6, P2Y1, P2X5 and P2X7 receptors on primary osteoblasts [for reviews of P2 receptor pharmacology, see Hoebertz et al, 2003; Burnstock & Knight, 2004]. ATPyS and 2-MeATP are also more universal purinergic receptor agonists; therefore, the increased intracellular Ca2+ levels observed probably involve the activation of several different P2 receptors, although from these data alone it is impossible to define which. Adenosine, AMP and the bisphosphonates, Zolendronate and Clodronate, were without effect, suggesting that these agents do not interact with cell surface receptors to cause increased intracellular Ca2+. This study did not determine activation of Ca2+-independent signalling pathways (such as cAMP) and would necessitate further investigation.

Several cell types including thymocytes (Koshiba et al, 1997), myeloid cells (Adrian et al, 2000) and leukocytes (Clifford et al, 1997) express purinergic receptors
in a differentiation dependant manner; a trend suggested but not investigated in osteoblasts (Dixon et al, 1997). In the present study, sharp augmentations (up to 6-fold) in the intracellular Ca\(^{2+}\) responses of osteoblasts to ATP and UTP occurred as cultures progressed towards bone nodule formation; these increases could not be accounted for by the modest increases (~ 2-fold) in cell number observed. These findings suggested changes in the expression of P2 receptors accompanied osteoblast differentiation. Subsequent RT-PCR and immunocytochemical analysis revealed a strong differentiation dependence of P2 receptor expression in long-term osteoblast cultures, characterised by a shift from early P2X expression to P2Y expression at later stages. Most notable was the striking increase in levels of the P2Y\(_2\) subtype during the second week of culture, coinciding with the onset of bone nodule formation. In some cases receptor protein expression appeared to persist somewhat longer than that of the respective mRNA; this could be due to heterogeneity between cultures, because the protein signal is sustained after degradation of the mRNA and/or because the mRNA level was too low for the RT-PCR to detect. Given that both RT-PCR and immunofluorescence are only qualitative, further work could involve quantitative techniques (such as western blotting or real time PCR) to confirm the findings presented here.

P2X\(_2\) receptor expression appeared strongest in proliferating osteoblasts. Expression of P2X\(_2\) receptor mRNA and protein decreased markedly with time in culture, with no receptor mRNA and only low levels of P2X\(_2\) receptor protein being detected beyond 11 days. The physiological role, if any, of P2X\(_2\) receptors in bone cell function requires further elucidation, although it has been implicated in mediating the activation of osteoclastic resorption by acid and ATP (Morrison et al, 1998). Given that this receptor displays the distinct property of potentiation at low pH (6.9-7.0), a role in the control of osteoblast function and differentiation during localised acidosis is possible. Activation of other P2 receptors negatively effects bone cell function by stimulating osteoclast formation and activity (Hoebertz et al, 2001) and inhibiting bone formation by osteoblasts (Hoebertz et al, 2002). P2X\(_2\) receptor knockout mice display age related increases in bone mass suggesting this purinoreceptor might also exert negative effects on bone cell function (Chapter 5).
The P2X₅ receptor is associated with proliferation in many cell types. Moreover, ATP-induced activation of P2X₅ receptors stimulates the proliferation of osteoblast-like cells in a Ca²⁺ independent, MAP-kinase dependent manner (Nakamura et al., 2000). In accordance with a proliferative role, P2X₅ receptor mRNA and protein expression was maximal during early stages of culture, a period characterised by pronounced proliferation and low OCN expression. With increasing culture time and decreasing proliferation, P2X₅ receptor expression decreased to near undetectable, whereas mRNA for OCN increased massively. Low-level, localised P2X₅ receptor staining was observed in the mature cultures (11-14 days), this likely reflects the uneven rates of cellular proliferation and differentiation across the osteoblast monolayer.

The P2X₇ receptor is widely expressed on immune cells including macrophages, lymphocytes and osteoclasts from several species (Modderman et al., 1994; Hoeberitz et al., 2000; Gartland et al., 2003b). These data provide confirmatory evidence for the constant expression of the P2X₇ receptor in primary rat osteoblasts, albeit at relatively low levels (Gartland et al., 2001). The function of this receptor in bone remains enigmatic. At high concentrations of extracellular ATP, the P2X₇ receptor mediates the formation of cytolytic pores (Murgia et al., 1992; Di Virgilio, 1995). Activation of osteoclast P2X₇ receptors has been reported to facilitate intercellular calcium signalling between osteoblasts and osteoclasts (Jorgensen et al., 2002), whilst blockade of this receptor inhibits osteoclast formation (Gartland et al., 2003a; Korcok et al., 2004). Deletion of P2X₇ receptors in mice leads to deficient periosteal bone formation together with excessive trabecular bone resorption (Ke et al., 2003). Despite the clear link between the P2X₇ receptor and ATP-mediated cell death, the low prevalence of apoptosis in osteoblasts suggests that this is an unlikely to be the sole function for P2X₇ receptors in bone cell metabolism (Jilka, 1998; Burnstock, 2002). As stimulation of P2X₇ receptors mediates the release of cytokines from non-skeletal cells, including IL-1β from macrophages (Ferrari et al., 1997; Solini et al., 1999), an alternative possibility is that pores induced by transient activation of P2X₇ receptors could play a role in the controlled release of certain cytoplasmic components from osteoblasts. Further work is required to determine exactly how the P2X₇ receptor modulates osteoblast activity and so influences skeletal remodelling.
In agreement with earlier findings, this study found strong evidence for the expression of the P2Y1 receptor on osteoblasts, together with significant intracellular Ca\(^{2+}\) responses to ADP (the ligand selective for this receptor as well as for the P2Y\(_{12}\) and P2Y\(_{13}\) receptors), particularly at higher concentrations (Maier et al, 1997; Bowler et al, 1999; Hoebertz et al, 2000; Hoebertz et al, 2003). However, ADP does not inhibit bone formation \textit{in vitro}, suggesting that stimulation of intracellular Ca\(^{2+}\) does not automatically lead to decreased bone formation. Data from this study demonstrated relatively constant, widespread expression of P2Y1 receptors on osteoblasts. Earlier work implicated this receptor in the potentiation of PTH receptor mediated Ca\(^{2+}\) signalling (Buckley et al, 2001; Bowler et al, 2001); this observation combined with the constant expression demonstrated here, suggests a general role for the P2Y1 receptor in modulating the actions of locally released nucleotides on osteoblast function.

The P2Y\(_{6}\) receptor had only previously been identified on transformed human osteoblast-like cells (Maier et al, 1997). These data clearly show that the P2Y\(_{6}\) receptor is expressed by normal rat osteoblasts, and that UDP, the agonist selective for this receptor evokes significant intracellular Ca\(^{2+}\) responses in osteoblasts. Receptor expression appeared to gradually increase, with high levels between 8-13 days, before decreasing slightly at later stages. Currently little is known of the role the P2Y\(_{6}\) receptor might play, if any, in osteoblast metabolism. A recent report has implicated P2Y\(_{6}\) receptors in osteoclast survival, whether the receptor has a similar function in osteoblasts is unknown (Korcok et al, 2005).

\textit{In vitro}, ATP and UTP significantly inhibit bone nodule formation by rat osteoblasts, whilst ADP and UDP are inactive (Chapter 3); this ligand specificity discounts any participation of the P2Y1 and P2Y\(_{6}\) receptors but suggests the involvement of either P2Y\(_{2}\) or P2Y\(_{4}\) receptor subtypes. As earlier \textit{in situ} hybridisation found no evidence for P2Y\(_{4}\) receptor mRNA expression on rat osteoblasts the P2Y\(_{2}\) receptor was hypothesized to mediate these functional effects (Hoebertz et al, 2000; Hoebertz et al, 2002). In this study, RT-PCR and immunofluorescence analysis revealed a striking increase in the levels of P2Y\(_{2}\) receptor mRNA and protein expression during the second week of culture, coinciding with the onset of nodule formation. Throughout the culture period, cells exhibited widespread P2Y\(_{2}\) receptor expression.
staining; at 11 and 14 days, the staining appeared notably more intense, suggesting that individual cells expressed increased amounts of receptor protein.

Previously, the P2Y4 receptor had only been identified in transformed osteoblast-like cell lines (Maier et al, 1997) and not in normal osteoblasts (Hoebertz et al, 2000). This study demonstrates clear expression of P2Y4 receptor mRNA and protein in rat osteoblasts. Several reasons potentially explain why the investigation by Hoebertz and colleagues (2000) failed to identify this receptor on rat osteoblasts. Firstly, the previous study only examined cells cultured for 2-4 days; given the low levels of P2Y4 receptor protein observed here at 4 days, expression of the receptor may have been undetectable before this point. Secondly, inherent differences in the sensitivities of the techniques employed may have contributed to the failure to identify P2Y4 receptor expression. In rat osteoblasts, expression of the P2Y4 receptor appeared differentiation dependent with the highest levels occurring at intermediate time points (~11 days), although in mature cultures, mRNA expression declined whilst protein persisted, albeit at a low level.

ATP and UTP equipotently activate both the rat P2Y2 and P2Y4 receptors, whilst UTP is more potent than ATP at human P2Y4 receptors. The finding that rat osteoblasts express the P2Y4 receptor raises the question of whether this receptor mediates the inhibitory effects of nucleotides on bone formation. In other cell types such as airway epithelial cells, the predominant P2Y2 receptor has been shown to mask the effects of P2Y4 and P2Y6 receptors and this may have occurred here (Lazarowski et al, 1997). In an attempt to address this issue, the P2 receptor antagonists reactive blue 2 and suramin were tested for potential effects. Reactive blue 2 consistently failed to have any effect on the inhibition of bone nodule formation caused by UTP suggesting no involvement of the P2Y4 receptor. Unfortunately, inhibitor studies using 0.1-100 µM suramin provided no further clarification because at ≥1 µM this agent was toxic to osteoblasts. An effect most likely due to the non-specific nature of suramin, as in addition to its role as a P2 receptor antagonist, suramin inhibits cell surface binding of many growth factors and is a potent anti-proliferative agent. Thus, these data suggest that the P2Y2 receptor is the chief mediator of the anti-osteogenic action of extracellular nucleotides, however, until a more specific P2Y2 receptor antagonist
becomes available the involvement of P2Y$_4$ receptors cannot be ruled out. The role of the P2Y$_4$ receptor in bone remodelling now requires further investigation.

The large increases in intracellular Ca$^{2+}$ responses to UTP and ATP in late stage osteoblast cultures, together with increased P2Y$_2$ receptor expression suggest these nucleotides may act to effectively switch off bone formation in mature cells. To test this hypothesis, osteoblasts were cultured with ATP or UTP for different periods of time and the effects on nodule formation quantified. Incubation with ATP and UTP from either 7 or 10 days to the culture end inhibited mineralised nodule formation to the same extent as culture from day 1. Conversely, treatment with nucleotide from 1–7 days failed to inhibit nodule formation whereas treatment from 1–10 days caused a slight decrease. During the first 7 days of culture, P2Y$_2$ expression is low and these data show exposure of osteoblasts to nucleotides during this period does not influence their ability to form mineralised nodules in the future. Moreover, treatment with ATP at day 14 of culture effectively switched off osteoblasts and prevented further bone formation, with all nodules present being formed before nucleotide addition.

Taken together these results suggest that the P2Y receptors, particularly the P2Y$_2$ subtype, expressed in differentiated, functional osteoblasts, could play an important role in the skeleton by acting as an "off-switch" for bone formation. In view of the diversity of P2 receptors expressed by bone cells, it is possible that some redundancy of function may exist, with some inhibitory effects mediated additionally, for example by the P2Y$_4$ or P2Y$_6$ receptor subtypes. The notion that ATP, the "universal agonist" at P2 receptors, could play a physiological role in regulating osteoblast function is supported by the recent observation that it is released from primary osteoblast cultures in amounts that could equate with local concentrations in the high nanomolar in vivo (Chapter 4). Detailed analysis of the skeletal phenotypes of knockout mice deficient in single or multiple P2Y receptors is now required to further elucidate the roles of extracellular nucleotides and their receptors in bone cell function (Cressman et al, 1999; Robaye et al, 2003).
CHAPTER 3

EXTRACELLULAR NUCLEOTIDES INHIBIT BONE FORMATION BY PREVENTING MINERALISATION

INTRODUCTION

Bone formation or osteogenesis involves two distinct stages firstly, synthesis and deposition of the organic matrix and, secondly, mineralisation. The organic matrix consists of ~ 90% type I collagen, and 10-15% bone matrix proteins such as osteocalcin (OCN), osteonectin, osteopontin, matrix gla protein (MGP) and bone sialoprotein (BSP). Collagen is essential for osteogenesis and defects in its production, such as in osteogenesis imperfecta, a group of conditions arising from mutations in the genes encoding type I collagen, result in the formation of brittle bones. Mature osteoblasts contain an extensive endoplasmic reticulum and Golgi apparatus enabling them to synthesize, modify and export via exocytosis the proteins needed for bone formation. Once deposited, the collagen fibres act as a structural template for mineralisation.

Osteoblasts mineralise osteoid by secreting matrix vesicles from specialised regions of the plasma membrane (Anderson et al, 1975). The polarised release of matrix vesicles into selected areas of the developing bone determines the non-random distribution of calcification (Anderson, 1995). Matrix vesicles vary in diameter from 1–100 nm and are highly enriched in phosphatases, annexins, matrix metalloproteinases (MMPs), phosphatidylserine, Ca\(^{2+}\) and orthophosphate (Pi) (Kirsch, 2005; Balcerzak, 2003). Accumulation of mineral ions within the matrix vesicle characterises the initial phase of mineralisation and is mediated by various ion channels and transporters; for example annexins II, V and VI, which are highly expressed in matrix vesicles, are thought to mediate Ca\(^{2+}\) uptake (Kirsch & Pfaffle, 1992; Kirsch et al, 2000). Annexin V also binds the type II and V collagen associated
with the outer leaflet of the matrix vesicle membrane, an interaction which anchors the vesicle to the extracellular matrix (Kirsch & Pfaffle, 1992). The initial nucleation or deposition of needle-like hydroxyapatite crystals \((\text{Ca}_{10}\text{(PO}_4\text{)}\text{(OH}_2\text{)})\) takes place on the inner surface of the matrix vesicle membrane and is regulated by phosphatases and annexins that control the concentration of inorganic ions. Continued addition of mineral ions causes the crystals to grow and upon reaching a certain length, they rupture the matrix vesicle membrane. Once released into the extracellular fluid, these hydroxyapatite crystals serve as a template for the formation of future crystals. Subsequent mineralisation is dependent on the external concentration of \(\text{Ca}^{2+}\) and \(\text{Pi}\) (Anderson, 2003). Bone matrix proteins can bind \(\text{Ca}^{2+}\) and hydroxyapatite with high affinity and appear to regulate mineralisation, although the mechanisms involved remain unclear (Boskey, 2003; Murshed et al, 2004). The release of matrix vesicles from the osteoblast membrane is regulated by factors such as vitamin \(\text{D}_3\) (Bonewald et al, 1992). A \(\text{Ca}^{2+}\), \(\text{Pi}\) or vitamin \(\text{D}_3\) deficiency can cause osteomalacia, a condition characterised by impaired mineralisation and soft bones with an increased tendency to fracture.

Hydroxyapatite crystal formation and propagation depends on a constant supply of \(\text{Pi}\). Inorganic pyrophosphate (PPI) potently antagonises the ability of \(\text{Pi}\) to crystallise with \(\text{Ca}^{2+}\) to form hydroxyapatite, whilst additionally suppressing crystal propagation (Fleisch & Neuman, 1961; Fleisch & Bisaz, 1962; Meyer, 1984). Thus, local PPI exclusion or degradation appears necessary for mineralisation to proceed and either an excess or deficit of PPI will profoundly affect crystal formation. Phosphatases, including alkaline phosphatase (ALP), plasma cell membrane glycoprotein-1 (also known as PC-1), ATPase and inorganic pyrophosphatase, hydrolyse organic phosphate-containing substrates to produce both \(\text{Pi}\) and PPI. Examples of the reactions mediated by these enzymes are as follows:

\[
\begin{align*}
\text{ATPase} / \text{ALP} \\
\text{ATP} + \text{H}_2\text{O} & \leftrightarrow \text{ADP} + \text{Pi} + \text{H}^+ \\
\text{Pyrophosphatase} \\
\text{PPI} & \leftrightarrow \text{Pi} + \text{Pi} \\
\text{PC-1} \\
\text{ATP} + \text{H}_2\text{O} & \leftrightarrow \text{AMP} + \text{PPI} + \text{H}^+
\end{align*}
\]
Recent work suggests that ALP and PC-1 are particularly important in the control of Pi and PPI levels both in vitro and in vivo (Johnson et al., 2000; Hessle et al., 2002). Of the four ALP isoforms (3 tissue-specific, 1 non-specific) mature osteoblasts express, the tissue non-specific form which, although covalently bound to the cell membrane via a phosphoinositol moiety, can be proteolytically cleaved to produce a soluble form. ALP hydrolyses a number of substrates including PPI, β-glycerophosphate, ATP and AMP to produce Pi (Hamade et al., 2003). Patients with mutations in the gene encoding tissue non-specific ALP first illustrated a direct role for this enzyme in mineralisation; these defects cause hypophosphatasia, a condition characterised by poorly mineralised cartilage and bones (Henthorn & Whyte, 1992). Further confirming this role, ALP knockout mice also display hypophosphatasia and defects in bone mineralisation that worsen with age (Narisawa et al., 1997; Fedde et al., 1999; Wennberg et al., 2000).

PC-1 belongs to the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family of enzymes, members of which hydrolyse the phosphodiesterase bond in purine or pyrimidine nucleoside triphosphates, such as ATP, to produce PPI (Goding et al., 1998). This enzyme is a class II homodimeric transmembrane protein and plays a direct role in the inhibition of mineralisation via facilitating PPI accumulation (Johnson et al., 1999). Mice with a naturally occurring PC-1 truncation mutation, termed “tiptoe walking”, display hypermineralisation especially around the intervertebral discs (Okawa et al., 1998); artificially generated homozygous PC-1 knockouts display an essentially identical phenotype to these natural mutants (Hessle et al., 2002).

The actions of ALP and PC-1 appear antagonistic, an idea supported by study of knockout mouse models. ALP-null mice display a hypophosphatasia and impaired mineralisation because of increased PPI and reduced Pi levels (Narisawa et al., 1997; Fedde et al., 1999). In contrast, PC-1 knockouts exhibit a hypermineralisation because of decreased extracellular PPI levels (Okawa et al., 1998; Hessle et al., 2002). However, if both ALP and PC-1 are inactivated a normal skeleton develops (Johnson et al., 2000; Hessle et al., 2002). Several factors, including vitamin D₃ (van Leeuwen et al., 2001), PTH, FGF, TGFβ (Oyajobi et al., 1994a; Oyajobi et al., 1994b; Mansukhani
et al, 2000) and acidosis (Brandao-Burch et al, 2005), modulate the expression and/or activity of ALP and PC-1, and therefore indirectly regulate the rate of mineralisation.

The extracellular nucleotides are important regulators of osteoblast function. In this chapter, the anti-osteogenic effects of extracellular nucleotides were studied in more detail. A number of techniques were employed (RT-PCR, histological staining, colorimetric assays) to determine whether nucleotides inhibit the deposition of the organic matrix and/or mineralisation, with particular attention given to the role of ALP and bone matrix proteins.
MATERIALS AND METHODS

Cell culture
Osteoblasts were isolated and cultured as described in Chapter 2. To determine the ability of nucleotides to influence bone nodule formation and ALP activity, osteoblasts were cultured in standard 24-well trays for up to 21 days; fresh nucleotides were added every 1-2 days, medium was exchanged every 2-3 days. For total RNA extraction, osteoblasts were cultured (with or without 10 μM nucleotide) in standard 6-well trays for up to 21 days; every 3-4 days total RNA (n = 2) was extracted as described in Chapter 2. Medium pH, pCO₂ and pO₂ were monitored throughout using a blood gas analyser, as described previously.

Quantification of bone nodule formation
Osteoblasts were cultured with 1-100 μM ATP, UTP, ADP or UDP (n = 6) and bone nodule formation quantified as described in Chapter 2.

Determination of alkaline phosphatase (ALP) activity
The alkaline phosphatase (ALP) activity of cell lysates was determined colorimetrically using commercially available kits. Cells were cultured in standard 24-well trays (with or without 10 μM ATP, UTP, ADP or UDP) and after 4, 7, 10 and 14 days selected cell layers were washed and cells harvested using a scraper (n = 6), followed by sonication at 4°C and centrifugation at 500 x g. The supernatant was collected and stored at 4°C until assaying in a 96-well tray. The ALP activity and Lowry protein contents of the supernatants were measured colorimetrically (Elx800 plate reader, Bio-tek International) at pH 9.8 using kits (Biotron Diagnostics, CA, USA and Sigma-Aldrich Dorset, UK, respectively) according to the manufacturers instructions. ALP activity was normalised for protein and expressed as 10⁻³ units/min/μg.

Masson's trichrome stain for collagen
Osteoblasts were cultured in 6-well trays for up to 17 days, with or without the addition of 10 μM ATP or UTP (n = 6); at 11 and 14 days cell layers were washed in
PBS and fixed with Bouin’s fixative (Sigma-Aldrich Dorset, UK) overnight at room temperature. After fixation, the monolayer was washed in running water (until yellow colour disappeared) and stained in Weigert’s haematoxylin (iron) solution (Sigma-Aldrich Dorset, UK) for 10 minutes. The cells were thoroughly washed in tap water and stained with 0.5% acid fuchsin, 0.5% glacial acetic acid for 5 minutes, rinsed in distilled water and treated with 1% phosphomolybdic acid solution for 5 minutes. The solution was then drained from the wells by tapping the plate upside down onto a tissue and cells stained for 5 minutes in 2% methyl blue, 2.5% glacial acetic acid, rinsed in distilled water and treated with 1% glacial acetic acid for 2 minutes. At the end of the procedure, the nuclei were stained black, the cytoplasm was red and the collagen purple/blue. Plates were visualised under an Olympus IMT-2 inverted microscope and images taken using a Leica digital camera.

RNA extraction, DNase treatment, cDNA generation and RT-PCR

These reactions were performed as described in Chapter 2. Specific primers were used to probe the expression of ALP, OCN, matrix gla protein (MGP), osteopontin (OPN), type I collagen (COL1) and PC-1 in cultures treated with ATP, UTP, ADP or UDP. Primer sequences and annealing temperatures are shown in Table 3.

Reagents

Unless mentioned, all chemicals were purchased from Sigma-Aldrich (Dorset, UK) and all molecular biology reagents from Promega UK (Southampton, UK). Primers were purchased from MWG Biotech (Ebersburg, Germany).

Statistical analysis

Analysis of data was carried out as described in Chapter 2.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β actin</td>
<td>S - gtt cgc cat gga tga cga t</td>
<td>53.0</td>
<td>332</td>
</tr>
<tr>
<td></td>
<td>AS - tct ggg tca tct ttc acg g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCN</td>
<td>S - gca gac acc atg agg acc ct</td>
<td>59.8</td>
<td>418</td>
</tr>
<tr>
<td></td>
<td>AS - gca gct tgt gcc gtc cat ac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>S - ctc att tgt gcc aga gaa</td>
<td>50.0</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td>AS - gtt gta cgt ctt gga gac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGP</td>
<td>S - ccc tgt cgt atg aat ctc</td>
<td>50.0</td>
<td>297</td>
</tr>
<tr>
<td></td>
<td>AS - gac tcc gta aca aag cga</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPN</td>
<td>S - cgg tga aag tgg cag atc</td>
<td>50.0</td>
<td>333</td>
</tr>
<tr>
<td></td>
<td>AS - gac tcg gga tac tgt tca</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COL1</td>
<td>S - gcg aag aag aca tcc ctag</td>
<td>50.0</td>
<td>377</td>
</tr>
<tr>
<td></td>
<td>AS - ctt gtc agg gat gcc atc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-1</td>
<td>S - gtc agt atg cgt gct aac</td>
<td>51.0</td>
<td>309</td>
</tr>
<tr>
<td></td>
<td>AS - tgg cac act gaa ctag</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. **Primer sequences and annealing temperatures for Chapter 3 RT-PCR reactions.**

Gene specific primers were designed in our laboratory; identity of PCR products was confirmed by sequencing.
Figure 3.2. Treatment with 10 μM UTP blocks matrix mineralisation by rat osteoblasts

(A) This image shows the bone nodules formed by rat osteoblasts cultured in standard 6-well trays for 21 days. (B) This phase contrast light microscopy image illustrates the typical trabecular morphology of alizarin red stained bone nodules formed by normal rat osteoblasts in vitro. (C) This image demonstrates the striking inhibition of bone formation that occurs in vitro following treatment with 10 μM UTP for 21 days. (D) Normal deposition of the organic matrix occurs in UTP-treated cultures (as highlighted by the arrow) however, the absence of alizarin red stained nodules indicates that mineralisation has been inhibited. Scale bars: A & C = 0.5 cm, B & D = 50 μm.
Figure 3.3. Culture with 10 μM ATP or UTP significantly inhibits ALP activity

Rat osteoblasts were treated with ATP, UTP, ADP or UDP (10 μM) and ALP activity quantified after 4, 7, 11 and 14 days of culture. At all time points, UTP inhibited ALP activity by 45-65%. ATP significantly inhibited ALP activity by up to 53% but only from 10 days of culture. ADP and UDP do not block bone nodule formation in vitro and in this assay did not affect ALP activity at any stage throughout the culture period (*** = p<0.001, ** = p<0.01). These results are typical of those seen in repeated ALP assays (n = 6).
Lack of effect of ATP and UTP on collagen formation

Synthesis and deposition of type I collagen is an essential part of osteogenesis and occurs prior to mineralisation. To determine whether extracellular nucleotides impair collagen deposition, histological staining was employed to examine the collagen fibres in control and nucleotide-treated cultures. The images in Figure 3.4 are representative of osteoblast monolayers treated with 10 μM ATP or UTP for 14 days. The osteoblast cytoplasm is stained dark red and the collagen fibres purple/blue. In both the control and nucleotide-treated cultures, the collagen fibres were orientated in an organised manner, although they were slightly less apparent in the controls because, following mineralisation, they have been incorporated into bone nodules. Moreover, the quantity of collagen in the ATP and UTP-treated cultures did not appear different from controls.

ATP and UTP downregulate ALP, COL1, PC-1 and OCN mRNA in osteoblasts but do not affect MGP and OPN expression; lack of effect of ADP and UDP

RT-PCR was employed to study ALP, COL1, OCN, OPN, MGP and PC-1 mRNA expression in osteoblasts treated with ATP, UTP, ADP or UDP (10 μM) at 4, 7 and 14 days of culture; the results are shown in Figure 3.5A & 3.5B. The dramatic increase in OCN and ALP expression illustrates the progression of osteoblast differentiation in control cultures. Treatment with ATP or UTP did not influence OPN and MGP mRNA expression at any stage (Figure 3.5A). OCN expression was not detected at day 4 and at 7 days there were no differences between the control and nucleotide treated cells. Conversely, after 14 days of culture OCN mRNA expression appeared lower in both the ATP and UTP-treated osteoblasts. Furthermore, unlike the controls, OCN expression did not increase from day 7 to day 14 in nucleotide-treated cells. In agreement with the colorimetric assay, ALP mRNA expression was downregulated in both the ATP and UTP-treated osteoblasts at all time points. Although the effects were less pronounced, COL1 expression was also slightly decreased in ATP and UTP-treated cells at days 7 and 14. Somewhat surprisingly, PC-1 mRNA levels were also decreased in both ATP and UTP-treated cells at 4, 7 and 14 days of culture.

Throughout the culture period, expression of ALP, COL1, OCN, OPN, MGP and PC-1 mRNA was unaffected by treatment with ADP or UDP (Figure 3.5B).
Figure 3.4. *Collagen formation appears unaffected by treatment with 10μM ATP or UTP*

These images, taken using light microscopy, illustrate the collagen formation in control and nucleotide-treated cultures and are representative of the staining observed in three different experiments (*n* = 6). The collagen fibres (purple/blue) were deposited in an organized fashion in all samples, with no perceptible differences in the volume of collagen. Because of mineralisation, the collagen fibres were less clear in the control images. In all images the scale bar = 10 μm
Figure 3.5. Treatment with 10 μM ATP or UTP inhibits the expression of ALP, PC-1 and OCN mRNA in rat osteoblasts; lack of effect of ADP or UDP.

Osteoblasts were cultured with ATP, UTP, ADP or UDP (10 μM) and ALP, OCN, OPN, MGP, COL1 and PC-1 mRNA expression studied after 4, 7 and 14 days of culture. In controls, the progression of osteoblast differentiation was illustrated by the increased OCN and ALP expression at 7 and 14 days. (A) Application of ATP or UTP did not affect OPN or MGP at any time point. OCN expression was unaffected at 4 and 7 days, but was downregulated in nucleotide treated cultures at 14 days. ALP, PC-1 and to a lesser degree COL-1 mRNA expression appeared consistently downregulated in the ATP and UTP-treated osteoblasts. (B) ADP and UDP did not affect the expression of any of the factors studied at any time point. Each PCR reaction was carried out in triplicate using cDNA from three separate experiments. The product sizes (in base pairs) for each gene amplified are: β-actin (332), ALP (238), OCN (418), OPN (333), MGP (297), COL1 (377) and PC-1 (309).
DISCUSSION

The investigation by Hoebertz et al (2002) described how low micromolar concentrations of ATP and UTP, inhibited bone formation by rat osteoblasts in vitro, a finding confirmed during the course of this research. Nevertheless, the mechanisms mediating this inhibition and the downstream signalling pathways activated following receptor stimulation have remained unclear. The work presented here has started to elucidate exactly how extracellular nucleotides act to prevent bone formation.

Osteoblasts were cultured with 1-100 μM nucleotide (ATP, UTP, ADP or UDP) before staining for mineralised nodules (alizarin red). In agreement with earlier findings, low micromolar concentrations of ATP and UTP significantly inhibited bone nodule formation, whereas ADP was inactive (Hoebertz et al, 2002). As shown in Chapter 2, mature rat osteoblasts express high levels of the P2Y₆ receptor; thus, the effect of UDP on bone formation was also investigated. At all concentrations tested, UDP failed to influence nodule formation suggesting no involvement of the P2Y₆ receptor in this process. Closer examination of stained osteoblast monolayers demonstrated that, despite a significant inhibition of mineralised nodule formation, both collagen formation and organic matrix deposition were relatively unaffected by treatment with ATP or UTP. Thus, the notion that nucleotides primarily block mineralisation was investigated further.

Mature osteoblasts express high levels of tissue non-specific ALP, an enzyme that hydrolyses phosphate-containing substrates to produce Pi necessary for mineralisation. ALP expression and activity is regulated by many local and systemic factors including localised acidosis (pH 6.90), another potent inhibitor of mineralisation in vitro (Brandao-Burch et al, 2005). Given the central role of ALP in mineralisation, the ability of extracellular nucleotides to influence its expression and activity was investigated colorimetrically and using RT-PCR. To ensure that medium acidification did not contribute to any of the effects observed, all experiments were controlled for pH (maintained at pH 7.40 throughout). Consistently, the P2Y₁ agonist, ADP, and the P2Y₆ agonist, UDP failed to influence ALP activity; conversely, ATP and UTP inhibited ALP activity by up to 65%. This ligand specificity correlates with observed effects on mineralised nodule formation. ATP and UTP are equipotent at rat P2Y₄
receptors, whereas at P2Y2 receptors UTP is a more potent agonist. Evidence suggests that the P2Y2 receptor subtype mediates the anti-osteogenic effects of nucleotides (see Chapter 2, Hoebertz et al, 2002) and in agreement, UTP consistently exerted a greater inhibitory effect on ALP activity. However, the lack of specific antagonists for the P2Y2 and P2Y4 receptors make it impossible to discount any involvement of the P2Y4 receptor. RT-PCR analysis demonstrated a striking decrease in ALP mRNA levels in osteoblasts treated with ATP or UTP, whereas application of ADP or UDP was without effect. Taken together, these findings suggest that stimulation of the P2Y2 receptor and possibly the P2Y4 receptor, leads to decreased ALP expression and activity, with the resulting decrease in Pi directly reducing the level of mineralisation.

The observation that ALP knockout mice still deposit bone mineral indicates that in vivo Pi can be generated from several sources (Johnson et al, 2000; Wennberg et al, 2000). In 10 μM ATP and UTP-treated cultures, up to a 90% reduction in mineralised nodule formation was observed; however, ALP activity was only inhibited by up to 65% and mRNA for the enzyme was still expressed, albeit at a low level. This suggests extracellular nucleotides also influence the expression and/or activity of other factors involved in mineralisation. PC-1 acts to suppress hydroxyapatite crystal formation and propagation by producing and regulating steady state levels of the inhibitory PPi. Thus, PC-1 is thought to act in opposition to ALP to regulate mineralisation (Johnson et al, 1999; Hessle et al, 2002). However, treatment with ATP or UTP decreased PC-1 mRNA expression in osteoblasts, whilst application of ADP or UDP was without effect; further work will be required to determine the reasons underlying this surprising observation.

Despite being the most abundant non collagenous protein in bone, the role of OCN in osteogenesis remains unclear, although evidence from in vivo (Ducy et al, 1996; Boskey et al, 1998) and in vitro (Hunter et al, 1996) studies suggest a role regulating mineralisation and matrix maturation. In this study, ATP and UTP decreased OCN mRNA expression in mature osteoblasts, whereas ADP and UDP were ineffective. This ligand specificity suggests, like ALP, the P2Y2 or P2Y4 receptor subtypes are mediating this inhibition, although how this decreased expression contributes towards the mineralisation block is unclear at present. OCN is a commonly used as marker of the mature osteoblast phenotype thus decreased mRNA expression could represent
impaired osteoblast differentiation in nucleotide-treated cultures. Both OPN and MGP act to inhibit mineralisation and consistently mRNA expression was unaffected by nucleotide treatment, indicating no involvement of these bone matrix proteins in the anti-osteogenic effects of extracellular nucleotides. Although no differences were observed in the histological staining, COL1 mRNA expression was slightly decreased in nucleotide treated cultures; these data suggest ATP/UTP may, in addition to inhibiting mineralisation, influence collagen formation in rat osteoblasts. Further work is now required to quantitatively measure collagen production, either by proline incorporation or by colorimetric assay, to determine the effects of extracellular nucleotides on organic matrix synthesis.

In contrast to the findings presented here, early investigations found ATP and other nucleoside triphosphates, at concentrations ≥ 0.2 mM, significantly increased Ca^{2+} deposition in bovine and rat matrix vesicle systems, an effect attributed to the actions of a specific ATPase rather than ALP (Hsu & Anderson, 1977; Hsu & Anderson, 1996). As nucleotides themselves can be hydrolysed to Pi, it is possible that short exposures to high ATP levels directly promote mineralisation by providing excess Pi for hydroxyapatite crystal formation. However, as this effect requires high micromolar or millimolar concentrations of ATP (which are unlikely to occur in vivo under normal conditions), this stimulatory action on mineralisation may not be physiologically relevant. Furthermore, ATP concentrations > 1mM induce smaller functional Ca^{2+} responses (Chapter 2) and may cause osteoblast apoptosis (Gartland et al, 2001). Thus, if such high levels do occur in vivo they are likely to be detrimental to bone cell function.

Information regarding the signalling cascades initiated following P2Y receptor activation is still limited. It is widely accepted that activation of P2Y_{1}, P2Y_{2}, P2Y_{4} and P2Y_{6} receptors leads to a rapid increase in intracellular Ca^{2+}, an effect observed here in primary rat osteoblasts (Chapter 2). Although ADP and UDP increase intracellular Ca^{2+}, they do not block bone formation or influence the expression of any of the factors studied here. Thus, it appears that increased intracellular Ca^{2+} is not the sole mediator of impaired mineralisation by ATP and UTP and factors further downstream confer the signalling specificity of individual P2 receptors. This study did not determine the effectors mediating the inhibition of ALP, OCN and PC-1 mRNA
expression. The promoters of both ALP and OCN genes contain consensus-binding sequences for a number of transcription factors including Runx2 (Ducy et al, 1996), Dlx5 (Kim et al, 2004) and c-fos (Ohta et al, 1992), thus there are several potential candidates for transcriptional regulation. Runx2 is commonly associated with stimulating the transcription of osteoblast marker genes and therefore inhibiting the expression and/or activation of this factor may well contribute to the decreased ALP and OCN expression observed here. However, a recent report (Costessi et al, 2005) found that ATP and UTP, signalling via P2Y2 receptors and the extracellular signal-related (ERK) cascade, enhanced the DNA binding activity of Runx2. Expression of common osteoblastic markers such as ALP and OCN was not investigated, although the authors reported increased expression of galectin-3, a protein implicated in cell proliferation. Moreover, this study employed a higher concentration of nucleotides (100 µM) and immortalised human osteoblast-like initial transfectant (HOBIT) cells. HOBIT cells do not express either P2Y4 or P2Y6 receptors, which, as shown here are highly expressed on primary rat osteoblasts (Chapter 2) and therefore these cells are inherently different. A previous study by the same authors also found that, 100 µM ATP, signalling via P2Y receptors and the transcription factor early growth response factor 1 (Egr1), acted to transiently increase COL1 expression (Pines et al, 2003). This finding is contradictory to those observed here and therefore it seems that HOBIT cells may behave in a different way to primary rat osteoblasts, thus it would be of interest to see if Runx2 expression and/or activity is affected by nucleotide stimulation in our in vitro system. ATP and ADP can stimulate, albeit weakly, the expression of c-fos in osteoblast-like cells (Bowler et al, 1999). As this effect is likely mediated by the P2Y1 receptor, activation of which did not influence OCN or ALP expression, c-fos seems an unlikely candidate for mediating the effects observed here. Although purinergic signalling has not been implicated in the induction of Dlx5 or its antagonistic factor Msx2, their ability to regulate osteoblastic gene expression makes them potential candidates for the effects observed here.

Taken together these data indicate that inhibition of ALP expression and activity mediates, at least in part, the mineralisation block observed in nucleotide-treated cultures. Moreover, RT-PCR analysis suggests that other factors such as PC-1 and OCN may also be involved, however, the nature of their participation remains unclear. Many other factors can also regulate mineralisation including annexins and MMPs.
Further work is now required to determine whether extracellular nucleotides influence the expression and/or activity of these factors.
CHAPTER 4

THE EFFECT OF ENVIRONMENTAL STRESSES ON OSTEOBLAST FUNCTION AND ATP RELEASE

INTRODUCTION

In vivo bone cells are exposed to numerous environmental stresses, including acidosis, hypoxia, hypothermia, hyperthermia and mechanical stimulation, all of which can influence their function and survival. Several groups have investigated the individual effects of these stimuli on cultured bone cells in vitro; however, in a physiological situation these events rarely occur in isolation. For example, the vascular disruption occurring in a localised tissue injury will cause a hypoxic microenvironment, the accompanying increase in anaerobic metabolism and the inflammatory response will result in a localised acidosis and hyperthermia, respectively.

In normal human tissues, the oxygen tension (pO₂) is between 5-12% and although relatively little accurate data exists regarding the pH of interstitial fluid, it is almost certainly lower than the pH 7.40 of arterial blood. Experimental limitations mean there are few studies into the pO₂ within the human skeleton; however, it has been reported that the oxygen tension in the bone marrow of healthy volunteers is 6.5% (Harrison et al, 2002) and in the synovial fluid of normal joints is 6-7% (Lund-Olesen, 1970). In diseased tissues, such as osteoarthritic joints, the pO₂ can fall to 1% or lower (Lewis et al, 1999).

Localised hypoxia occurs when blood supply is reduced or disrupted, and is associated with fracture, tissue damage, inflammation and tumours. Because of the vascular disruption, one of the most fundamental processes in wound healing is angiogenesis, a process that ensures the delivery of oxygen (O₂) and nutrients to the regenerating tissue. A number of cytokines are angiogenic, the most important being
vascular endothelial growth factor (VEGF). VEGF acts to stimulate the formation of new vessels whilst critically regulating the budding and growth of new capillaries from existing vascular structures. Osteoblasts constitutively produce VEGF, expression of which is markedly upregulated during hypoxia, possibly representing a basic chemostatic response to low oxygen (Steinbrech et al., 1999; Steinbrech et al., 2000; Akeno et al., 2001). In vitro exposure to hypoxia for up to 96 hours also increases the osteoblastic expression of other genes involved in fracture repair, including TGF-β, IGF-2 and collagen (I and III) (Steinbrech et al., 2000; Warren et al., 2001); conversely, expression of the osteoblast differentiation factor Runx2 is markedly decreased (Park et al., 2002). These findings suggest transient hypoxia after injury acts as a stimulus for extracellular matrix synthesis and fracture repair but more prolonged hypoxia will be detrimental to bone matrix formation.

Hypoxia also regulates osteoclast formation and activity. Using cells derived from mouse marrow, work performed in our laboratory demonstrated that culture in 2% O₂ caused a significant 3.5-fold and 9.5-fold increase in osteoclast formation and resorption, respectively (please see paper in Appendix II by Arnett et al. 2003).

Core body temperature in humans varies between 36.4-37.1°C, depending on location, for example a healthy knee joint has a reported temperature of 36.45-36.58°C (Kenny et al., 2002). Normally, body temperature is carefully maintained as even small deviations can affect cellular metabolism by influencing reaction kinetics, protein and nucleic acid stability and lipid bilayer properties. Nevertheless, changes in body temperature occur during many pathological situations. Inflammation and infection increase both local and systemic temperatures and these events at sites close to or within bone surfaces have long been associated with bone loss. Hyperthyroidism is characterised by an increased metabolic rate and heat production (or thermogenesis) as well as accelerated bone remodelling, increased bone loss and fracture risk (Lakatos, 2003). In contrast, hypothyroidism is associated with decreased body temperature, delayed bone formation and short stature. Ageing is associated with attenuated vasoconstrictor responses to cold stress and decreased thermogenesis; this inhibits heat retention and can lead to a lower core body temperature (Kenney et al., 2003). At present, whether these changes in ambient temperature directly affect the skeleton in vivo remains to be determined.
Previous work has demonstrated that prolonged heat or cold can have a striking effect on bone cell function; an extreme example of this occurs in frostbite when ice crystal formation in the extracellular fluid and direct vascular endothelial injury cause marked cellular damage leading to bone necrosis, particularly in the hand and foot (Leonard et al, 2001). In vitro work indicated osteoblast-like cells are thermosensitive, as culture at 40°C decreases DNA synthesis and proliferation (Flour et al, 1992; Shui & Scutt, 2001). Even transient exposure (10 minutes) to severe hyperthermia (48°C) causes disruption of the osteoblast cytoskeleton and increased apoptosis and necrosis. Moreover, Hsp70 levels were increased dramatically at 45°C and 48°C, indicating the activation of protection mechanisms; however, at 48°C given the increased osteoblast apoptosis these mechanisms appear to be ineffective (Li et al, 1999). Hypothermia is also detrimental to bone cell function as osteoblasts cultured at 33°C for 96 hours exhibited decreased DNA synthesis and mineralisation (Shui & Scutt, 2001). Recent work in our own lab, using mononuclear cells derived from human peripheral blood, has shown that culture at 40°C causes a 4-fold decrease in osteoclast formation and 10-fold decrease in resorption per osteoclast. Contrastingly, 34°C caused a 2-fold increase in osteoclast formation but was accompanied by a 30% decrease in resorption (Utting et al, 2005).

ATP is a ubiquitous intracellular energy molecule, found within the cytoplasm at concentrations between 2-5 mM (Hoebertz et al, 2003). Consequently, following membrane damage or necrosis, all cells can potentially release ATP into the extracellular environment and influence local purinergic signalling. Numerous excitatory and non-excitatory cells including neurons (White, 1984), platelets (Beigi et al, 1999), epithelial and endothelial cells (Bodin & Burnstock, 2001a; Knight et al, 2002), fibroblasts (Gerasimovskaya et al, 2002), astrocytes (Coco et al, 2003), chondrocytes (Graff et al, 2000), and erythrocytes (Sprague et al, 1998) release ATP in a controlled manner. In many cell types, ATP release is enhanced by stressful stimuli including hypoxia (Bergfeld & Forrester, 1992; Bodin & Burnstock, 1995; Gerasimovskaya et al, 2002), osmotic shock, fluid shear stress (Bodin & Burnstock, 2001a; Genetos et al, 2005) and mechanical stimulation (Romanello et al, 2001).

The exact mechanisms mediating constitutive ATP release remain unclear but differences between cell types are emerging. Current theories involve: (1) ATP
binding cassette (ABC) transporters, (2) vesicular exocytosis, and (3) gap junctions and hemichannels. Extensive investigation of the transmembranous ABC proteins has yielded several potential candidates for mediating ATP release, including the cystic fibrosis transmembrane conductance regulator (CFTR) and the sulfonylurea receptor (SUR). Functionally, these proteins regulate ion channel activity; for example, CFTR is an ATP-dependant, cAMP-activated Cl channel, which also regulates K+ channels. The CFTR protein has been implicated in ATP release from several cell types including erythrocytes (Sprague et al, 1998; Braunstein et al, 2001). Controlled vesicular exocytosis is implicated in ATP release from epithelial and endothelial cells (Bodin & Burnstock, 1998; Knight et al, 2002). Finally, connexin hemichannels and gap junctions, which allow the movement of molecules less than 1 Kda, reportedly act as conductive pathway to mediate ATP release from astrocytes (Cotrina et al, 1998; Coco et al, 2003). For a detailed review on ATP release mechanisms, see Lazarowski et al, 2003.

Constitutive ATP release has been reported from various modified osteoblast-like cell lines including: SaOS-2 cells (Buckley et al, 2003), human osteoblast-like initial transfectant (HOBIT) cells (Romanello et al, 2001; Romanello et al, 2005) and MC3T3-E1 cells (Genetos et al, 2005). Furthermore, both mechanical stimulation and fluid shear stress are reported to enhance this basal ATP release (Romanello et al, 2001; Genetos et al, 2005).

ATP release from primary osteoblasts has not previously been investigated; therefore, to obtain results that maximise physiological relevance, the research presented here utilised normal, bone forming osteoblasts derived from neonatal rat calvaria. The work in this chapter falls into three main areas: (1) the properties and mechanisms of constitutive ATP release from osteoblasts, (2) the effect of transient exposure to low oxygen, hypothermia or hyperthermia on ATP release, and (3) the effects of continuous culture under these conditions on osteoblast function and ATP release.
MATERIALS AND METHODS

Cell culture

Primary rat osteoblastic cells were isolated as described previously and cultured for up to 24 days in standard 6-well trays. To determine the effect of long-term culture at low O₂ plates were transferred to gas-tight fuse ("hypoxia") boxes 24 hours after subculture and gassed daily for 90 seconds with the appropriate O₂ (2% or 20% O₂, 5% CO₂; balance N₂) concentration. Cylinders containing custom mixtures of O₂, CO₂ and N₂ were purchased from BOC (London, UK). To examine the effects of temperature change on osteoblast function, plates were transferred to humidified incubators running at 34°C or 40°C (5% CO₂-95% air) 24 hours post-seeding. Medium pH, pO₂ and pCO₂ were monitored throughout, using a blood gas analyser as described previously.

Quantification of ATP release

To measure ATP release, culture medium was removed, cell layers were washed with PBS, serum free DMEM added (1ml/well) and plates incubated at 5%CO₂-95% air, 37°C for 30-60 minutes prior to sample collection (n = 12). To investigate release mechanisms, several agents (monensin, brefeldin A, n-ethylmaleimide (NEM) and gadolinium (Gd³⁺) at 1-1000 μM) were added to the serum free DMEM and the effects measured. Previous reports implicated increased intracellular Ca²⁺ in ATP release, so the calcium ionophore ionomycin was also tested (0.1-10 μM) for potential effects. In all cases, samples were frozen immediately after collection for later ATP quantification.

To examine the affects of long-term hypoxia on ATP release, plates containing osteoblasts were positioned into gas-tight "hypoxia" boxes 24 hours after seeding and gassed daily for 90 seconds with 20% or 2% O₂. Cell layers were prepared for ATP determination as described above before regassing to ensure the correct O₂ concentration. To determine the effect of transient hypoxia on ATP release test plates minus lids were sealed in hypoxia chambers and exposed to 20% or 2% O₂ for between 30 seconds and 11.5 minutes. A similar method was employed to study the effects of transient and continuous hypothermia and hyperthermia on ATP release. Finally, in all cases osteoblast monolayers were fixed and stained as described.
previously to demonstrate the presence of mature osteoblasts and mineralised bone nodules.

**Luciferin-luciferase assay**

ATP release is measured luminetrically using the *luciferin-luciferase* assay, which exploits the ability of firefly luciferase to catalyse its substrate D-luciferin to oxyluciferin and light, in the presence of ATP. The equation for this reaction is shown below:

\[
\text{ATP + D-luciferin} \rightarrow \text{AMP + PPi + oxyluciferin + CO}_2 + \text{light}
\]

ATP standards (10 pM to 1 μM) and test samples (50 μl) were pipetted into a white (nonphosphorescent) 96-well plate (Nunc, Fisher Scientific, UK) and sited in the luminometer (Lucy 1, Anthos Labtec, Salzburg, Austria). The luciferin-luciferase reagent (100 μl) was automatically injected into each well and measured for 10 seconds at ~ 560 nm. All standards, blanks and the lyophilised ATP reagent containing D-luciferin and luciferase were dissolved in serum free DMEM. A calibration curve was constructed using the ATP standards and used to calculate ATP levels in test samples.

**Assessment of plasma membrane integrity**

Cell viability at 3, 7 and 14 days of culture was investigated using the Promega CytoTox 96® non-radioactive cytotoxicity assay. This assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released on cell lysis. LDH oxidises lactate into pyruvate, generating NADH, which is then used to convert a tetrazolium salt into a red formazan product. The amount of colour formed is proportional to the number of lysed cells.

Culture medium was removed, serum free DMEM added (1ml/well) and plates incubated for 1 hour at 37°C before sample collection (*n* = 12); all test samples were kept at 4°C until assaying. To establish total cellular LDH levels, cell layers, bathed in serum-free DMEM, were harvested using a scraper followed by sonication at 4°C and centrifugation at 500 x g. The LDH content of the supernatants and cell lysates were
measured colorimetrically (490 nm) (Elx800 plate reader, Bio-tek International, Fisher Scientific UK, Loughborough) as per manufacturer’s instructions. By expressing released LDH as a percentage of the total cellular LDH cell viability could be calculated.

**Proliferation, alkaline phosphatase (ALP) and bone nodule formation assays**

Osteoblast proliferation, ALP and bone nodule formation were quantified using the methods described previously. To rule out direct effects of temperature or oxygen tension on reaction kinetics, all assays were carried out at 37°C or atmospheric O₂. To study the effects of continuous culture at low O₂, hypothermia or hyperthermia on osteoblast function, cells were cultured for 15-24 days, depending on the onset of mineralisation, which varied between cultures.

**Reagents**

All tissue culture reagents were purchased from Gibco (Paisley, UK). Unless mentioned all chemicals were purchased from Sigma-Aldrich (Dorset, UK) and dissolved in distilled water with the exception of monensin and Brefeldin A, which were dissolved in ethanol and methanol, respectively. The CytoTox 96® Non-radioactive cytotoxicity assay was obtained from Promega UK (Southampton, UK) and ATP monitoring reagent from Biothema (Handen, Sweden).

**Statistical analysis**

All data was analysed as described in Chapter 2.
RESULTS

Replacement of medium stimulates ATP release

The foetal calf serum (FCS) in normal growth medium contains factors inhibitory to the luciferin-luciferase assay; consequently, it was necessary to bathe cells in serum-free DMEM for the duration of the experiment. As the absence of serum is inherently stressful to osteoblasts, time in this medium was kept to a minimum. Shear stress is a known stimulator of ATP release; therefore, it is highly likely the mechanical disturbances arising during medium exchange exert a similar effect. Medium change was found to have a dramatic effect on ATP release; within 30 seconds ATP levels had increased approximately 3-fold (Figure 4.1A). The ATP levels remained significantly elevated for up to 5 minutes post medium exchange returning to basal levels within 20 minutes. Following this observation, in all subsequent ATP release experiments, plates were left for 30-60 minutes prior to sample collection, to enable complete degradation of any ATP released because of mechanical perturbations.

Constitutive ATP release increases with osteoblast differentiation

Using primary bone forming rat osteoblasts, ATP release and cell proliferation were measured throughout the culture period, allowing expression of data as ATP release per osteoblast. Compared to 4 days, ATP release per osteoblast was significantly increased by up to 7-fold at 8, 11 and 15 days of culture, with the maximum level of release occurring at 11 days (Figure 4.1B). A quantitative ALP assay illustrated the progression of osteoblast differentiation in these cultures and between 4-18 days enzyme activity increased up to 12-fold (Figure 4.1B). Measurement of osteoblast viability consistently showed no significant changes in the level of cell lysis throughout the culture period.
Figure 4.1. **Constitutive ATP release from primary rat osteoblasts increases with differentiation and is stimulated by medium exchange**

(A) Medium exchange \((t = 0)\) induced a rapid 3-fold increase in ATP release from osteoblasts; levels returned to basal within 20 minutes \((n = 12)\). **(B)** ATP release per osteoblast increased up to 7-fold at 8, 11 and 15 days of culture compared to day 4 \((n = 12)\). This indicates that osteoblast differentiation may influence ATP release, an idea confirmed by the concurrent increase in ALP activity \((** = p<0.001, * * = p<0.01, * = p<0.05)\). These data are representative of the results obtained from three separate experiments.
Constitutive ATP release from osteoblasts involves vesicular exocytosis

Previous reports indicate basal ATP release from osteoblast-like cells occurs, at least in part, by vesicular mechanisms (Genetos et al., 2005; Romanello et al., 2005); to establish whether this is the case in primary rat osteoblasts the exocytosis inhibitors monensin, NEM and brefeldin A (1–1000 µM) were tested. When compared to untreated controls, addition of 1, 10 or 100 µM monensin to culture medium significantly decreased the level of ATP detected by between 40–50%. At concentrations of 100 µM and 1000 µM NEM also reduced ATP release by 90% and 99%, respectively. Although 1 µM and 10 µM NEM elicited a small (~15%) decrease in ATP release these values were not statistically significant. Brefeldin A caused an 18–30% decrease in ATP release; however, at all concentrations these inhibitory effects were non-significant (Figure 4.2A).

In other cell types, increased intracellular Ca\(^{2+}\) is thought to mediate the release of ATP from intracellular stores (Knight et al., 2002). The trivalent lanthanide gadolinium (Gd\(^{3+}\)) inhibits several stretch-activated ion channels including the mechanosensitive selective cation channel (MSCC); this channel, which is expressed on osteoblasts, facilitates rapid Ca\(^{2+}\) entry into the cell and is implicated in increased intracellular Ca\(^{2+}\) in response to shear stress. Cells were treated with a range of Gd\(^{3+}\) concentrations (1–100 µM), but even at the highest levels, this agent had no effect on constitutive ATP release (Figure 4.2B).

The Ca\(^{2+}\) ionophore, ionomycin, acts to increase intracellular Ca\(^{2+}\) by decreasing plasma membrane integrity. When tested, all concentrations (0.1–10 µM) of ionomycin caused enhanced ATP release with 1-10µM causing a significant up to ~2-fold increase (Figure 4.2).
Figure 4.2. ATP release from osteoblasts is mediated by vesicular exocytosis.

(A) Vesicular exocytosis inhibitors 1–100 μM Monensin and 100 μM NEM significantly blocked ATP release from osteoblasts by 50% and 90% respectively, whereas brefeldin A was without significant effect (n = 12). (B) The MSCC channel blocker Gd³⁺ did not affect constitutive ATP release from osteoblasts (n = 12). (C) The Ca²⁺ ionophore ionomycin increased ATP release by up to ~ 2-fold (n = 12) (*** = p<0.001, ** = p<0.01, * = p<0.05). All inhibitor studies were performed a minimum of three times.
**Continuous hypoxia inhibits osteoblast proliferation and function, and reduces cell viability**

The effect of continuous culture in low oxygen (2% O₂) on osteoblast proliferation and viability was quantified at regular intervals over a 14-day period. In control cultures (20% O₂), cell number increased progressively between day 4 and day 14; in contrast, in 2% O₂ osteoblast proliferation appeared to arrest around day 11 with culture beyond this point associated with a slight reduction in cell number (Figure 4.3A). Osteoblast numbers were consistently lower in 2% O₂, increasing by only ~20% over the 14 day culture period; concurrently cell number increased by approximately 100% in control cultures. At 11-14 days osteoblast numbers were significantly reduced by up to 52% in 2% O₂ compared to 20% O₂. Using the LDH cytotoxicity assay, the medium of hypoxic cultures was found to contain higher levels of cellular LDH (up to 3-fold) compared to controls, indicating continuous culture at low oxygen decreases osteoblast viability (Figure 4.3B). Limitations of this particular assay mean it is not possible to determine whether the decreased viability in 2% O₂ occurs because of increased necrosis, apoptosis or a combination of the two. LDH levels in the 20% O₂ controls exhibited no significant variation, indicating osteoblast viability remains relatively unchanged throughout the culture period.

Following culture for 21 days, osteoblast cell layers were fixed, stained with alizarin red and mineralised bone nodule formation was quantified. Repeated experiments demonstrated a marked inhibition (up to 90% decrease) of bone nodule formation in the 2% cultures, compared to 20% controls (Figure 4.3C). Osteoblasts cultured under normoxia displayed abundant nodule formation with structures exhibiting the typical trabecular morphology seen in vitro (Figure 4.4A & 4.4C). In contrast, the 2% O₂ cultures exhibited significantly fewer nodules, and those that were formed appeared small and rounded (Figure 4.4B & 4.4D).
Figure 4.3. Long-term hypoxia inhibits osteoblast proliferation, viability and function

(A) Continuous culture in 2% $O_2$ impairs osteoblast proliferation leading to a 50% decrease in cell number at 11 and 14 days ($n = 6$). (B) Levels of cellular LDH in the culture medium were increased up to 3-fold in 2% $O_2$ compared to 20% controls, indicating a reduced osteoblast viability following prolonged exposure to hypoxia ($n = 6$). (C) Long-term culture in 2% $O_2$ caused up to 90% inhibition of mineralised bone nodule formation ($n = 6$) (** = $p<0.01$, *** = $p<0.001$). All experiments into the effect of hypoxia on osteoblast function were performed at least three times.
Figure 4.4. *Hypoxia inhibits bone nodule formation by rat osteoblasts*

(A & B) Representative scanned images of osteoblast monolayers following prolonged culture in 20% (A) and 2% (B) O₂ in a standard 6-well tray. Osteoblasts cultured in 2% O₂ form significantly fewer bone nodules than the 20% controls. (C) This image represents the typical trabecular morphology of bone nodules formed *in vitro* by osteoblasts cultured in 20% O₂. (D) In contrast, the bone nodules formed in hypoxic cultures are fewer, smaller and rounded in appearance. Images C & D were taken using phase contrast light microscopy with a digital camera attachment. Scale bars in images A & B = 0.5 cm, in images C & D = 50 μm.
Transient exposure to hypoxia stimulates ATP release from osteoblasts without affecting cell viability

Hypoxia is a known stimulator of ATP release from numerous cell types, including endothelial cells (Bodin et al., 1995), fibroblasts (Gerasimovskaya et al., 2002) and erythrocytes (Bergfeld et al., 1992). Primary rat osteoblasts, cultured until the initiation of bone nodule formation (10-14 days), were exposed to 20% or 2% O₂ for 30 seconds to 11.5 minutes. Exposure to 2% O₂ for up to 90 seconds caused a rapid significant increase in ATP release; after 30 seconds and 90 seconds, ATP levels were ~ 2.5-fold and 2-fold higher, respectively (Figure 4.5A). Exposure to hypoxia for periods in excess 2 minutes resulted in smaller but insignificant increases in ATP release. Repeated experiments demonstrated the length of elevated ATP release varied between cultures, however, generally it did not exceed 3 minutes. For all control samples gassed with 20% O₂, the level of ATP release did not vary greatly; indicating the movement of gas over the culture medium was not stimulating the increased release. Levels of cellular LDH in the culture medium were measured to determine whether transient hypoxia caused increased cell lysis, therefore accounting for the increased ATP levels detected. Repeatedly, transient hypoxia did not influence the amount of LDH in the medium (Figure 4.5B).

Hypoxia induced ATP release involves vesicular exocytosis

To establish whether hypoxia-induced ATP release occurred via a vesicular mechanism, cells were incubated in serum free DMEM containing either NEM or monensin (100 μM) before transient exposure to 20% or 2% O₂. In untreated cells, 2% O₂ induced a significant 2-fold increase in ATP secretion compared to 20% O₂ controls. Treatment with monensin and NEM decreased ATP release from control samples by 40% and 65%, respectively (Figure 4.6). Hypoxia-induced ATP release was also inhibited by these agents, being decreased by 62% and 80% following application of monensin and NEM, respectively (Figure 4.6).
Figure 4.5. Transient hypoxia stimulates ATP release from osteoblasts without affecting cell viability

(A) Transient exposure to 2% O₂ for 30-90 seconds caused a rapid increase in ATP release from osteoblasts (2.5-fold) (n = 12). (B) Medium LDH levels were unchanged in 2% O₂ cultures compared to controls suggesting hypoxia induced ATP release was not caused by increased cell lysis (n = 12) (** = p<0.001, * = p<0.05). These data are representative of the results from three separate experiments.
Figure 4.6. *Hypoxia-induced ATP release occurs via vesicular exocytosis*

Exposure to 2% O\textsubscript{2} for 30 seconds caused a 2-fold increase in ATP release from osteoblasts (# = p<0.05). Addition of monensin and NEM to the culture medium (100 µM) significantly inhibited both hypoxia-induced and constitutive ATP release (n = 12) from primary rat osteoblasts by up to 80% (*** = p<0.001, ** = p<0.01, * = p<0.05). All experiments were performed in triplicate.
Continuous hypoxia decreases total ATP release from osteoblasts during the early stages of culture

Since long-term culture in low oxygen dramatically impairs osteoblast proliferation and function, basal ATP release was quantified after 8 and 15 days from cells cultured in 20% or 2% O₂. At day 8 of culture, total ATP levels were significantly lower at 2% O₂ (67% decrease) but by day 15 although lower at 2% O₂ (30% decrease) the level of total ATP release was not significantly different (Figure 4.7A). Consistent with earlier observations, osteoblast proliferation was impaired in the 2% O₂ cultures and therefore ATP secretion was normalised for cell number. ATP release per osteoblast, although lower was not significantly different at 2% O₂ compared to 20% controls (Figure 4.7B).

Figure 4.7. Chronic hypoxia does not influence the level of ATP release per osteoblast.
(A) Total ATP release from osteoblasts cultured for 8 days in 2% O₂ (n = 12) was decreased by ~ 67%, but following 15 days of culture although reduced (30%) the level of total ATP release did not vary significantly. (B) When normalised for cell number the level of ATP release per osteoblast in 2% O₂ did not vary significantly from 20% controls (** = p<0.01). These data are representative of at least three separate experiments.
Continuous culture in hypothermic or hyperthermic conditions inhibits osteoblast function.

Osteoblasts were cultured for up to 18 days to determine the effects of hypothermia (34°C) and hyperthermia (40°C) on cell proliferation (measured every 3 days). In 34°C cultures, a significant reduction in osteoblast number was observed at most time points; cell number being decreased by up to 33% (Figure 4.8A). Chronic exposure to 40°C also reduced osteoblast proliferation by up to 25% (Figure 4.8B). At both 34°C and 40°C, a greater inhibitory affect was observed at the early stages of culture (4-7 days). Osteoblast proliferation, at all three temperatures, followed a typical ‘S’ shaped curve, with a log phase of steady growth followed by a stationary phase, characterised by virtually no proliferation. However, in the 34°C and 40°C cultures, this growth curve was shifted to the right, reflecting the slower rate of cell proliferation in hypothermia or hyperthermia.

At the end of the experiments, osteoblast monolayers were fixed, stained with alizarin red and mineralised nodule formation quantified. Continuous exposure to both 34°C and 40°C caused a striking inhibition of osteoblast activity, with a 95% and 75% reduction in bone nodule formation observed, respectively (Figure 4.8C). Prolonged hypothermia reproducibly, exerted a greater inhibitory effect on osteoblast function than hyperthermia. The images in Figure 4.9 were acquired using a high-resolution scanner (Epson Perfection Photo, 3200) and by light microscopy with digital camera attachment. They are representative of the mineralised bone nodules formed in 34°C, 37°C and 40°C following 21 days in culture. At both 34°C and 40°C, there are significantly fewer bone nodules and those formed do not display the typical curved morphology observed at 37°C. The nodules formed in hypothermic or hyperthermic temperatures are similar to those observed in hypoxic cultures (Figure 4.4), being small and rounded in appearance.
Figure 4.8. Prolonged exposure to hypothermia or hyperthermia inhibits osteoblast proliferation and function

(A) Long-term culture (up to 18 days) at 34°C inhibited osteoblast proliferation by up to 33% ($n = 6$). (B) Continuous culture in hyperthermic temperatures (40°C) reduced osteoblast number by up to 25% ($n = 6$). (C) Prolonged exposure to 34°C or 40°C significantly impaired osteoblast function, inhibiting bone nodule formation by 95% and 75%, respectively ($n = 6$) (*** = $p<0.001$, ** = $p<0.01$, * = $p<0.05$). All experiments into the effect of temperature on osteoblast proliferation and function were performed in triplicate.
Figure 4.9. *Prolonged hypothermia or hyperthermia inhibits bone nodule formation by rat osteoblasts*

Representative images of bone nodules formed by primary rat osteoblasts cultured at 34°C, 37°C or 40°C for 21 days. The low power images on the left were acquired using a high-resolution scanner. Osteoblasts cultured at 37°C form bone nodules displaying the typical trabecular morphology shown in the higher power image on the right (taken using phase contrast light microscopy). Bone formation was significantly reduced at both 34°C and 40°C, and those nodules that were formed appear considerably smaller and rounded in appearance. Scale bars: low power images = 0.5 cm, high power images = 50 μm.
Transient hyperthermia stimulates ATP release from osteoblasts without affecting cell viability

Figure 4.5 shows how transient hypoxia stimulates ATP release from osteoblasts; in vivo localised hypoxia is likely to occur in association with inflammation, consequently the effects of temperature on ATP release were also investigated. At the onset of bone formation (10-14 days of culture at 37°C), osteoblasts were exposed to 34°C or 40°C for 1-120 minutes and the level of ATP release compared to 37°C controls. Consistently, decreasing the temperature to 34°C had no effect on ATP release from osteoblasts (Figure 4.10A). In contrast, increasing the ambient temperature to 40°C for 30, 60 or 120 minutes caused a significant 2-fold increase in the level of ATP release (Figure 4.10B and 4.10C). Exposure to 40°C for periods shorter than 30 minutes did not have any stimulatory effects on ATP release. Assessment of cell viability using the LDH cytotoxicity assay demonstrated that exposure to hyperthermia for up to 120 minutes did not affect the level of cellular LDH detected in the medium (Figure 4.10C). Therefore, transient increases in temperature do not induce membrane damage and instead, the enhanced ATP release is likely to occur via controlled mechanisms.

Long-term culture at 34°C or 40°C decreases constitutive ATP release

After 7 and 14 days of continuous culture in 34°C, 37°C or 40°C, medium samples were collected to determine the effect of prolonged hypothermia and hyperthermia on constitutive ATP release from osteoblasts. Culture at 34°C or 40°C significantly impaired ATP secretion from osteoblasts, levels being decreased by 35-40% in 34°C and 60-70% in 40°C (Figure 4.11). Prolonged hyperthermia reproducibly inhibited constitutive ATP release per osteoblast to a greater extent than hypothermia. Taken together these data show that long-term culture at 34°C or 40°C has a marked negative effect on constitutive ATP release from osteoblasts.
Figure 4.10. *Transient hyperthermia stimulates ATP release from osteoblasts without affecting cell viability*

(A) Incubation at 34°C for 20-60 minutes had no effect on ATP release (n = 12). (B) Exposure to 40°C for 30-60 minutes increased ATP release from osteoblasts up to 2-fold (n = 12). (C) Transient hyperthermia (t = 120 minutes) enhanced ATP release approximately 2.5-fold without affecting cell viability (n = 12) (*** = p<0.001, ** = p<0.01). These data are representative of those observed in three separate experiments.
Figure 4.11. Chronic hypothermia or hyperthermia inhibits ATP release from osteoblasts.

After 7 and 14 days of culture, medium samples were collected for ATP quantification via the luciferin-luciferase assay (n = 12). Results were normalised for cell number and compared to 37°C controls; continuous culture at 34°C or 40°C significantly reduced the amount of ATP released per osteoblast by up to 40% and 70% respectively (**p<0.001, ***p<0.01). All experiments were performed in triplicate.
DISCUSSION

This study aimed to investigate constitutive ATP release from osteoblasts and how it is affected by the stresses hypoxia, hypothermia and hyperthermia (both chronic and acute). Earlier work examined the consequences of relatively short hypoxic exposures on osteoblast function (up to 96 hours) and found they exerted a striking effect on proliferation, differentiation and gene expression (Steinbrech et al., 1999; Steinbrech et al., 2000; Park et al., 2002). Likewise, short-term culture (96 hours) at 33°C and 40°C significantly impaired proliferation in osteoblast-like cells (Flour et al., 1992; Shui & Scutt, 2001). However, the effects of chronic exposure to low oxygen, hypothermia or hyperthermia on osteoblast function and ATP release remained poorly investigated.

In this study, prolonged hypoxia exerted a marked effect on proliferation with up to a 52% reduction in cell number observed. Chronic hypothermia or hyperthermia were also inhibitory decreasing osteoblast number by up to 33%. In control (37°C or 20% O₂) cultures, osteoblast proliferation followed the usual in vitro growth kinetics with a log period of steady growth followed by a stationary phase, characterised by low proliferation. Culture at 34°C or 40°C shifted the growth curve to the right, indicating that prolonged hypothermia or hyperthermia retard osteoblast proliferation. In contrast, osteoblasts cultured for up to 14 days in 2% O₂ exhibited only minimal changes in cell numbers, suggesting that long-term oxygen deprivation arrests proliferation. Moreover, prolonged hypoxia significantly affected osteoblast viability, particularly at the later stages of culture, although whether this was caused by increased necrosis, apoptosis and/or decreased membrane integrity remains to be determined. Conversely, the effect of long-term hypothermia or hyperthermia on osteoblast viability provided inconsistent results.

Quantification of mineralised nodule formation demonstrated the effect of hypoxia, hypothermia and hyperthermia on osteoblast function in vitro. Prolonged exposure to 2% O₂ reproducibly inhibited mineralised matrix formation by up to 90%. Bone formation is dependent on the correct synthesis and deposition of collagen; recent data from our group demonstrated impaired collagen formation at 2% O₂ (Utting et al., submitted). Several post-translational modifications are required for the formation of mature collagen including the hydroxylation of proline residues by prolyl-4-
hydroylase, a reaction that enables the formation of stabilising intrastrand hydrogen
bonds. Inhibition of this reaction prevents the formation of a stable triple helix and
produces collagen that is more susceptible to proteolytic degradation and denatures at
lower temperatures (Fessler & Fessler, 1974). Prolyl-4-hydroxylase, is oxygen
dependent; therefore, impaired proline hydroxylation in hypoxia may explain the
impaired collagen and bone nodule formation observed here in vitro.

Continuous culture at 34°C and 40°C inhibited mineralised bone nodule formation
by 95% and 75%, respectively. Ambient temperature may also effect collagen
formation as early studies demonstrated that the thermal stability of procollagen is
proportional to the hydroxyproline content, with low temperatures (20°C-30°C) being
associated with a marked reduction in proline hydroxylation reactions (Jimenez et al,
1974). Moreover, reports suggest fully hydroxylated procollagen denatures at 37.9°C
(Rosenbloom et al, 1973), preventing the formation of stable, mature collagen at
higher temperatures. These observations suggest that the impaired nodule formation
observed here in vitro at 34°C and 40°C may occur because of disrupted collagen
production. In agreement, unlike the noticeable mineralisation block seen in the
nucleotide-treated cultures (Chapter 3), unmineralised matrix was not apparent in the
osteoblast monolayers cultured at 34°C or 40°C.

This investigation has provided the first evidence for constitutive ATP release from
primary rat osteoblasts. Earlier reports demonstrated ATP release from modified
osteoblast-like MC3T3-E1 (Genetos et al, 2005) and SaOs-2 cells (Buckley et al,
2003) in the nanomolar range, however, this study only detected release in the
picomolar range. The transformed MCT3T3-E1 cell line is derived from murine
neonatal calvaria and expresses many characteristics of the mature osteoblast
phenotype including bone formation in vitro. In contrast, SaOS-2 cells are derived
from human osteosarcomas and, despite producing mineralised matrix in vitro, lack
some characteristics of the mature osteoblast phenotype. The inherent differences
between primary cells and these immortalised osteoblast-like cell lines, combined with
the different species of origin may explain the lower levels of ATP release detected
here. Constitutive ATP release from rat osteoblasts changed with time, increasing up
to 7-fold in the second week of culture; this was accompanied by increased ALP
activity, confirming the progression of osteoblast maturation. Over the same period
cell viability was unchanged indicating that increased cell lysis is unlikely to contribute towards the elevated levels. Combined these data suggest that cellular differentiation influences the basal level of ATP release and potentially local purinergic signalling.

At present, the exact processes mediating controlled ATP release remain unclear but there are several different hypotheses. The transmembranous ABC proteins, including CFTR and SUR, bind and hydrolyse ATP to provide energy for the active translocation of substrates including inorganic ions and amino acids. Currently, there is evidence for the involvement of ABC proteins, particularly CFTR, in both basal and stimulated ATP release from erythrocytes and fibroblasts (Pasyk & Foskett, 1997; Sprague et al, 1998; Braunstein et al, 2001). However, the exact mechanisms involved require further definition and may involve the modulation of a separate ATP permeable channel. The involvement of these channels in ATP efflux was not studied here but current evidence indicates that ABC proteins do not mediate ATP release from osteoblast-like cells (Romanello et al, 2001).

In other cell types, including astrocytes ( Cotrina et al, 1998), gap junctions and hemichannels have been implicated in ATP secretion, however, current evidence suggests they are unlikely to mediate ATP release from osteoblasts. HOBIT cells engineered to over-express a major component of the gap junction, connexin 43, displayed no differences in either basal or stimulated ATP release compared to controls (Romanello et al, 2001). Additionally, gap junction inhibition failed to effect fluid shear stress induced ATP release from osteoblasts (Genetos et al, 2005).

Purinergic neurotransmission is mediated by the controlled release of ATP-filled synaptic vesicles, making vesicular exocytosis a major ATP release mechanism in neuronal cells (Bodin & Burnstock, 2001b). Certain non-neuronal cells, including epithelial and endothelial cells (Bodin & Burnstock, 2001a; Knight et al, 2002) and mechanically stimulated osteoblast-like cells (Genetos et al, 2005; Romanello et al, 2005) have also been shown to release ATP via vesicular mechanisms. The inhibitors monensin, brefeldin A and NEM disrupt different components of the vesicular exocytosis pathway and thus are widely utilised in the study of ATP release mechanisms. Monensin inhibits vesicular formation at the golgi apparatus and whilst concentrations of 100 μM inhibit ATP secretion in other systems (Knight et al, 2002),
in this study, concentrations of ≥ 1 μM blocked ATP release from osteoblasts. By interfering with vesicle-associated NSF proteins, NEM inhibits vesicular fusion with the plasma membrane and it inhibits ATP secretion at concentrations between 1-10 μM in some systems but 100 μM in others (Bodin & Burnstock, 2001a; Genetos et al, 2005). In this investigation, 100–1000 μM NEM almost completely blocked ATP release, whereas lower concentrations were ineffective. Brefeldin A disrupts vesicular trafficking by blocking protein transport from the endoplasmic reticulum to the Golgi apparatus. Although inhibitory at 10 μM in other cell types, brefeldin A did not significantly affect ATP release from osteoblasts.

Gadolinium (Gd³⁺) is a potent blocker of mechanosensitive ion channels, including the MSCC channel present on osteoblasts; these channels, following activation by membrane stretch and mechanical perturbations, facilitate Ca²⁺ influx and have been implicated in ATP release from cells including hepatocytes (Roman et al, 1999). A previous study reported concentrations of 10 μM Gd³⁺ as sufficient to block cation channels (Caldwell et al, 1998). Here, 1-100 μM Gd³⁺ failed to affect constitutive ATP release, suggesting that mechanosensitive ion channels, including MSCC, do not mediate the increase in intracellular Ca²⁺ required for ATP secretion under normal conditions. This is in agreement with a report by Genetos et al (2005), which found Gd³⁺ failed to affect basal or shear stress stimulated ATP release from osteoblast-like cells. The Ca²⁺ ionophore, ionomycin, significantly increased constitutive ATP release from rat osteoblasts agreeing with earlier findings implicating increased intracellular Ca²⁺, either by influx from the extracellular environment or release from internal stores, in mediating ATP release (Knight et al, 2002). The inhibitory actions of monensin and NEM suggest an involvement of vesicular exocytosis in constitutive ATP release from primary rat osteoblasts. However, in view of the high concentrations of NEM required to inhibit ATP release, it is possible the effects observed were non-specific. Moreover, the inability of monensin and NEM to completely block ATP release and the lack of activity of Brefeldin A, suggest another pathway may be involved. Taken together this investigation illustrates further the complex mechanisms involved in controlled ATP release from cells.

Many cell types, including endothelial cells (Bodin & Burnstock, 1995), fibroblasts (Gerasimovskaya et al, 2002) and erythrocytes (Bergfeld & Forrester, 1992; Bozzo et
release ATP in response to hypoxia, a response which has been linked to ATP-induced vasodilatation of the vascular endothelium (Marshall, 2000). Transient exposure to 2% O₂ caused a rapid increase in ATP release from osteoblasts (up to 2.3-fold) that was sustained for up to 180 seconds. Lactate dehydrogenase (LDH) levels in the culture medium were consistently unaffected by transient hypoxia. Taken together, these data indicate sudden exposure to low oxygen prompts the release of ATP by controlled mechanisms rather than by increased cell lysis or membrane damage. Since basal ATP release appears to involve vesicular exocytosis, inhibitor studies were performed to determine the processes mediating the increased secretion following transient exposure to low O₂. Both monensin and NEM inhibited hypoxia-induced ATP release by up to 80%, indicating that vesicular exocytosis is involved in the increased ATP secretion from osteoblasts following a stressful stimulus. In agreement, osteoblast-like cells exposed to fluid shear displayed increased ATP release with the peak secretion occurring after 1 minute, a response that was also inhibited by monensin and NEM (Genetos et al, 2005). Transient hypoxia (50 seconds) also stimulates ATP release from erythrocytes, however, this efflux was inhibited by the channel blockers, dipyridamole and 4,4'-diisothiocyanatostilbene-2,2'disulphonic acid (DIDS), illustrating the different mechanisms employed by cells to secrete ATP (Bergfeld & Forrester, 1992).

Following acute exposure to hypoxia one of the first cellular responses is a sharp increase in intracellular Ca²⁺, the duration of which depends on the intensity of the hypoxic event (Toescu, 2004). Since increased intracellular Ca²⁺ is a prerequisite for ATP secretion, this response may well be the stimulus for the hypoxia-induced release observed here. The increase in Ca²⁺ levels, is primarily mediated via influx from the external environment, and may involve three different families of ion channels: (1) voltage sensitive calcium channels (VSCCs); (2) ligand operated ion channels such as glutamatergic receptor channels; (3) transient receptor potential (TRP) channels (Toescu, 2004). A number of these ion channels are expressed on osteoblasts including L-VSCC (Genetos et al, 2005), both ionotrophic glutamatergic receptors (AMPA and NMDA) (Itzstein et al, 2001; Hinoi et al, 2002;) and TRPV6 (Weber et al, 2001). Consequently, there are several potential candidates for mediating the increased intracellular Ca²⁺ and subsequent ATP secretion from osteoblasts following exposure to acute hypoxia.
No published data exist at present on the effect of acute hypothermia or hyperthermia on ATP release from cells. In this study, whilst transient cold shock (1 minute to 2 hours) did not affect the level of ATP release, exposure to 40°C for 30-120 minutes caused a sustained elevation in ATP release from primary rat osteoblasts. A study of patients with mandibular fractures demonstrated a ~ 2.5°C increase in temperature within the fracture callus (Kourie et al, 2000), consequently the heat-shock induced ATP release measured here may well occur in vivo. Ion channels belonging to the TRP superfamily sense and respond to local temperature changes and are widely expressed in the nervous system. These thermosensitive channels, including TRPV1, TRPV2 and TRPV3, are stimulated by a range of temperatures (Patapoutian et al, 2003; Bender et al, 2005) and once activated cause a significant increase in cation conductance and intracellular Ca²⁺ (Xu et al, 2002). Of these, TRPV3 is potentially the best candidate by which increased local temperature could result in elevated ATP release because its response range is 37-39°C. However, with the exception of the non-thermosensitive TRPV6 (Weber et al, 2001) there are no published data on the expression of TRPV channels on osteoblasts; but, this does present an interesting area for further study.

Prolonged hypoxia, hypothermia and hyperthermia exerted a striking inhibitory effect on ATP release from osteoblasts. Hypoxic cultures displayed up to a 65% decrease in total ATP release. Since culture at 2% O₂ impairs proliferation, this reduction is probably due to fewer cells being present. Conversely, when normalised for cell number ATP release per osteoblast was not significantly different. Chronic hypoxia causes cells to switch from mitochondrial-based metabolism (the citric acid cycle and the electron transport chain) to anaerobic glycolysis. Since the glycolytic pathway only produces two ATP molecules per glucose molecule hydrolysed, prolonged exposure to low oxygen strongly reduces ATP synthesis. Consequently, under these conditions cells would be expected to preserve their energy stores, thus the observation that cellular ATP release remains unchanged in 2% O₂ seems surprising. Assessment of LDH levels demonstrated a marked reduction in osteoblast viability in 2% O₂, especially during the later stages of culture. Therefore, it is possible that increased cell lysis and/or membrane damage may have contributed to the hypoxia-induced ATP release. Additionally, chronic hypoxia is reported to reduce ectonucleotidase activity, and thus, decrease the rate of extracellular ATP hydrolysis.
(Gerasimovskaya et al, 2002). The increased cell lysis combined with decreased hydrolysis could potentially explain why ATP release per osteoblast appeared unaffected by chronic exposure to hypoxia.

Prolonged hypothermia and hyperthermia reduced constitutive ATP release per osteoblast by ~ 40% and ~ 70% respectively. Since temperature influences enzyme tertiary structure, activation energies and consequently reaction kinetics, the effect of temperature changes on the enzymes responsible for ATP synthesis and hydrolysis might help to explain the observed results. Moreover, temperature regulates the properties of biological membranes, with subsequent influences on vesicle formation and the function of integral membrane proteins (Hazel, 1995).

The data presented in this chapter indicate how environmental stresses modulate osteoblast function and ATP release. Chronic hypoxia was shown to be inhibitory to both osteoblast proliferation and activity. The basic effects of hypoxia on osteoblasts are examined in more detail in a recent manuscript (Utting, Robins, Behar, Brandao-Burch, Orriss & Arnett, submitted). Furthermore, other data from our laboratory has shown osteoclast formation and activity is dramatically increased at low oxygen (Arnett, Gibbons, Utting, Orriss, Hoeberitz, Rosendaal & Meghji, 2003). Combined these findings suggest that a prolonged hypoxic environment in vivo, would negatively effect bone remodelling, shifting the balance in favour of net bone loss. These findings can potentially explain the bone loss occurring in many pathological situations characterised by low oxygen including tumours, ischemia and inflammation.

Prolonged exposure to temperatures only a few degrees above or below 37°C also exerted striking effects on osteoblast proliferation and function. Moreover, recent work performed in our laboratory demonstrated that ambient temperature also affects osteoclast formation and activity in vitro, with osteoclast formation being decreased in hyperthermia but increased in hypothermia (Utting et al, 2005). These results suggest strongly that local temperature changes occurring because of inflammation, infection, ageing or conditions such as hypothyroidism, could contribute to the low bone formation, remodelling and increased bone loss observed in these situations.

Transient exposure to hypoxia or hyperthermia caused increased ATP release, whereas more prolonged exposures reduced ATP secretion. Enhanced concentrations
of extracellular nucleotides following these stressful stimuli is likely to directly influence local purinergic signalling and therefore may contribute towards the initial repair processes. This idea will be discussed further in Chapter 6.
CHAPTER 5

THE BONE PHENOTYPE OF P2X$_2$, P2X$_3$, P2X$_{2/3}$ AND P2Y$_1$ RECEPTOR KNOCKOUT MICE

INTRODUCTION

The development of transgenic ("knockout") mouse models has facilitated the detailed study of preselected gene products \textit{in vivo}. Now widely utilised, these animals have provided vital information regarding the physiological roles of many hormones, growth factors and proteins. Generation of knockout mice is a complex, multi-step process taking many months, the first step being the synthesis of a DNA construct designed to introduce a mutation into the mouse genome at the desired locus. Following transfection and colony expansion, mouse embryonic stem cells undergo southern blot analysis to determine which cells contain the construct DNA integrated at the correct location. Suitably modified embryonic stem cells are used to generate the knockout animals via injection into mouse blastocysts and implantation in a surrogate mother. Selective interbreeding between offspring can eventually generate homozygous knockout mice. These animals, known as "constitutive knockouts", each contain the same mutated gene that is expressed throughout development and in all cells of the adult. In some cases, disruption of the target genes fails to produce viable offspring, often termed embryonically lethal. When this occurs, the above technique is modified to enable the production of a "conditional knockout"; these animals contain mutations inducible at different stages of development or in selected cell types.

Use of knockout animals in bone research has provided fundamental information regarding the transcription factors, cytokines and signalling molecules involved in skeletal development and bone remodelling. Disruption of some genes exerts gross effects on the skeleton; for example, animals lacking the transcription factor Runx2, which is a prerequisite for osteoblast development, demonstrate an almost complete lack of ossification (Komori \textit{et al}, 1997), whilst RANKL knockout mice exhibit severe
osteopetrosis (Kong et al., 1999). Removal of less essential factors has slighter skeletal effects, for example mice lacking FGF-2, which is a potent mitogen for osteoblasts and osteoprogenitors, develop a skeleton but exhibit decreased bone mass and a decreased rate of bone formation (Montero et al., 2000).

To date, mice deficient in P2X1, P2X2, P2X3, P2X2/3, P2X7, P2Y1, P2Y2 and P2Y4 receptors have been created. These animals have provided useful information regarding purinergic signalling in vivo, but with the exception of P2X7 receptor knockouts (Ke et al., 2003; Gartland et al., 2003c; Jorgensen et al., 2003), there are no reports of skeletal alterations in these animals. This study investigates whether P2X2, P2X3, P2X2/3 and P2Y1 receptor knockout mice display a changed bone phenotype. Background information regarding the expression profile and pharmacology of these P2 receptors is provided in Chapter 1.

Both P2X2 knockout (P2X2−/−) and P2X3 knockout (P2X3−/−) mice display normal viability with no overt phenotypic differences (Cockayne et al., 2000; Zhong et al., 2001; Cockayne et al., 2005). However, approximately 90% of P2X2/P2X3 double receptor knockouts (P2X2/3−/−) die between 5 days and weaning. These animals fail to thrive because of stunted growth, lymphohematopoietic hypoplasia in the bone marrow and thymus and die because of bacterial pneumonia and multiple organ dysfunctions. Both P2X2−/− and P2X3−/− mice exhibit deficits in pain-related behaviour and normal sensory processes such as urinary bladder reflexes and gastrointestinal peristalsis (Cockayne et al., 2000; Zhong et al., 2001; Cockayne et al., 2005). P2X2−/− and P2X2/3−/− mice also demonstrate impaired ventilatory responses to hypoxia (Rong et al., 2003) and lack responses to agonist stimulation in parasympathetic otic ganglion nerves (Ma et al., 2004), suggesting a role for the P2X2 subunit in the sensory effects of ATP in the carotid body and nerve transmission in parasympathetic neurones. Additionally, P2X2−/− mice also exhibit abnormalities in the neuromuscular junction and in skeletal muscle function (Ryten et al., 2004).

P2Y1 knockout (P2Y1−/−) mice are viable, with no gross external abnormalities, and display normal development, survival and reproduction (Leon et al., 1999). However, these animals are resistant to acute thromboembolism and exhibit increased bleeding. Moreover, functional studies of platelets derived from P2Y1−/− mice demonstrated a loss of ADP-induced aggregation, suggesting an important role for the P2Y1 receptor in
platelet aggregation and blood clotting (Fabre et al, 1999; Leon et al, 1999; Leon et al, 2001).

Comparison of several murine strains demonstrated that skeletal maturity is reached by approximately 4 months of age, with large changes in both total and femoral bone mineral density (BMD) and bone mineral content (BMC) observed between 2–4 months; from 6 months upwards, only small changes in BMD and BMC occur (Beamer et al, 1996). Investigations into the mechanical properties of mouse femoral bones demonstrated peak bone strength is achieved at approximately 20 weeks of age (Brodt et al, 1999). The skeletons of juvenile animals (2-3 months) exhibit rapid growth remodelling and contain abundant osteoclasts; marrow cultures from young mice efficiently generate large numbers of osteoclasts in vitro when stimulated by M-CSF and RANKL. Comparative studies using several different mouse strains, all raised in identical environments, demonstrated marked differences in bone density, indicating genetic variation influences bone mass: for example the C57BL/6 inbred strain has a lower BMD compared to other strains such as the C3H/HEJ strain (Beamer et al, 1996; Chen & Kalu, 1999). Thus, it is important that experimental comparisons are made between knockouts and wild type controls derived from the same genetic background.

Previous investigations have shown that the P2X2 receptor and the P2Y1 receptor, but not the P2X3 receptor, are expressed on both osteoblasts and osteoclasts (Chapter 2; Hoebertz et al, 2003). From the experimental data available, it is possible to hypothesize the effects of receptor removal on the skeletons of P2X2−/−, P2X3−/−, P2X2/3−/− and P2Y1−/− animals. In vitro evidence has implicated both the P2Y1 receptor (Hoebertz et al, 2001) and the acid-activated P2X2 receptor (Morrison et al, 1998) in the nucleotide-mediated stimulation of osteoclast formation and activity. Given this ability to shift the balance of bone remodelling in the favour of resorption, removal of either the P2X2 receptor or the P2Y1 receptor may well lead to increased bone mass. In contrast, as P2X3 receptors are not expressed on bone cells, receptor removal would not be expected to have any direct skeletal effects. The effect of P2X2/3 receptor removal on the skeleton is harder to predict because although heteromeric P2X2/3 receptors are not present on bone cells, P2X2/3−/− mice will also lack homomeric P2X2 and P2X3 receptors. The present study employed the non-invasive
technique of dual-energy x-ray absorptiometry (or DEXA) scanning to investigate whether these proposed effects of P2X2, P2X3, P2X2/3 or P2Y1 receptor removal occur in vivo.
MATERIALS AND METHODS

Principles of DEXA scanning

Dual-energy x-ray absorptiometry (DEXA) scanning is widely used both clinically and experimentally to determine bone density. This study employed the PIXIImus DEXA scanner (Lunar, GE Healthcare, USA) specifically designed to study the skeleton of small rodents (Figure 5.1).

DEXA scanning works on the principle that x-rays reflect from bone in a density dependent manner. The PIXIImus system exposes the entire animal to a cone shaped beam of high energy (80 kV) and low energy (55 kV) x-rays. A CCD (charged couple device) camera detects the radiation hitting a luminescent panel below the test subject. The varied compositions of bone mineral, fat and lean tissue means they differentially absorb or reflect the dual energy x-rays; thus by digitally processing the detected radiation, the PIXIImus can calculate the relative quantities of bone, fat and lean tissue along the x-ray path. During each scan, the dual x-ray exposure is performed four times and on completion, the data is collated to produce a ‘summary’ image, the density of which is compared to a standard object (mouse phantom) used to calibrate the instrument.

In this study, the PIXIImus system was used to measure the bone mineral content (BMC), bone mineral density (BMD), total fat and lean tissue and percentage fat of test subjects. The BMC, a parameter directly calculated by the DEXA scanner, is measured in grams and represents the total amount of bone mineral present in a defined area. BMD is calculated by dividing the BMC by projected bone area and represents the amount of bone mineral present in a cm². However, the BMD, which is measured in grams/cm², is not a true density measurement because it does not take into account the volume in which the bone mineral is contained.

Generation of knockout animals

The knockout mice used in this study originated from two separate sources and have slightly different genetic backgrounds; the P2X2', P2X3' and P2X2/3' mice are from a C57BL/6 x 129/01a lineage, whilst the P2Y' mice are derived from a
C57BL/6 J lineage. These strains, particularly C57BL/6, are widely used in knockout generation, have a long lifespan and display no inherent bone defects. The P2X knockouts were a kind gift from Dr D Cockayne (Roche Bioscience, USA) and the P2Y1−/− mice from Dr C Gachet (Strasbourg, France). P2X2−/− mice were generated via introduction of a deletion encompassing exons 2-11 in the mouse P2X2 gene, whilst a 1-kb deletion encompassing exon 1 and the ATG initiation codon produced the P2X3−/− animals. Breeding of P2X2−/− with P2X3−/− mice to eventually produce offspring carrying a deletion in both P2X2 and P2X3 genes generated the P2X2/3−/− knockouts. P2Y1−/− animals were generated via insertion of a poly(A) cassette into exon 1. All P2X knockouts scanned were female, whilst the P2Y1−/− animals were male. All knockouts were derived from homozygous breeding pairs, in which each mutant line was compared with its own filial generation-matched wild type controls, thus eliminating any strain related effects on bone mass. The P2X receptor knockouts were housed in a designated animal facility and routinely maintained on a 12 hour light/12 hour dark cycle and given ad libitum access to food and water.

**Bone mineral measurements**

The P2X2−/− and P2X3−/− animals (n = 10) were received aged 2 months, and changes in bone mass monitored over a 7-month period; however the P2X2/3−/− mice (n = 10) were 6 months old on arrival and so were only monitored for a 2-month period. Two month old mice are experiencing puberty, rapid growth, rapid bone remodelling and are considered juveniles, by 4 months they are classified as young adults and ≥ 6 months corresponds to full adult maturity.

Following weighing, anaesthesia was induced by exposure to isofluorane for up to 2 minutes (exact time dependent on subject size); an intraperitoneal injection of 2.5% tribomoethanol in 2-methylbutan-2-ol at 0.16 ml/10g (Sigma-Aldrich, Dorset, UK) was administered to fully anaesthetize each mouse for approximately 15-20 minutes. Subjects were carefully positioned with their limbs extended and the head excluded, on plastic trays designed specifically for the PIXIImus system (Figure 5.1); all live animals were scanned in triplicate with repositioning between scan. On completion, animals were carefully monitored until fully recovered from the anaesthesia. All procedures were performed according to Home Office guidelines and regulations (Animals for Scientific Procedures Act, 1986).
Unlike the P2X receptor knockouts, the 2-month-old P2Y1−/− mice (n = 10) were frozen when received for scanning, thus any age-related changes in bone mass could not be measured. P2Y1−/− mice and their wild type controls were thoroughly defrosted before being positioned on plastic trays for scanning as described above. Since initial triplicate scans of three P2Y1−/− animals showed no differences in the bone mineral measurements it was only necessary to scan each test subject once.

All data was analysed using the PIXIImus software (version 1.8, Lunar, GE Healthcare, USA) (Figure 5.1) and for P2X receptor knockouts the three scans were averaged to give final values for total BMD, BMC, fat and lean tissue. Localised analysis was additionally carried out to determine the BMD and BMC of the spine, femora and tibiae/fibulae.

Statistical analysis

Data were analysed as described in Chapter 2
RESULTS

**P2X$_2^{−/−}$ and P2X$_3^{−/−}$ mice: general observations**

The P2X$_2^{−/−}$ and P2X$_3^{−/−}$ animals did not exhibit any obvious changes in phenotype; however, previous behavioural analysis found P2X$_2^{−/−}$ mice had a tendency to hyperactivity (Cockayne *et al.*, 2005) (Figure 5.1A-C). Throughout the study, all subjects were healthy, the only exception being at 9 months of age when some P2X$_3^{−/−}$ mice developed an autoimmune skin condition ($n = 4$). Before developing this condition animals experienced hair loss on the back and shoulders, which eventually lead to excessive scratching and the development of skin lesions. As the condition was untreatable, animals developing lesions were culled. This pathology was not present in any of the test subjects throughout the duration of the experiment and only developed after completion of the final scans. Many disorders of bone and mineral metabolism, such as osteopetrosis, present dental manifestations including impaired tooth eruption. Normal tooth eruption is dependent on bone resorption surrounding the developing tooth; inadequate resorption can hinder this process leading to unerupted and malformed teeth or delayed eruption (Krebsbach & Polverini, 1999). However, in both P2X$_2^{−/−}$ and the P2X$_3^{−/−}$ mice tooth eruption was unaffected indicating no gross effects of receptor deletion on bone resorption.

**P2X$_2^{−/−}$ and P2X$_3^{−/−}$ mice: total bone mineral measurements**

Total BMD and BMC measurements from P2X$_2^{−/−}$, P2X$_3^{−/−}$ and wild types at 2-9 months of age are summarised in Figure 5.2. In all animals, the largest change in both bone mineral parameters took place between 2-4 months of age with only marginal changes occurring at 6-9 months. Throughout the test period P2X$_2^{−/−}$, but not P2X$_3^{−/−}$ animals displayed a significantly enhanced total BMD compared to age matched controls (Figure 5.2A). The P2X$_2^{−/−}$ mice exhibited a 9% increase in total BMD at 2 months of age, decreasing to 6% at 4 months and 5% at 6-9 months, indicating the effect of P2X$_2$ receptor removal is most prevalent in juvenile animals. P2X$_2^{−/−}$ animals also displayed an age related increase in total BMC. In the 2-month old mice, total BMC was increased by 18% at 2 months, decreasing to 9% at 4 months and 10% at 6-9 months. The P2X$_3^{−/−}$ animals did not display variations in total BMC at any age (Figure 5.2B).
Figure 5.1. \(P2X_{2}^{-}\) and \(P2X_{3}^{-}\) mice and the PiXImus system

Representative images of female (A) wild type, (B) \(P2X_{2}^{-}\) and (C) \(P2X_{3}^{-}\) mice aged 4 months. These pictures show that the knockout animals have no gross abnormalities. (D) An image showing the PiXImus analysis software used to calculate levels of bone mineral and soft tissues. (E) The PiXImus system. (F) This image represents the typical image of a mouse produced following a 5 minute DEXA scan. The scale bar in images A-C = 2 cm.
Figure 5.2. Total BMD and BMC was increased in P2X2 +/- mice

Compared to controls, P2X2 +/- mice displayed increased (A) total BMD (up to 9%) and increased (B) total BMC (up to 18%). The largest differences between wildtypes and P2X2 +/- occurred in the 2-month-old animals. Total BMD and BMC were unchanged in female P2X3 +/- animals (n = 10, all animals female) (*** = p<0.001, ** = p<0.01, * = p<0.05).
**P2X2−/− and P2X3−/− mice: total weight, fat and lean tissue content**

Total body weight was monitored throughout the experimental period and at all ages the P2X2−/− mice were significantly heavier than the wild types (Figure 5.3A). In contrast to the total BMD/BMC, these differences in body weight increased slightly with age; the animals being ~ 9% heavier at 2 months, increasing to 12% at 4 and 6 months before reducing to 10% at 9 months. The body weight of P2X3−/− mice did not vary from age-matched controls.

The PIXImus was also employed to measure soft tissues. Compared to wildtypes, P2X3−/− animals demonstrated no differences in lean tissue at any point. Conversely, the P2X2−/− knockouts displayed an age related increase in lean tissue (Figure 5.3B); with levels being enhanced by 8%, 10%, 11% and 14% at 2, 4, 6 and 9 months of age, respectively.

P2X2−/− and P2X3−/− mice displayed no differences in percentage fat at any time point, compared to age matched controls (Figure 5.3C). Total fat levels in P2X2−/− animals were generally unchanged (Figure 5.3D).

**P2X2−/− and P2X3−/− mice: bone mineral measurements of the femora and tibiae/fibulae**

To determine whether the age-related trend observed in the whole body scans was reflected in individual bones, the PIXImus was also used to quantify the BMD and BMC of the long bones in the hind legs (femora, tibiae and fibulae). Due to limitations of resolution, the PIXImus system cannot distinguish between the tibia and fibula, so the readings for these bones are combined. In keeping with the whole body results, the P2X3−/− mice displayed no differences in any of these measurements at any age.

The P2X2−/− mice displayed significantly increased tibiae/fibulae BMD at 2 and 4 months (5–6 % increase) but not 6 and 9 months (Figure 5.4A). A large 24% increase in tibiae/fibulae BMC was apparent at 2 months, decreasing to 18%, 10% and 13% at 4, 6 and 9 months, respectively (Figure 5.4B).

Femoral bone mineral measurements were also increased in the P2X2−/− animals, although the differences were smaller than the tibiae/fibulae readings. The P2X2−/− mice exhibited a significantly higher femoral BMD at 2-6 months but not 9 months of
age; the values being increased by 7%, 6%, 5% and 4% at 2, 4, 6 and 9 months, respectively (Figure 5.4C). Femoral BMC displayed a larger increase than the BMD. P2X<sup>2</sup> animals display a significant 15% increase in femoral BMC at 2 months and 7% increase at 4 months of age (Figure 5.4D). In 6 and 9-month P2X<sup>2</sup> mice, the femoral BMC was no different from the controls.

**P2X<sup>2</sup> and P2X<sup>3</sup> mice: bone mineral measurements of the spine**

In keeping with the other measurements, P2X<sup>3</sup> mice displayed no differences in either spinal BMD or BMC. The P2X<sup>2</sup> knockouts did display differences in spinal BMD and BMC but they were smaller than those observed in the long bones. Spinal BMD was significantly increased by 9% in the 2-month-old P2X<sup>2</sup> mice, but from 4 months upwards, no differences between the knockouts and wild types were observed (Figure 5.5A). However, the 2-month P2X<sup>2</sup> knockouts displayed a large 27% increase in spinal BMC, which decreased to 15% at 4 months. The 6 and 9 month old animals showed little or no variation in spinal BMC compared to controls (Figure 5.5B).
Figure 5.3. *Total weight and lean tissue content was increased in P2X₂⁺ mice*

(A) At all ages, P2X₂⁺ but not P2X₃⁺ mice displayed an increased weight (up to 12%) compared to wildtypes (n = 10, all female). (B) P2X₂⁺ mice displayed up to a 14% increase in the level of lean tissue compared to age matched controls, whereas P2X₃⁺ animals showed no differences. (C) Percentage fat was unchanged in both P2X₂⁺ or P2X₃⁺ knockout animals. (D) Throughout the study, total fat was generally unchanged, with the exception of the 4-month-old P2X₂⁺ mice who had slightly increased fat levels (** = p<0.01, * = p<0.05).
Figure 5.4. The long bones of P2X$_2^+$ mice display age-related increases in BMD and BMC

(A) Tibiae/fibulae BMD was significantly enhanced by 5-6% in P2X$_2^+$ mice aged 2 and 4 months but not 6 and 9 months. (B) Tibiae/fibulae BMC was increased by up to 15% in P2X$_2^+$ animals; P2X$_3^+$ mice exhibited no differences in tibiae/fibulae BMC and BMD at any age. (C) Femoral BMD was increased by 5-7% in 2-6 month old P2X$_2^+$ knockouts. (D) Femoral BMC was increased by 7-15% in 2-4 month old P2X$_2^+$ mice; P2X$_3^+$ mice exhibited no differences in femoral BMC and BMD at any age (n = 10, all animals female) (**=p<0.01, * = p<0.05).
Figure 5.5. Spinal BMD and BMC was increased in young P2X_2^+/− mice

(A) Spinal BMD was significantly increased by 9% in 2-month old P2X_2^+/− mice, whereas 4–9 month old animals displayed no differences. (B) P2X_2^+/− knockouts displayed a significantly increased spinal BMC at 2 and 4 months, being increased by 27% and 15% respectively. Spinal BMD and BMC were unchanged in the P2X_3^+/− animals at all ages (n = 10, all animals female) (** = p<0.001, * = p<0.05).
**P2X$_{2/3}^{dbl-/-}$ mice: general observations**

The double knockouts appeared slightly runty and were somewhat smaller than their corresponding age matched controls. Tooth eruption was unimpaired in the P2X$_{2/3}^{dbl-/-}$ mice. Following day-to-day observations it became apparent that the double knockouts displayed some behavioural differences; they appeared less active and were less inclined to move around the cages than the wild types. The images in Figure 5.6 are representative of these animals at 6 months and demonstrate the slight size difference between P2X$_{2/3}^{dbl-/-}$ mice and their controls.

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**Figure 5.6.** *P2X$_{2/3}^{dbl-/-}$ mice were somewhat smaller than the age-matched controls*

These images are representative of the female P2X$_{2/3}^{dbl-/-}$ mice are their corresponding female wild types (WT) at 6 months of age. In both images scale bar = 2 cm
**P2X2\textsubscript{2/3}\textsuperscript{dbb/-} mice: total bone mineral measurements**

As these animals were mature adults when received for study, there is no data available on the effect of receptor deletion on the skeleton of younger animals. When compared to age matched controls, the P2X2\textsubscript{2/3}\textsuperscript{dbb/-} mice displayed a significant 6-7% decrease in total BMD at both 6 and 8 months; this is contrary to the single P2X\textsubscript{2} knockouts, which exhibited a 5-9% increase in total BMD (Figure 5.7A). Somewhat surprisingly, the P2X2\textsubscript{2/3}\textsuperscript{dbb/-} mice displayed no differences in the total BMC at either 6 or 8 months of age (Figure 5.7B). These data suggest P2X2\textsubscript{2/3} knockouts possess similar levels of bone mineral, but a reduced bone density. Because the murine skeleton has reached maturity by 6 months, there was little variation in both total BMD and BMC between the two scans.

![Figure 5.7](image-url)  
**Figure 5.7.** Total BMD was decreased but total BMC was unchanged in P2X2\textsubscript{2/3}\textsuperscript{dbb/-} animals  
(A) At 6 and 8 months, P2X2\textsubscript{2/3}\textsuperscript{dbb/-} mice displayed a 6-7% decrease in total BMD compared to wild types. (B) There were no differences in the total BMC of the P2X2\textsubscript{2/3}\textsuperscript{dbb/-} and wild type animals. Only minimal changes in BMD and BMC were observed between the scans at 6 and 8 months (n = 10, all animals female) (** = p<0.01).
**P2X2/3^d^b^l^l^- mice: total weight, lean tissue and fat content**

Total body weight of the P2X2/3^d^b^l^l^- animals was ~ 10% and ~ 16% lower than the wildtypes at 6 and 8 months, respectively (Figure 5.8A). Quantification of total lean tissue in P2X2/3^d^b^l^l^- mice demonstrated an 8% decrease at 6 months and 10% reduction at 8 months. For both these values, the observed differences were only statistically significant in the 8-month old mice (Figure 5.8B).

P2X2/3^d^b^l^l^- mice displayed reduced levels of total fat (11%-22%) (Figure 5.8C) and percentage fat (22%-38%) (Figure 5.8D) at both 6 and 8 months; however, none of these decreases were statistically significant.

**P2X2/3^d^b^l^l^- mice: bone mineral measurements of femora and tibiae/fibulae**

Quantification of femora and tibiae/fibulae bones from P2X2/3^d^b^l^l^- mice and corresponding wild types revealed marked changes in the local BMD and BMC. P2X2/3^d^b^l^l^- animals exhibited a 9% and 12% reduction in femoral BMD at 6 and 8 months respectively (Figure 5.9A). P2X2/3^d^b^l^l^- mice displayed no changes in total BMC, however femoral analysis demonstrated a significant ~ 16% decrease in femoral BMC at both 6 and 8 months of age (Figure 5.9B). Similar effects were seen in tibiae/fibulae BMD and BMC; at 6 and 8 months, P2X2/3^d^b^l^l^- animals displayed a 11% and 14% reduction in BMD (Figure 5.9C), accompanied by an 11% and 15% decrease in BMC (Figure 5.9D).

In both cases, the local decreases in BMD and BMC were greater than the total measurements. Comparable to the whole body readings, the local scans showed nominal changes between 6 and 8 months in both knockouts and wild types.
Figure 5.8. Changes in weight, lean tissue and fat content in P2X$_{23}^{\text{db/l-}}$ mice

(A) Total body weight was significantly reduced at 8 months (16% decrease) but not 6 months (10% decrease) in P2X$_{23}^{\text{db/l-}}$ animals. (B) Lean tissue content was only decreased in the 8-month P2X$_{23}^{\text{db/l-}}$ mice, being reduced by 10%. Although lower, there were no significant differences in (C) percentage fat and (D) total fat between P2X$_{23}^{\text{db/l-}}$ animals and corresponding wild types at 6 or 8 months (n = 10, all animals female) (* = p<0.05).
Figure 5.9. The long bones of P2X2\textsubscript{2/3}\textsuperscript{dih-/-} mice display decreased BMD and BMC

(A) Femoral BMD was reduced in the P2X2\textsubscript{2/3}\textsuperscript{dih-/-} mice at both 6 and 8 months, by 9% and 12% respectively. (B) P2X2\textsubscript{2/3}\textsuperscript{dih-/-} animals displayed a ~16% decrease in femoral BMC at both ages. (C) Tibiae/fibulae BMD was reduced by 11-14% in the P2X2\textsubscript{2/3}\textsuperscript{dih-/-} mice. (D) P2X2\textsubscript{2/3}\textsuperscript{dih-/-} knockouts displayed a decreased tibiae/fibulae BMC at both 6 and 8 months being reduced by 11% and 15%, respectively (n = 10, all animals female) (*** = p<0.001, ** = p<0.01, * = p<0.05).
**P2X\textsubscript{2/3}\textsuperscript{dbl+} mice: bone mineral measurements of the spine**

In contrast to the long bone analysis, quantification of the spinal BMD and BMC in P2X\textsubscript{2/3}\textsuperscript{dbl+} mice demonstrated little effect of receptor removal on this region of the skeleton. The spinal BMD of the P2X\textsubscript{2/3}\textsuperscript{dbl+} animals did not vary from the wild types at 6 or 8 months of age (Figure 5.10A); spinal BMC appeared similarly unchanged varying by > 2% in test subjects (Figure 5.10B).

**Figure 5.10. Lack of effect of P2X\textsubscript{2/3} receptor removal on spinal BMD and BMC**

P2X\textsubscript{2/3}\textsuperscript{dbl+} animals displayed no differences in (A) spinal BMD or (B) spinal BMC at either 6 or 8 months of age, compared to wildtypes (n = 10, all animals female).
**P2Y₁<sup>−/−</sup> mice: general observations**

The P2Y₁<sup>−/−</sup> receptor deficient mice displayed no gross physical abnormalities and tooth eruption was unaffected. The images in **Figure 5.11** are representative of these animals when received aged 2 months.

![Figure 5.11](image_url)

**Figure 5.11.** *P2Y₁<sup>−/−</sup> mice do not display any overt changes in phenotype*

Representative images of the male, 2-month old P2Y₁<sup>−/−</sup> mice and their controls scanned in this study. In both images scale bar = 2 cm.
**P2Y* mice: total bone mineral measurements**

Quantification of the 2-month old P2Y* mice and corresponding wild types revealed receptor removal causes a significant, albeit small, change in bone phenotype; however, in contrast to the P2X2* animals, the P2Y* mice exhibited decreases in both total BMD and BMC. A 5% reduction in total BMD (Figure 5.12A) and a 7% decrease in total BMC (Figure 5.12B) was observed; these differences were similar in magnitude to those observed in the P2X2/3* animals but smaller than those observed in the P2X2* animals.

**P2Y* mice: total body weight, fat and lean tissue content**

Analysis of total body weight (Figure 5.12C), lean tissue (Figure 5.12D), fat (Figure 5.12E) and percentage fat (Figure 5.12F) in P2Y* mice demonstrated no differences in any of these parameters.

**P2Y* mice: femora, tibiae/fibulae and spinal bone mineral measurements**

Analysis of individual bones from wild type and P2Y* animals revealed some small decreases in the bone mineral values. Quantification of the femoral bones demonstrated a non-significant 4% decrease in BMD (Figure 5.13A) but a significant 14% reduction in the BMC (Figure 5.13B).

The tibiae/fibulae bones of the P2Y* mice displayed a decreased BMD (Figure 5.13C) and BMC (Figure 5.13D), being in both cases, reduced by 9%. Finally, spinal analyses revealed an 8% reduction in BMD (Figure 5.13E) but only a non-significant 5% reduction in BMC (Figure 5.13F).
Figure 5.12. P2Y₁⁺ mice display decreased total BMD and BMC but unchanged weight, lean tissue and fat content

(A) Total BMD was reduced by 5% in the P2Y₁⁺ animals. (B) P2Y₁⁺ mice displayed a significant 7% decrease in total BMC. P2Y₁ receptor deletion did not influence (C) total weight, (D) lean tissue content, (E) fat and (F) percentage fat (n = 10, all animals male) (* = p < 0.05).
Figure 5.13. Changes in BMC and BMD in the spine, femora and tibiae/fibulae of P2Y₁⁺ mice

(A) Femoral BMD was unchanged in P2Y₁⁺ mice compared to wild type, whereas (B) femoral BMC was decreased by 14%. Both Tibiae/fibulae (C) BMD and (D) BMC were decreased by 9% in P2Y₁⁺ animals. (E) P2Y₁⁺ mice displayed an 8% decrease in spinal BMD, (F) but spinal BMC was no different to controls (n = 10, all animals male) (** = p < 0.01, * = p < 0.05).
DISCUSSION

Previous study of P2 receptor knockout mice highlighted the important role of P2X2, P2X3 and P2Y1 receptor-mediated purinergic signalling in vivo (Leon et al, 1999; Cockayne et al, 2000; Vlaskovska et al, 2001; Rong et al, 2003; Cockayne et al, 2005). The work carried out here provides the first indication that P2X2, P2X2/3 and P2Y1 but not P2X3-receptor knockout mice display changes in bone phenotype.

The acid-activated P2X2 receptor is expressed on osteoclasts and proliferating osteoblasts (Chapter 2; Hoebertz et al, 2000). In this study, DEXA analysis demonstrated a significantly increased total BMD and BMC in the P2X2+/− mice. Moreover, closer investigation of selected regions indicated similar changes occurred at a local level, with the femora, vertebrae and tibiae/fibulae all showing increases in BMD and BMC. Of the other parameters measured, both body weight and lean tissue levels also appeared increased in the P2X2+/− mice, whereas total and percentage fat were generally unaffected. In younger P2X2+/− animals (2-4 months), although weight and lean tissue were increased, these changes were smaller than the increases in bone mass. Conversely, in older P2X2+/− animals (6-9 months), there were bigger changes in weight and lean tissue than bone mass. Developing mammalian skeletal muscle, but not adult muscle fibres express the P2X2 receptor, and earlier work demonstrated P2X2-receptor deficient animals display abnormalities in neuromuscular junction structure and skeletal muscle function (Ryten et al, 2004b). These muscle defects are not observed in P2X3 knockouts, which in this study displayed no changes in lean tissue; therefore, these skeletal muscle abnormalities may have contributed to the increased lean tissue seen in the P2X2-receptor deficient mice. It reasonable to suppose that these changes in lean tissue may have contributed indirectly, to some extent, to the bone phenotype observed in these animals by affecting loading on the skeleton.

The increased bone mass, particularly in the younger P2X2+/− animals, suggests that receptor deletion is also having direct effects on bone cell function and is consistent with a role for the P2X2 receptor in the negative regulation of bone remodelling. In agreement, previous work implicated the P2X2 receptor and extracellular ATP in stimulating the resorptive activity of osteoclasts (Morrison et al, 1998); thus, the
increase in BMD and BMC observed here could be the result of a small decrease in resorption. Since osteoblasts also express the P2X2 receptor, impaired cell function may also contribute to the changes in bone phenotype observed in the P2X2 knockouts.

The local and total differences in BMC and BMD between the P2X2−/− mice and wild type controls reduced with age, however, why this occurred remains unclear. Bone cells express a number of purinergic receptors (Chapter 2; Hoebertz et al, 2003) thus, it is possible that ATP responses are mediated, via increasing compensation, by other P2 family members in the older P2X2−/− animals. Conversely, as the mouse skeleton does not reach maturity until about 4 months and the largest effects of receptor deletion were observed in the 2-month old animals, these data could indicate a role for the P2X2 receptor in modulating bone cell function in the developing skeleton.

Numerous studies have failed to detect P2X3 receptor expression on either osteoblasts or osteoclasts; thus receptor deletion is unlikely to influence bone cell function directly [see review Hoebertz et al, 2003]. DEXA analysis of P2X3−/− mice demonstrated no significant differences, from juveniles through to mature adults, in total BMD, total BMC, weight, lean tissue and fat content; additionally, closer investigation of the femoral, tibial and vertebral bones indicated no significant changes in local BMC or BMD. The absence of a skeletal phenotype in these animals suggests P2X3 receptors do not play a role, either directly or indirectly via other cell types, in modulating bone remodelling in vivo.

To date there are no reports identifying the expression of heteromeric P2X2/3 receptors on bone cells (Hoebertz et al, 2003). The P2X2/3−/− animals, in addition to lacking P2X2/3 receptors, do not possess functional homomeric P2X2 and P2X3 receptors. DEXA analysis demonstrated that the P2X2/3−/− animals exhibited a decreased total BMD whilst total BMC was unchanged. Local analysis showed BMD and BMC was decreased in the long bones but unaffected in the spine. P2X2/3 receptor deletion had a small influence on the other parameters measured as both lean tissue and body weight were decreased in 8-month animals. Fat content was lower in P2X2/3−/− mice but not significantly so. Thus, decreased levels of soft tissues may help to explain the decreased weight observed in 8-month P2X2/3−/− mice.
Removal of both P2X2 and P2X3 receptor subunits disrupts a number of physiological processes and produces animals with an unhealthy constitution and high rate of mortality (Cockayne et al, 2000; Vlaskovska et al, 2001; Cockayne et al, 2005). Thus, deficits in the nervous system and general illness may act indirectly to influence bone remodelling in P2X2/3<sup>dbl</sup>-/animals. During the course of this study, it was also noted that movement and activity appeared considerably reduced in P2X2/3<sup>dbl</sup>-/mice. Since mechanical loading is necessary for the maintenance of bone mass it is possible that reduced activity and bone loading may have also contributed towards the altered skeletal phenotype of the P2X2/3<sup>dbl</sup>-/animals. Therefore, the effects of decreased body weight and activity combined with the increased level of illness may outweigh any positive effects that removal of P2X2 receptors has on bone mass. This can potentially explain why the P2X2/3<sup>dbl</sup>-/ mice displayed alterations in skeletal phenotype opposite to that of the P2X2<sup>-/-</sup> animals.

Several investigations have provided <i>in vitro</i> evidence for the involvement of P2Y<sub>1</sub> receptors in the modulation of bone cell function. In osteoclasts, ATP and ADP can signal via the P2Y<sub>1</sub> receptor to stimulate formation and activity (Hoebertz et al, 2001). Whereas in osteoblasts, extracellular nucleotides can act to modulate local responses to systemic factors such as PTH (Bowler et al, 2001; Buckley et al, 2001). Surprisingly, the 2-month-old P2Y<sub>1</sub>-/ mice displayed small decreases in total and local BMC and BMD. No differences were observed in total body weight, lean tissue or fat content. However, P2Y<sub>1</sub>-/ mice are known to display defects in platelet aggregation and blood clotting (Leon et al, 1999; Leon et al, 2001); thus, other systemic perturbations may indirectly influence bone cell function and counteract any positive effects P2Y<sub>1</sub> receptor deletion has on bone mass.

Three separate studies have described skeletal changes in P2X7 knockout animals, albeit with conflicting results (Gartland et al, 2003c; Jorgensen et al, 2003; Ke et al, 2003). Garland et al (2003) reported no differences in total and local BMD, as measured by DEXA analysis, but did report increased cortical bone thickness in the tibial shaft. Jorgensen and colleagues (2003) reported increased bone mass in female but not male P2X7 knockouts, whilst Ke et al (2003) found that skeletal changes occurred in both sexes (including decreased cortical bone thickness), with a greater effect in male animals. These opposing data may reflect the different genetic
backgrounds of the mice studied. In the present investigation, all the P2X knockouts were female and all P2Y₁ knockouts were male, so no gender-related effects could be determined. Given the sex differences seen in the P2X₇-null mice, it would be of interest to see if male P2X and female P2Y₁ knockouts exhibited similar changes in BMD and BMC.

The long bones and vertebrae contain differing proportions of cortical and trabecular bone. By providing localised measurements for the spine and long bones, the data in this present study may give a rough indication of the differential effects of P2 receptor deletion on cortical and trabecular bone. Generally, the long bones were more affected by receptor deletion, suggesting a greater effect on cortical bone. However, more detailed information regarding changes in bone structure on the microscopic scale, for example cortical/trabecular area and density, can be obtained using techniques such as quantitative micro computed tomography. To determine how receptor deletion affects bone resorption, formation or mineralisation would require invasive techniques such as bone histomorphometry.

The uses of a PIXImus densitometer are limited by its relatively low resolution and because the parameters measured are static, it provides no information regarding potential effects on bone remodelling. Conversely, DEXA scanning is very rapid (each scan ~ 5 minutes) and can be used on live animals. For these reasons, this technique is commonly employed prior to carrying out more invasive and time-consuming techniques, to screen knockout animals for the presence of a changed bone phenotype. Despite its limitations, DEXA scanning has found quite widespread usage with a number of published studies employing PIXImus densitometers. For example, van’t Hof et al (2004) demonstrated 10-week old neuronal nitric oxide synthase (nNOS) knockouts displayed a 13% increase in total BMD and 26% increase in total BMC. These effects are slightly better than the biggest effects observed in this study, as P2X₂⁺ mice displayed a 9% increase in total BMD and 18% increase in total BMC. In addition, work by Idris and colleagues (2005) demonstrated an 18% increase in femoral BMD in cannabinoid type 1 (CB₁) receptor knockouts. In both of these investigations, further study using peripheral quantitative computed tomography (pQCT) illustrated similar sized defects in excised bones, indicating that defects detected by DEXA scanning are reproducible via other techniques.
In conclusion, this study provides the first indication of an altered bone phenotype in P2X2, P2X2/3 and P2Y1 but not in P2X3-receptor deficient mice. The effects described here are moderate in size and may reflect the potential redundancy of this primitive signalling system in vivo. Given the findings from earlier chapters (2 & 3), which demonstrated widespread expression of the P2Y2, P2Y4 and P2Y6 receptors on osteoblasts and the ability of extracellular nucleotides to “switch off” mineralisation analysis of these receptor knockouts is now required.
CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

ATP is a ubiquitous molecule with a fundamental role in many intracellular biochemical processes. The "purinergic" concept was first introduced by Burnstock in 1972; it is now widely recognised that signalling by extracellular nucleotides and their receptors plays an important role in both neuronal and non-neuronal cells (Burnstock & Knight, 2004). Over recent years, the involvement of extracellular nucleotides in the regulation of bone cell function has become more apparent [see review by Hoebertz et al, 2003]. The aim of this thesis was to investigate the effects of extracellular nucleotides on bone cell function during normal and stress situations.

This chapter contains a brief summary of the key experimental findings of this thesis and suggests potential future work. Additionally, several issues arising from the experimental chapters will be discussed in a broader purinergic context with particular reference to bone pathophysiology.

Bone cells express multiple P2 receptor subtypes [see review by Hoebertz et al, 2003], and despite earlier indications that purinergic receptor expression changes with osteoblast differentiation (Dixon et al, 1997), this idea remained poorly investigated. In the first experimental chapter, I demonstrated for the first time that expression of P2 receptors on primary rat osteoblasts is strongly dependent on differentiation and time in culture by using RT-PCR and immunofluorescence. These changes were characterised by a shift from P2X to P2Y receptor expression, with mature osteoblasts strongly expressing P2Y2, P2Y4 and P2Y6 receptors. The effect of osteoclast differentiation on P2 receptor expression was not studied here having already been investigated to some extent; a previous report found that, with the exception of the P2Y6 receptor, no changes in P2 receptor mRNA expression occurred during human osteoclast formation in vitro (Buckley et al, 2002). Some cell types preferentially
express particular P2 receptors for example; vascular smooth muscle cells express high levels of P2X<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors but lower levels of P2X<sub>4</sub>, P2X<sub>7</sub>, P2Y<sub>1</sub> and P2Y<sub>4</sub> receptors (Wang et al, 2002). Since both osteoblasts and osteoclasts express several P2 receptors, future work should involve quantitative analysis, by employing techniques such as real time PCR and western blotting, of the relative levels of each P2 receptor subtype on bone cells as they proliferate and differentiate.

Research into purinergic signalling presents some special difficulties; firstly, the fundamental role of nucleotides in cellular biochemistry means that, unlike other signalling factors, ATP (and other nucleotides) cannot be removed from an in vitro system. Secondly, the multiplicity of P2 receptors, the lack of information regarding receptor tertiary structure and the overlap of receptor actions has meant that there are few specific agonists and antagonists available (Burnstock & Knight, 2004). For these reasons, only a small proportion of the P2 receptors expressed on bone cells (P2X<sub>5</sub>, P2X<sub>7</sub>, P2Y<sub>1</sub>, P2Y<sub>2</sub>) have assigned functional effects. In this thesis, the inhibitory effects of ATP and UTP on osteoblast function were shown to be late acting, effectively “switching off” mineralised bone formation in mature cells. Although the P2Y<sub>2</sub> receptor is the most likely mediator of these anti-osteogenic effects in vitro, because there is no selective P2Y<sub>2</sub> receptor antagonist, involvement of the P2Y<sub>4</sub> receptor is possible. However, two approaches could be employed to clarify this issue; either by culture of calvarial cells from knockout animals or by using the technique of RNA interference (RNAi) it should be possible to study the functional responses of osteoblasts lacking the P2Y<sub>2</sub> or the P2Y<sub>4</sub> receptor in vitro. Moreover, RNAi could also be employed to investigate how activation of other P2 receptors (especially where a knockout model is currently unavailable such as the P2Y<sub>6</sub> receptor) influences both osteoblast and osteoclast development and function.

In Chapter 3, closer study of nucleotide-treated osteoblast cultures indicated that ATP and UTP primarily inhibit mineralisation rather than organic matrix deposition. Collagen expression, as assessed by RT-PCR, was slightly decreased by nucleotide treatment; however, collagen formation and deposition seemed relatively unaffected. Further clarification of the effects of extracellular nucleotides on collagen production is now required and could be obtained by electron microscopy or quantitatively by measuring proline incorporation. The impaired mineralisation observed here was due,
at least in part, to decreased alkaline phosphatase (ALP) expression and activity, however, other mechanisms are likely to be involved and this presents an interesting area of future study. Mineralisation involves the coordinated actions of many enzymes (including ALP), ion transporters (annexins) and bone matrix proteins (osteocalcin, osteopontin) (Kirsch, 2005; Balcerzak, 2003), which regulate the levels of Ca\(^{2+}\) and Pi as well as the calcification inhibitor PPI. One potential target of purinergic signalling is the transmembrane protein ANK, which mediates the active transport of PPI across the plasma membrane. Truncation of this protein, as seen in ank/ank null mice, causes hypermineralisation (Ho et al, 2000) and suggests a potential role for ANK in the regulation of mineralisation. Microarray analysis could be employed to investigate changes in osteoblast gene expression following treatment with nucleotide agonists, thus indicating which genes are the target of purinergic signalling and, potentially, other factors (such as ANK) involved in the nucleotide-mediated inhibition of mineralisation. Additionally, microarray could be employed to study the gene expression of nucleotide-treated osteoclasts, thereby providing information regarding the factors mediating the functional effects of nucleotides (including increased resorption) in these cells.

Many cell types, including epithelial and endothelial cells (Bodin & Burnstock, 2001a; Knight et al, 2002), fibroblasts (Gerasimovskaya et al, 2002), chondrocytes (Graff et al, 2000) and osteoblast-like cells (Romanello et al, 2001; Buckley et al, 2003; Genetos et al, 2005) have been shown to release ATP constitutively. In Chapter 4, ATP release from osteoblasts, under basal and stimulated conditions, was investigated using the luciferin-luciferase assay. For the first time, primary rat osteoblasts were shown to constitutively release ATP, probably via vesicular exocytosis, in a differentiation-dependent manner. Transient hypoxia or hyperthermia increased ATP release from osteoblasts 2-3-fold, whereas hypothermia was without effect. Conversely, more prolonged exposures to low oxygen, hyperthermia or hypothermia impaired osteoblast proliferation, function and ATP release. The mechanisms by which osteoblasts sense the sudden change in oxygen tension or temperature, leading to the increased ATP release are unknown at present. Some potential candidates for this role include L-VSCC and TRP channels, which have been discussed in detail in Chapter 4. Since inflammation and hypoxia are commonly associated with a localised acidosis, which is deleterious to the skeleton, the ability of
decreased pH to influence ATP release from osteoblasts presents an interesting
extension to the work performed here. Other cells derived from the
monocyte/macrophage lineage, including macrophages (Beigi & Dubyak, 2000),
release ATP constitutively; to date, basal release of ATP and UTP from osteoclasts has
not been reported but presents an area for future study.

Quantification of ATP release in vitro is complicated by a number of issues; firstly,
the volume of fluid bathing cells is significantly larger in vitro than it is in vivo and,
secondly, rapid hydrolysis or transphosphorylation reactions by ecto-enzymes can
influence the local nucleotide concentration (Lazarowski et al, 2003). Consequently,
ATP levels measured in vitro are likely to underestimate the actual concentration at the
cell membrane; an idea confirmed by studies using luciferase anchored to the platelet
plasma membrane, which measured ATP release in the range 10-15 μM (Beigi et al,
1999). With these factors in mind, ATP release from osteoblasts in vivo could be in
the high nanomolar or low micromolar range rather than the high picomolar/low
nanomolar range measured in this investigation. This could result in a local
concentration sufficient to activate, in an autocrine/paracrine manner, purinergic
signalling in the bone microenvironment.

Uridine-containing nucleotides potently activate a subset of P2Y receptors, with a
broad spectrum of cell responses resulting from receptor stimulation. Although UTP
can be generated from other nucleotides by the activity of extracellular nucleoside
diphosphate kinase (E-NDPK), cellular release of UTP remains poorly researched
(Lazarowski et al, 2000). Using the UDP-glucose pyrophosphorylase assay, UTP
release has been reported from endothelial cells, epithelial cells and platelets
(Cressman et al, 1999; Lazarowski & Harden, 1999). To date there are no reports of
UTP release from osteoblasts but given the widespread expression of several uridine-
sensitive P2Y receptors (Chapter 2) and the ability of UTP to enhance ATP release
(Bowler et al, 2001) and inhibit mineralisation (Chapter 3), it may present a
worthwhile area for future research.

The aim of Chapter 5 was to evaluate, by employing bone densitometry to screen
selected P2 receptor knockout mice, the significance of purinergic receptors on bone
metabolism in vivo. This investigation demonstrated for the first time that P2X2
receptor knockouts displayed an age-related increase in bone mass, whereas P2X2/3
\textsuperscript{dbl/-}. 

174
and P2Y1−/− mice had a decreased bone mass. P2X3 receptor knockout mice exhibited no skeletal changes. The most common application of the PIXImus system, as employed here, is to screen knockout animals for an altered bone phenotype prior to more detailed analysis (see the discussion of Chapter 5 for advantages and disadvantages of DEXA scanning). Since P2X2, P2X2/3 and P2Y1-receptor deficient mice experienced skeletal changes future work could involve bone histomorphometry to determine changes in bone structure on a microscopic scale. Additionally, in vitro culture of cells derived from knockout animals may help to establish whether receptor removal influences osteoblast and/or osteoclast development and/or function. A standard model used to amplify the skeletal effects of a particular factor is to study it in mice already stressed by ovariectomy. This technique has been employed to illustrate the involvement of many molecules in bone metabolism including nitric oxide (Wimalawansa et al, 1996) and osteopontin (Yoshitake et al, 1999). Considering the protective effect oestrogens exert on the skeleton and the negative effects extracellular nucleotides have on bone remodelling, it would be of interest to challenge the P2 receptor knockouts with ovariectomy to determine whether differential effects on bone mass occur.

Expression of multiple P2 receptor subtypes is now considered the norm in most tissues including bone [see review by Burnstock & Knight, 2004]. It is well known that P2X receptor subunits interact to form homomultimers or heteromultimers, with differing pharmacological activities (see Chapter 1 for overview); however, at present, expression of heteromeric P2X receptors in bone cells has not been reported. Over recent years, experimental evidence using immunoprecipitation and ligand-binding analysis challenged the view that G-protein coupled receptors (GPCRs) exist and function as monomers; instead it is now widely accepted that many GPCRs form functional homodimers, heterodimers or oligomeric complexes [see review by Milligan, 2004]. The first purine receptor reported to form heteromeric complexes was the adenosine A1 receptor, which forms functional heteromultimers with dopamine receptors (Gines et al, 2000). The ability of the adenosine A2A receptor to also form heteromeric complexes with dopamine receptors was reported recently (Fuxe et al, 2005). The ability of P2Y receptors to form homo- or heteromultimeric assemblies is less well studied, however, one report by Wang et al (2002) reported the presence of a 180-kd protein, which could correspond to a P2Y1 receptor tetramer, in smooth muscle
cells. Moreover, it was shown recently that the A<sub>1</sub> receptor forms a heteromeric complex with P2Y<sub>1</sub> receptors in rat brain tissue, producing an adenosine receptor with P2Y-like-agonist pharmacology (Nakata <i>et al</i>, 2005; Yoshioka <i>et al</i>, 2002). Given that bone cells express many GPCRs, including at least four P2Y receptor subtypes, it is possible that they form heteromultimers (such as P2Y<sub>2</sub> and P2Y<sub>4</sub>) that influence bone cell function in a different manner to homomeric complexes. Investigation into the expression of heteromeric P2Y and P2X receptor complexes on bone cells is now required.

To be physiologically relevant regulators of bone remodelling, extracellular nucleotides must be delivered into the bone microenvironment in a controlled manner. Once released nucleotides are rapidly broken down by an extracellular hydrolysis cascade that results in the formation of the respective nucleoside and free phosphate (Pi). Molecular and functional characterisation has shown that there are several families of ecto-nucleotidases; these include (1) the E-NTPDase (ecto-nucleoside triphosphate diphosphohydrolase) family, (2) the E-NPP (ecto-nucleotide pyrophosphatase/ phosphodiesterase) family, (3) alkaline phosphatases and, (4) ecto-5'-nucleotidase (Zimmerman, 2000). Ecto-nucleotidases are usually membrane bound, but can be cleaved to produce soluble isoforms often referred to as exo-nucleotidases. Many of these ecto-nucleotidase families have overlapping specificities; for example members of the E-NTPDase (also referred to as ecto-ATPase or ecto-apyrase) family catalyse reactions including NTP $\rightarrow$ NDP + Pi and NDP $\rightarrow$ NMP + P Pi, whereas members of the E-NPP family hydrolyse NTP $\rightarrow$ NMP + P Pi or NDP $\rightarrow$ NMP + Pi. Thus, the combined activities of these ecto-enzymes limit the actions of extracellular nucleotides to cells within close proximity of the release site.

The expression of ecto-nucleotidases in bone remains poorly investigated; therefore little is known about ATP hydrolysis in bone under normal and pathological conditions, although <i>in vitro</i>, ATP has a reported half-life of between 50 seconds (Bowler <i>et al</i>, 2001) and ~ 5 minutes (Chapter 4). Osteoblasts are known to express tissue non-specific alkaline phosphatase and PC-1 (E-NPP1), both of which are important in the regulation of mineralisation (Johnson <i>et al</i>, 2000). P Pi is a potent inhibitor of mineralisation and can be produced by the hydrolysis of extracellular nucleotide triphosphates. Both ATP and UTP inhibit mineralisation at concentrations.
of 1-100 μM (Chapter 3; Hoebertz et al, 2002), an effect thought to be mediated via
the P2Y₂ and possibly the P2Y₄ receptor. However, an intriguing possibility is that
hydrolysis of extracellular nucleotides by PC-1 results in excess PPI, which then acts
directly to inhibit hydroxyapatite crystal formation and mineralisation. Although
quantitative physiologically relevant data are scarce; using femurs derived from 9-day-
old chick embryos, Fleisch et al (1965) demonstrated that low micromolar
concentrations of PPI inhibited calcification. Moreover, a recent investigation reported
that 30 μM ATP produces ~ 4 μM PPI in vitro (Zhong et al, 2005). In an attempt to
clarify whether these inhibitory effects are direct or P2 receptor mediated, studies are
ongoing to determine the levels of PPI that block mineralisation and the extracellular
levels of PPI following treatment with nucleotides in our in vitro nodule system.

The rapid hydrolysis of extracellular nucleotides means that, once released, they
must act relatively quickly on their target cells to induce signalling events and
functional effects. The duration of cellular responses to nucleotide stimulation is less
clear, although, as shown in Chapter 2, a single application of ATP to mature rat
osteoblasts had lasting and significant effects on bone formation. Due to differential
rates of release, breakdown and diffusion, ATP concentrations will vary in both time
and space, thus the local environment surrounding one cell is likely to differ to that
experienced even a few cell diameters away. Furthermore, as osteoblasts express the
interconversion enzyme E-NDPK (Buckley et al, 2003), relative levels of different
nucleotide agonists are also likely to vary in the bone local environment. Since the
effects of extracellular nucleotides on bone cell function and apoptosis are
concentration and agonist dependent, the rate of release, interconversion and
breakdown will directly influence purinergic signalling and make any functional
effects highly localised.

In many cell types, including erythrocytes (Bergfeld & Forrester, 1992),
endothelial cells (Bodin & Burnstock, 1995), fibroblasts (Gerasimovskaya et al, 2002)
and osteoblasts (Chapter 4; Genetos et al, 2005), ATP release is enhanced by stressful
stimuli including hypoxia. Pathological situations such as fracture are commonly
characterised by both inflammation (including hyperthermia) and hypoxia and thus are
likely to be associated with an increased extracellular nucleotide concentration.
Moreover in such situations, ATP levels can be further enhanced by the ability of
extracellular nucleotides to induce their own release, leakage from damaged cells and/or release from other cell types (including immune cells and platelets) in the vicinity (Bodin & Burnstock, 1996).

Once released, extracellular nucleotides can potentially act in several ways, either by directly modulating bone cell function or indirectly by stimulating the release of other local factors important in bone metabolism. For example, ATP secreted in response to shear stress mediates enhanced PGE$_2$ release from osteoblast-like cells (Genetos et al, 2005) and endothelial cells (Ostrom et al, 2000). The localised acidosis associated with inflammation and hypoxia will provide the low pH necessary for osteoclast activation; thus, ATP and its degradation product ADP can act through an autocrine/paracrine loop to directly stimulate osteoclastic resorption of damaged bone (Hoebertz et al, 2001). On mature osteoblasts, ATP can signal to down-regulate ALP expression and activity, arresting mineralisation and preventing further bone formation (Chapter 2 and 3). Moreover, if the extracellular concentration is high enough ATP may function via the P2X$_7$ receptor to induce osteoblast apoptosis (Gartland et al, 2001).

The inflammatory response following tissue injury recruits cells such as macrophages, dendritic cells and monocytes to the damaged region. Extracellular ATP can enhance the release of proinflammatory cytokines, including IL-1$\alpha$/-1$\beta$ (Ferrari et al, 1997; Perregaux & Gabel, 1998; Ferrari et al, 2000), IL-6 (Ihara et al, 2005; Hanley et al, 2004) and TNF-$\alpha$ (Ferrari et al, 2000), from many of these cells. Once released, these cytokines could act directly on bone cells to modulate their function. For example, IL-1 can promote mature osteoclast survival and resorption (Jimi et al, 1998; Xing et al, 2003), whilst also suppressing osteoblast function (Kwan et al, 2004). Thus, the primary event following a sudden stress situation could be increased osteoclastic resorption and removal of any damaged bone (an important process in fracture healing). ATP can act synergistically with TNF-$\alpha$ in the activation and maturation of dendritic cells (Schnurr et al, 2000); whether the same happens during the stimulation of osteoclastogenesis and/or resorption is unknown at present but presents an interesting area of future study. Based on the evidence presented here, it is possible that extracellular nucleotides play a role in cellular responses after an initial
exposure to a stress situation, a model for this involvement is summarised in Figure 6.1.

Many pathological situations characterised by chronic hypoxia, hypothermia and/or hyperthermia are also associated with increased bone loss and include inflammatory conditions such as rheumatoid arthritis (Schett & Smolen, 2005). Long-term exposure to hypoxia decreases the expression of common osteoblastic markers such as osteocalcin and ALP and is likely to impair osteoblast differentiation (Utting et al, submitted), whilst increasing osteoclast formation and activity (Arnett et al, 2003). Furthermore, both hyperthermia and hypothermia have marked effects on osteoblast (Chapter 4) and osteoclast function (Utting et al, 2005). However, because nucleotide release from osteoblasts is generally decreased following longer-term exposures to these stressful stimuli, purinergic signalling is less likely to mediate the bone loss occurring in these situations.

As shown in this thesis, P2 receptor expression on osteoblasts is dependent on cellular differentiation. Given that chronic hypoxia impairs osteoblast differentiation, the P2 receptor expression profile of cells exposed to these conditions may well be different to normal. Therefore, it would be of interest to study expression of P2 receptors in bone cells subjected to hypoxia, hypothermia, hyperthermia and acidosis. An effective way to screen for changes in gene expression in these situations would be to use microarray analysis.

In summary, the work presented in this thesis has helped to elucidate the functions of nucleotides on bone cell function. A speculative model for the role and interactions of P2 receptors on bone cells, based on the present results, is shown in Figure 6.1.
Figure 6.1. A speculative role for ATP once released from osteoblasts following transient hypoxia or hyperthermia or loading

Purinergic signalling following increased ATP from osteoblasts following transient hypoxia or hyperthermia. Potential cellular mechanisms for sensing hypoxia and temperature include HIF, L-VSCC channels and TRP channels (see Chapters 1 and 4).


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# APPENDIX I

## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>2-MeSADP</td>
<td>2-MethylthioADP</td>
</tr>
<tr>
<td>2-MeSATP</td>
<td>2-MethylthioATP</td>
</tr>
<tr>
<td>αβ-meATP</td>
<td>α,β-methylene ATP</td>
</tr>
<tr>
<td>ABC proteins</td>
<td>ATP binding cassette proteins</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylate cyclase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AM</td>
<td>Acetomethoxyester</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One way analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activating protein 1</td>
</tr>
<tr>
<td>ARNT</td>
<td>Arylhydrocarbon nuclear transporter</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPγS</td>
<td>Adenosine 5'-O-(3-thiotriphosphate)</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone mineral content</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BMU</td>
<td>Bone multicellular unit</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone sialoprotein</td>
</tr>
<tr>
<td>Bz-ATP</td>
<td>2',3'-O-(benzoyl-4-benzoyl)-ATP</td>
</tr>
<tr>
<td>cAMP</td>
<td>3'-5' cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CRE</td>
<td>Cyclic AMP response element</td>
</tr>
<tr>
<td>CB₁</td>
<td>Cannabinoid type I receptor</td>
</tr>
<tr>
<td>Cbfα1</td>
<td>Core binding factor α1</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged coupled device</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>CCAAT/enhancer binding protein alpha</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis conductance regulator</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Cl</td>
<td>Chloride ions</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COL1</td>
<td>Type I collagen</td>
</tr>
<tr>
<td>Csps</td>
<td>Cold shock proteins</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual energy x-ray absorptiometry</td>
</tr>
<tr>
<td>Dhh</td>
<td>Desert hedgehog</td>
</tr>
<tr>
<td>DIDS</td>
<td>4,4'-diisothiocyanostilbene-2,2'disulphonic acid</td>
</tr>
<tr>
<td>Dlx5</td>
<td>Distal-less gene 5</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Essential Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>E-NPP</td>
<td>Ecto-nucleotide pyrophosphatase/ phosphodiesterase</td>
</tr>
<tr>
<td>E-NTPDase</td>
<td>Ecto-nucleoside triphosphate diphosphohydrolase</td>
</tr>
<tr>
<td>E-NDPK</td>
<td>Ecto-nucleoside diphosphokinase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Erg-1</td>
<td>Early growth response factor 1</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FLIPR</td>
<td>Fluorescence imaging plate reader</td>
</tr>
<tr>
<td>Gd³⁺</td>
<td>Gadolinium</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>H⁺</td>
<td>Protons</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks balanced salt solution</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HOBIT</td>
<td>Human osteoblast-like initial transfectant cells</td>
</tr>
<tr>
<td>HREs</td>
<td>Hormone response element</td>
</tr>
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<td>HRE</td>
<td>Hypoxia response element</td>
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<tr>
<td>HSE</td>
<td>Heat shock element</td>
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<td>HSFs</td>
<td>Heat shock factors</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Hsps</td>
<td>Heat shock proteins</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin growth factor</td>
</tr>
<tr>
<td>Ihh</td>
<td>Indian hedgehog</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of NFκB</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon-γ</td>
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<tr>
<td>IP₃</td>
<td>Inositol triphosphate</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>K⁺</td>
<td>Potassium ions</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>LH</td>
<td>Lysyl hydroxylase</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
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<tr>
<td>MGP</td>
<td>Matrix gla protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
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<td>MRS2179</td>
<td>N⁶-methyl-2'-deoxyadenosine-3',5'-bisphosphate</td>
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<td>2-chloro-N⁶-methyl-(N)-methanocarba-2'-deoxyadenosine-3',5'-bisphosphate</td>
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<td>MSCC</td>
<td>Mechanosensitive cation selective channel</td>
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<td>Muscle segment homologue 2</td>
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<tr>
<td>N₂</td>
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<tr>
<td>Na⁺</td>
<td>Sodium ions</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
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<td>NCP</td>
<td>Non collagenous proteins</td>
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<tr>
<td>NEM</td>
<td>n-ethylmaleimide</td>
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<tr>
<td>NFATc1</td>
<td>Nuclear factor of activated T cells 1</td>
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<td>NFκB</td>
<td>Nuclear factorκB</td>
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<td>NHS</td>
<td>Normal horse serum</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
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<td>Nanometre</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
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<tr>
<td>O₂</td>
<td>Oxygen</td>
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<tr>
<td>OCN</td>
<td>Osteocalcin</td>
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<td>ODF</td>
<td>Osteoclast differentiation factor</td>
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<td>ON</td>
<td>Osteonectin</td>
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<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
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<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>OSE</td>
<td>Osteoblast-specific cis-acting elements</td>
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<td>Osx</td>
<td>Osterix</td>
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<td>P₂X₂⁻⁻</td>
<td>P₂X₂ receptor knockout</td>
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<tr>
<td>P₂X₂/P₂X₃⁻⁻</td>
<td>P₂X₂/P₂X₃ double receptor knockout</td>
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<td>P₂X₃⁻⁻</td>
<td>P₂X₃ receptor knockout</td>
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<tr>
<td>P₂Y₁⁻⁻</td>
<td>P₂Y₁ receptor knockout</td>
</tr>
<tr>
<td>PC-1</td>
<td>Plasma membrane glycoprotein 1</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PGE₂</td>
<td>Prostaglandin E₂</td>
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<tr>
<td>Pi</td>
<td>Orthophosphate</td>
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<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<td>PO₂</td>
<td>Oxygen tension</td>
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<td>Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid</td>
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<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor gamma</td>
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<tr>
<td>PPI</td>
<td>Pyrophosphate</td>
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<tr>
<td>pQCT</td>
<td>Peripheral quantitative computed tomography</td>
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<td>PTH</td>
<td>Parathyroid hormone</td>
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<tr>
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<td>Parathyroid hormone related peptide</td>
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<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa B ligand</td>
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<td>Integrin binding consensus sequence Arg-Gly-Asp</td>
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<td>Ribonucleic acid</td>
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<td>RNAi</td>
<td>RNA interference</td>
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<td>Definition</td>
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<tr>
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<td>------------</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<td>Runx2</td>
<td>Runt related transcription factor 2</td>
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<td>Shh</td>
<td>Sonic hedgehog</td>
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<td>SIBLINGS</td>
<td>Small integrin binding ligand N-linked glycoproteins</td>
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<td>SRE</td>
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<td>Sulfonylurea receptor</td>
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<td>Transforming growth factor beta</td>
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<td>TM</td>
<td>Transmembranous domain</td>
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<td>TNFα</td>
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<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
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<td>TNP-ATP</td>
<td>2',3'-O-(2,4,6-trinitrophenyl)-ATP</td>
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<td>TRANCE</td>
<td>TNF-related activation induced cytokine</td>
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<td>TNFR-associated factor</td>
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<tr>
<td>TRACP</td>
<td>Tartrate-resistant acid phosphatase</td>
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<tr>
<td>TRP</td>
<td>Transient receptor potential</td>
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<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<tr>
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<td>Micromolar</td>
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<tr>
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<td>Uridine triphosphate</td>
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<td>Vascular endothelial growth factor</td>
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<td>VSCC</td>
<td>Voltage sensitive calcium channels</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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</table>
APPENDIX II

LIST OF PUBLICATIONS

Papers


Orriss IR, Knight GE, Ranasinghe S, Burnstock G and Arnett TR. 2005. Osteoblast responses to nucleotides increase during differentiation with time in culture. Submitted to Bone.


Abstracts


