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Hypoxia Stimulates Vesicular ATP Release From Rat Osteoblasts

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Many neuronal and non-neuronal cell types release ATP in a controlled manner. After release, extracellular ATP (or, following hydrolysis, ADP) acts on cells in a paracrine manner via P2 receptors. Extracellular nucleotides are now thought to play an important role in the regulation of bone cell function. ATP (and ADP), acting via the $P2Y_1$ receptor, stimulate osteoclast formation and activity, whilst $P2Y_2$ receptor stimulation by ATP (or UTP) inhibits bone mineralization by osteoblasts. We found that rat calvarial osteoblasts released ATP constitutively, in a differentiation-dependent manner, with mature, bone-forming osteoblasts releasing up to sevenfold more ATP than undifferentiated, proliferating cells. The inhibitors of vesicular exocytosis, monensin, and *N*-ethylmaleimide (1–1,000 μM) inhibited basal ATP release by up to 99%. The presence of granular ATP-filled vesicles within the osteoblast cytoplasm was demonstrated by quinacrine staining. Exposure to hypoxia (2% O_2) for up to 3 min increased ATP release from osteoblasts up to 2.5-fold without affecting cell viability. Peak concentrations of ATP released into culture medium were >1 μM, which equates with concentrations known to exert significant effects on osteoblast and osteoclast function. Monensin and *N*-ethylmaleimide (100 μM) attenuated the hypoxia-induced ATP release by up to 80%. Depletion of quinacrine-stained vesicles was also apparent after hypoxic stimulation, indicating that ATP release had taken place. These data suggest that vesicular exocytosis is a key mediator of ATP release from osteoblasts, in biologically significant amounts. Moreover, increased extracellular ATP levels following acute exposure to low O_2 could influence local purinergic signaling and affect the balance between bone formation and bone resorption.

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It is well recognized that extracellular nucleotides, signaling via the P2 receptors, participate in a wide number of biological processes in both neuronal and non-neuronal tissues (see reviews by Burnstock and Knight, 2004; Burnstock, 2007a). P2 receptors are subdivided into the P2X ligand-gated ion channels and P2Y G-protein-coupled receptors (Kennedy and Burnstock, 1985; Abbracchio and Burnstock, 1994). Currently, seven P2X receptors ($P2X_{1-7}$) and eight P2Y ($P2Y_{1, 2, 4, 6, 11-14}$) receptors have been identified; each of these receptors has been cloned, characterized and displays distinct tissue expression and pharmacology (Ralevic and Burnstock, 1998; Burnstock, 2007b). P2 receptors respond to a range of adenine and uridine-containing nucleotides including adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine triphosphate (UTP), and uridine diphosphate (UDP).

In recent years, it has become evident that extracellular nucleotides may play a significant role in bone biology modulating both osteoblast and osteoclast function (see reviews by Gallagher and Buckley, 2002; Hoebertz et al., 2003). Osteoblasts, the bone-forming cells, express at least seven different P2 receptor subtypes ($P2X_2$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$) in a differentiation-dependent manner (Orriss et al., 2006). Osteoclasts, the bone resorbing cells, also express multiple P2 receptors ($P2X_2$, $P2X_4$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) (Hoebertz et al., 2003). Functional effects of purinergic signaling on bone cells include increased osteoblast proliferation (Nakamura et al., 2000), decreased bone mineralization by osteoblasts (Orriss et al., 2007), induction of osteoblastic membrane blebbing (Panupinthu et al., 2007), enhanced osteoclast formation and activity (Hoebertz et al., 2001), and the production of lipid mediators such as lysophosphatidic acid (Panupinthu et al., 2008).

ATP is present in cell cytoplasm at concentrations between 2 and 5 mM. Following membrane damage or necrosis, all cells can potentially release ATP into the extracellular environment, which can then act in an autocrine/paracrine manner to influence local purinergic signaling. In addition, numerous excitatory and non-excitatory cells including epithelial and endothelial cells (Bodin and Burnstock, 2001a; Knight et al., 2002), platelets (Beigi et al., 1999), fibroblasts (Gerasimovskaya et al., 2002), chondrocytes (Graff et al., 2000), erythrocytes (Sprague et al., 1998), and astrocytes (Coco et al., 2003) release ATP in a controlled manner. Constitutive ATP release has also been reported from a number of osteoblast-like cells lines (Romanello et al., 2001; Buckley et al., 2003; Genetos et al., 2005).

The mechanisms mediating constitutive ATP release remain unclear but differences between cell types are emerging. Current hypotheses involve: (1) ATP binding cassette (ABC) transporters, (2) vesicular exocytosis, perhaps involving lysosomes (Zhang et al., 2007), (3) gap junctions and connexion or pannexin hemichannels (Lazarowski et al., 2003; Spray et al., 2006), (4) maxi anion channels (Sabirov and Okada, 2005), and (5) the $P2X_7$ receptor (Suadicani et al., 2006). Several ABC proteins are potential candidates for mediating ATP release including the cystic fibrosis transmembrane conductance regulator (CFTR). The CFTR has been implicated in ATP release from several cell types including erythrocytes (Sprague et al., 1998). Controlled vesicular exocytosis is implicated in ATP release from epithelial and endothelial cells (Bodin and

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Burnstock, 2001a; Knight et al., 2002). Additionally, it has been suggested that ATP release from osteoblast-like cells occurs, at least in part, via vesicular mechanisms (Genetos et al., 2005; Romanello et al., 2005). Finally, connexin hemichannels and gap junctions, which allow the movement of molecules less than 1 kDa, reportedly act as a conductive pathway to mediate ATP release from astrocytes (Cotrina et al., 1998; Coco et al., 2003). In non-neuronal cells, controlled ATP release is significantly increased by pathophysiological stimuli including hypoxia (Bergfeld and Forrester, 1992; Bodin and Burnstock, 1995; Gerasimovskaya et al., 2002), fluid shear stress (Bodin and Burnstock, 2001a; Genetos et al., 2005), mechanical perturbations (Romanello et al., 2001), and some agents such as veratridine (Bodin and Burnstock, 2001b).

Hypoxia, which is associated with tissue damage, inflammation, bone fracture and tumors, strongly inhibits osteoblast function (Utting et al., 2006) whilst stimulating osteoclast formation (Arnett et al., 2003). The objective of this study was firstly to examine ATP release from primary rat osteoblasts at all stages of differentiation and, secondly, to determine whether hypoxia could enhance ATP release from osteoblasts and thus potentially contribute to the potent effects of reduced O_2 on bone cells.

Methods

Reagents

All tissue culture reagents were purchased from Gibco (Paisley, UK); unless otherwise mentioned, all chemicals were purchased from Sigma–Aldrich (Poole, Dorset, UK). The CytoTox 96® non-radioactive cytoxicity assay was obtained from Promega UK (Southampton, UK) and ATP monitoring reagent from Biothema (Handen, Sweden). Cylinders containing custom mixtures of O_2, CO_2, and N_2 were purchased from BOC (London, UK).

Cell culture

Primary rat osteoblast cells were obtained by sequential enzyme digestion of excised calvarial bones from 2-day-old neonatal Sprague–Dawley rats using a three-step process (1% trypsin in PBS for 10 min; 0.2% collagenase type II in Hanks balanced salt solution (HBSS) for 30 min; 0.2% collagenase type II in HBSS for 60 min). The first two digests were discarded and the cells resuspended in Dulbecco's Modified Essential Medium (Gibco) supplemented with 10% fetal calf serum (FCS), 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% CO_2–95% air at 37°C in 75 cm^2 flasks until confluent. Upon confluence, cells were sub-cultured into 6-well trays in DMEM supplemented with 2 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and 10 nM dexamethasone (mixture abbreviated to "supplemented DMEM"), with half-medium changes every 3 days. Throughout the culture, medium pH, pCO_2, and pO_2 were monitored using a blood gas analyzer (ABL-705, Radiometer, Crawley, UK).

Measurement of ATP release under normal and hypoxic conditions

Prior to measurement of ATP release, culture medium was removed, cell layers washed, and cells incubated with serum-free DMEM (1 ml/well). With the exception of the experiment in Figure 1A, samples were collected after 1 h and immediately snap-frozen on dry ice for later ATP quantification. In the experiments examining release mechanisms, vesicular inhibitors (1–1,000 μM monensin, brefeldin A, *N*-ethylmaleimide (NEM), and gadolinium (Gd^{3+})) were added to the serum-free DMEM. Since increased intracellular Ca^{2+} has also been implicated in ATP release, the calcium ionophore ionomycin (0.1–10 μM) was tested for potential effects.

To examine the effects of long-term hypoxia on basal ATP release, plates containing osteoblasts were positioned into gas-tight "hypoxia" boxes 24 h after seeding and gassed daily for 90 sec with 20% or 2% O_2 (with half-medium changes every 3 days). Cell layers were prepared for ATP determination as described above before regassing to ensure the appropriate O_2 concentration; samples were collected 1 h after medium exchange and gassing.

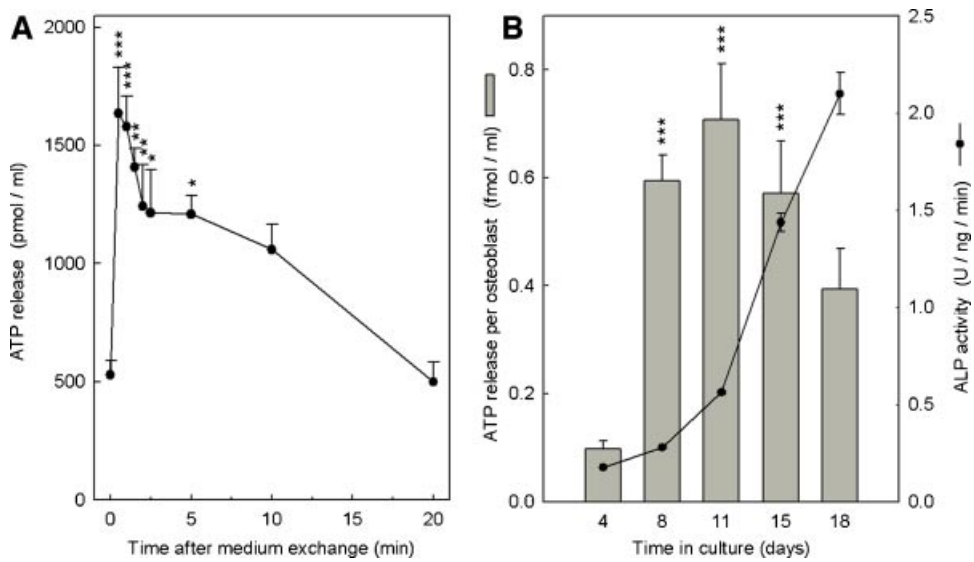

Fig. 1. The effects of medium exchange and osteoblast differentiation on constitutive ATP release. ATP release was measured at regular intervals up to 30 min after exchange to serum-free DMEM. A: Medium exchange (t = 0) induced a rapid threefold increase in ATP release from osteoblasts; levels returned to baseline within 20 min. B: ATP release per osteoblast increased up to sevenfold at 8, 11, and 15 days of culture compared to day 4 (samples were collected 1 h after medium exchange). ALP activity was used as a marker of osteoblast differentiation; highlighting the progression from precursor to mature osteoblast. ALP activity increased 12-fold between days 4 and 18 of culture. Values are means ± SEM (n = 12); significantly different from controls: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

To determine the effect of transient hypoxia on ATP release, test plates minus lids were sealed in "hypoxia" chambers 1 h after exchange to serum-free DMEM and gassed with 20% or 2% O_2. Samples were collected at regular intervals between 0.5 and 11.5 min. All determinations were repeated 12 times.

Luciferin-luciferase assay

ATP release was measured luminetrically using the *luciferin-luciferase* assay. ATP standards (10 pM–1 μM) and test samples were pipetted into a white (non-phosphorescent) 96-well plate and sited in the luminometer (Lucy 1, Anthos Labtec, Salzburg, Austria). The luciferin-luciferase reagent was automatically injected into each well and measured for 10 sec at 560 nm. All standards, blanks, and the lyophilized ATP reagent containing D-luciferin and luciferase were dissolved in serum-free DMEM. A calibration curve was constructed using ATP standards and used to calculate ATP levels in test samples.

Determination of alkaline phosphatase (ALP) activity

The alkaline phosphatase (ALP) activity of cell lysates was determined colorimetrically (Bio-Tek EL_X800 plate reader, Fisher Scientific, Loughborough, UK) using commercially available kits (Biotron Diagnostics, Hemet, CA and Sigma–Aldrich): this assay uses *p*-nitrophenyl phosphate as a substrate, which in the presence of ALP, is converted to the yellow chromogen *p*-nitrophenyl. Osteoblasts were cultured in 6-well trays and enzyme activity measured after 4, 8, 11, 15, and 18 days. To assay ALP activity, cell layers were washed and cells harvested using a scraper ($n = 6$) followed by sonication at 4°C and centrifugation at 500*g*. The supernatant was collected and stored at 4°C until assaying at pH 9.8. Total protein in cell lysates was determined using the Bradford assay (Sigma–Aldrich). Determinations were repeated six times.

Assessment of plasma membrane integrity

Cell viability following exposure to hypoxia was assessed using the Promega CytoTox 96® cytoxicity assay. The assay measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released on cell lysis. LDH oxidizes lactate into pyruvate, generating NADH, which is then used to convert a tetrazolium salt into a red formazan product. The amount of color formed is proportional to the number of lysed cells.

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*g*. The LDH content of the supernatants and cell lysates were measured colorimetrically (490 nm) (EL_X800 plate reader, Biotek Elx800 plate reader, Fisher Scientific, Loughborough, UK) as per manufacturer's instructions. By expressing released LDH as a percentage of the total cellular LDH cell viability could be calculated.

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. MTT reagent (100 μg dissolved in 200 μl DMEM) was added to each cell layer and plates were incubated in the dark at 37°C for 5 h; culture medium was then removed carefully and formazan crystals were solubilised by the addition of 200 μl dimethyl sulphoxide (DMSO). Absorbance was read at 550 and 650 nm in a plate reader (EL_X800, Bio-Tek International); a standard curve for determination of cell numbers was constructed using cells seeded at 10^2–10^6/well.

Quinacrine staining

The acridine derivative, quinacrine, is a weak-base that binds ATP with a high affinity. When excited by light at 476 nm it emits fluorescence in the 500–540 nm range and is widely used to visualize ATP-containing sub-cellular compartments in live cells (Irvin and Irvin, 1954; Olson et al., 1976). Osteoblasts were seeded onto sterile 1 cm diameter discs, cut from Melinex (Du Pont Teijin Films, Dumfries, UK) clear polyester film, in 24-well trays at 2.5×10^4 cells/disc and cultured in supplemented DMEM for 7–10 days. To visualize ATP-filled vesicles, Melinex discs were twice washed with PBS before incubation with 30 μM quinacrine for 1 h; discs were washed twice more and mounted onto microscope slides. The cells were immediately observed using fluorescence microscopy with a digital camera attachment (AxioCam MRc5, Imaging Associates Ltd, Bicester, UK). Inhibitor effects were investigated by simultaneous incubation with quinacrine (30 μM) and either monensin (100 μM) for 1 h or NEM (100 μM) for 15 min. To examine the effects of hypoxia, cells were exposed to 20% or 2% O_2 for 0.5–3 min immediately prior to visualization.

Statistical analysis

Statistical comparisons were made by one-way analysis of variance and adjusted using the Bonferroni method (In Stat 3, GraphPad, San Deigo, CA). Representative data are presented as means ± SEM for 6–12 replicates. Results presented are for representative experiments that were each repeated at least three times ($P < 0.05$ was taken as significant).

Results

Medium replacement stimulates ATP release from osteoblasts

The FCS in osteogenic growth medium contains factors inhibitory to the *luciferin-luciferase* assay. Therefore, it was necessary to transfer osteoblasts to serum-free DMEM for experimental measurement of ATP release. Since shear stress is a known stimulator of ATP release from cells, we first investigated whether the mechanical disturbances arising during exchange of culture medium could exert a similar stimulatory effect. ATP release was observed to increase approximately threefold within 30 sec of medium exchange (Fig. 1A). The ATP levels in culture medium remained significantly elevated for up to 5 min, returning to basal levels within 20 min. Therefore in all subsequent experiments, culture plates were left for 1 h following the change to serum-free medium before measurement of ATP levels, so as to ensure complete degradation of ATP released due to mechanical perturbation.

Constitutive ATP release increases with osteoblast differentiation

When cultured in osteogenic growth medium, cells derived from rat calvaria differentiate from an immature, proliferative phenotype into mature, bone-forming osteoblasts. To determine whether cellular differentiation influenced constitutive ATP release from osteoblasts, ALP activity (a marker of osteoblast differentiation), cell number, and ATP release were measured after 4, 8, 11, 15, and 18 days of culture. ALP activity progressively increased throughout the culture, peaking at day 18, at which time levels were 12-fold higher than day 4 (Fig. 1B). Abundant bone formation was normally observed from day 10 onwards. Compared to day 4, ATP release per osteoblast was increased six- and sevenfold at days 8 and 11, respectively; from day 15 onwards osteoblastic ATP release began to decline but remained at least fourfold

higher than at day 4 (Fig. 1B). Measurement of osteoblast viability consistently showed no significant changes in the level of cell lysis throughout the culture period (data not shown).

ATP release from osteoblasts involves vesicular exocytosis

To investigate whether exocytosis mediates ATP release from primary rat osteoblasts, the inhibitors monensin, NEM, and brefeldin A (1–1,000 μM) were added to the culture medium 1 h prior to assay. Treatment with 1, 10, and 100 μM monensin decreased ATP release by 40%, 45%, and 50%, respectively (Fig. 2A). NEM, at concentrations of 100 and 1,000 μM inhibited ATP release by 90% and 99%, respectively (Fig. 2B). Although a downward trend was observed, brefeldin A did not significantly affect constitutive ATP release from osteoblasts (Fig. 2C).

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+}) blocks several cation channels including the mechanosensitive cation-selective channel (MSCC). This channel, which is expressed on osteoblasts (Ryder and Duncan, 2001), facilitates rapid Ca^{2+} entry and is implicated in increased intracellular Ca^{2+} in response to shear stress. Treatment with 1–100 μM Gd^{3+} had no effect on constitutive ATP release from osteoblasts (Fig. 2D). Ionomycin, which increases intracellular Ca^{2+} by acting as a cation carrier, increased ATP release by 40% and 50%, respectively when applied to osteoblasts at concentrations of 1 and 10 μM (Fig. 2E).

Quinacrine staining of ATP vesicles

In untreated osteoblasts, abundant cytoplasmic quinacrine staining, with a clear granular–vesicular appearance was observed (Fig. 3A,B). Treatment with 100 μM monensin (Fig. 3C) resulted in a marked reduction in cytoplasmic fluorescence. After treatment with NEM small reductions only in fluorescence were evident (Fig. 3D).

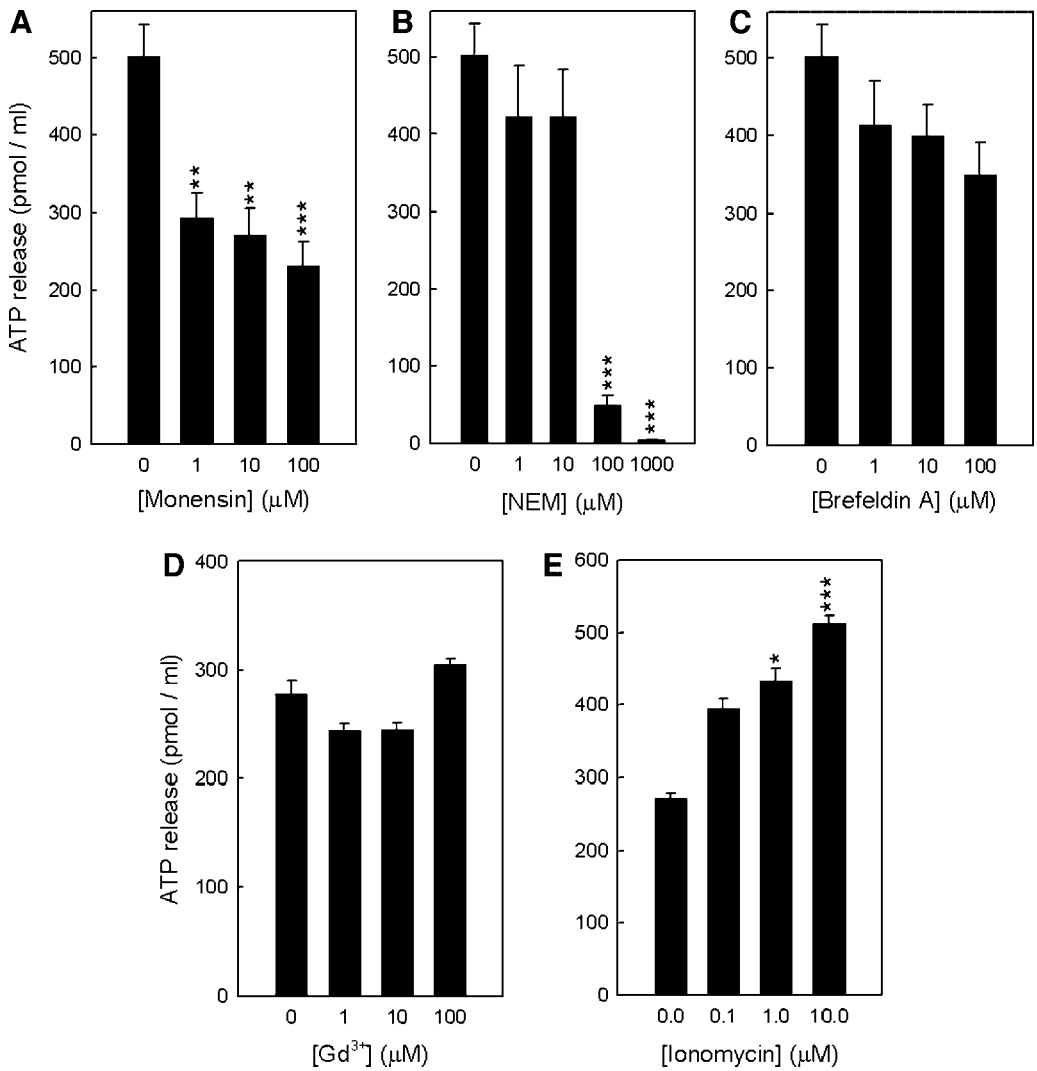

Fig. 2. ATP release from osteoblasts is mediated by vesicular exocytosis. Vesicular exocytosis inhibitors (A) 1–100 μM monensin and (B) 100–1,000 μM NEM blocked ATP release from osteoblasts by 50% and 90%, respectively, whereas (C) brefeldin A was without effect. D: The MSCC channel blocker Gd^{3+} did not affect ATP release from osteoblasts. E: The Ca^{2+} ionophore ionomycin increased ATP release by up to 50%. Samples were collected for measurement of ATP levels 1 h after exchange to serum-free medium. Values are means ± SEM (n = 12); significantly different from controls: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

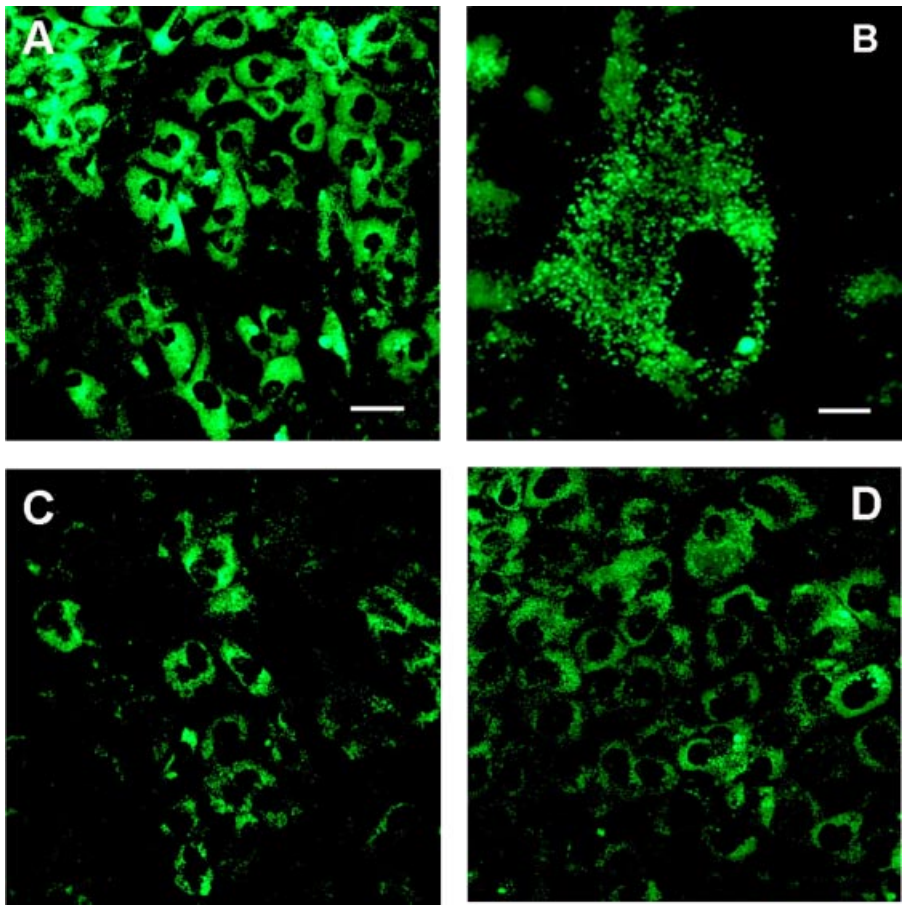

Fig. 3. Quinacrine staining of ATP-filled vesicles in osteoblasts. A: The high level of fluorescence distributed within the cytoplasm of all osteoblasts demonstrates intracellular stores of ATP. The high power image in (B) shows the fluorescence within osteoblasts is mainly distributed in granular vesicles. (C) The level of fluorescence within the cytoplasm was decreased following treatment with 100 μM monensin. D: Treatment with NEM only resulted in small decreases in cytoplasmic fluorescence. Scale bars: A, C, D = 50 μm, B = 5 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Transient exposure to hypoxia stimulates ATP release from osteoblasts

Primary rat osteoblasts were cultured until the onset of bone nodule formation (~10 days) before exposure to 20% or 2% O_2 for 0.5–11.5 min. Two percent O_2 for up to 1.5 min caused a rapid, transient increase in ATP release; after 0.5 and 1.5 min, ATP levels were 2.5- and 2-fold higher, respectively (Fig. 4A). Hypoxia for periods in excess of 2 min resulted in smaller non-significant increases in ATP secretion. In control cultures gassed with 20% O_2, the level of ATP release did not vary significantly. Short-term exposure to 20% or 2% O_2 did not affect osteoblast viability (determined by assaying culture medium LDH levels) at any stage (Fig. 4B). Exposure to 2% O_2 for 3 min consistently reduced cytoplasmic quinacrine fluorescence, compared to 20% O_2 controls (Fig. 4C).

To determine the effects of continuous exposure to hypoxia, osteoblasts were cultured in 20% or 2% O_2 for 8 and 15 days. Since 2% O_2 strongly inhibits osteoblast proliferation (Utting et al., 2006), ATP secretion was normalized for cell number. We found that constitutive ATP release per osteoblast was not significantly different in 2% O_2 compared to 20% controls at either 8 or 15 days (data not shown).

Hypoxia-induced ATP release involves vesicular exocytosis

To establish whether hypoxia-induced ATP release occurred via vesicular exocytosis, cells were incubated in medium containing 100 μM NEM or monensin prior to exposure to 20% or 2% O_2. In 20% O_2 controls, NEM and monensin decreased basal ATP release by up to 65%. In untreated cells, transient hypoxia induced a twofold increase in ATP secretion; however, in cells pre-incubated with monensin and NEM the hypoxia-induced ATP release was decreased by 62% and 80%, respectively (Fig. 5).

Discussion

The aim of this investigation was to study the mechanisms of ATP release from primary rat osteoblasts under normal and hypoxic (2% O_2) conditions. The culture system we used enabled osteoblast differentiation to be monitored and confirmed, not only by the large increases in ALP activity but by abundant formation of "trabecular-shaped" mineralized nodules, as previously described (Orriss et al., 2007).

Osteoblasts constitutively released ATP at all times and the quantity released was dependent on time in culture. Osteoblast maturation was accompanied by up to a sevenfold increase in basal ATP release per cell. Since osteoblast viability was unchanged the increased levels of ATP detected were not due to cell lysis or membrane damage. Intracellular ATP levels have been shown to be up to fivefold higher in differentiated osteoblasts (Komarova et al., 2000). Thus, the increased ATP release from mature cells observed here could occur as a direct result of a higher intracellular ATP concentration; however, an

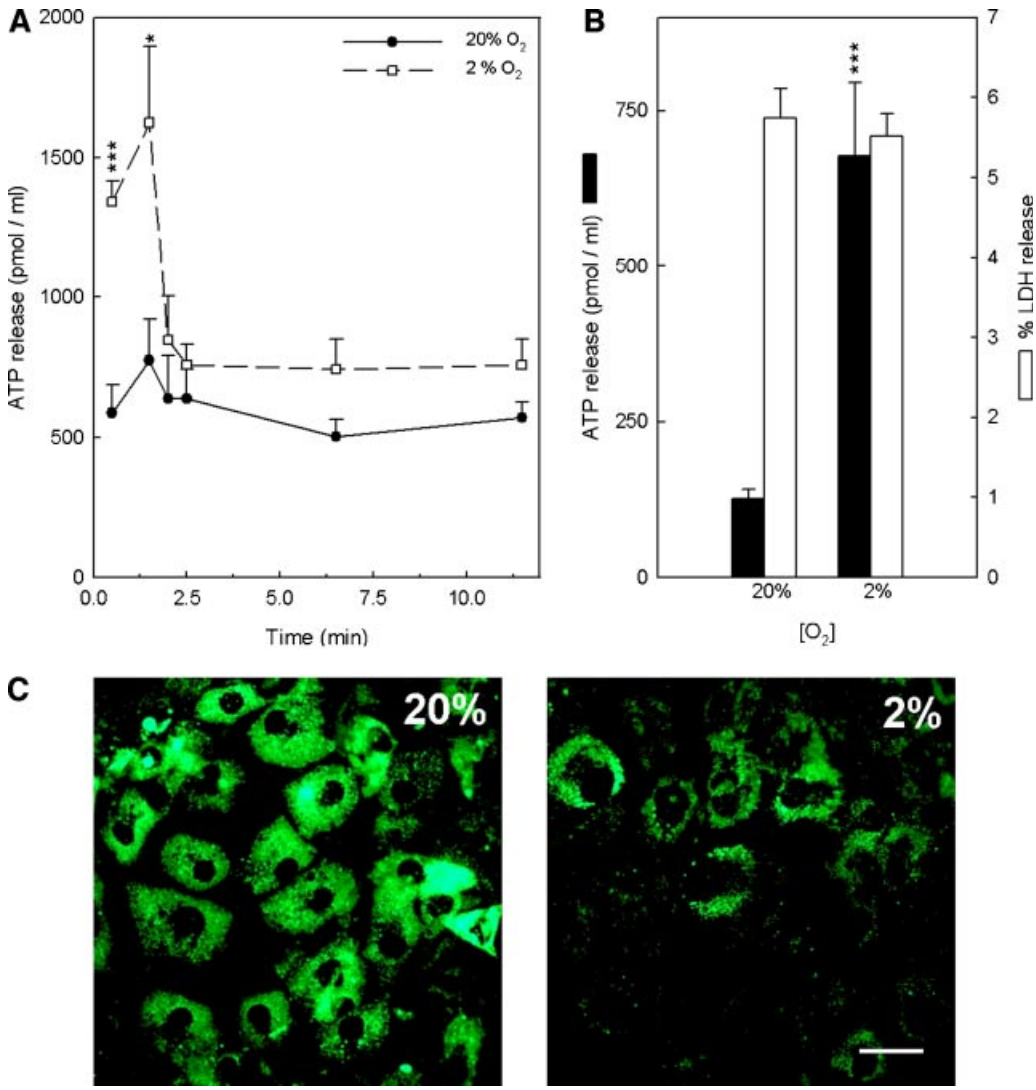

Fig. 4. Transient hypoxia stimulates ATP release from osteoblasts without affecting cell viability. Osteoblasts were exposed to acute hypoxia 1 h after exchange to serum-free medium; samples were collected for measurement of ATP levels at regular intervals between 0.5 and 11.5 min after exposure to 2% or 20% O_2. A: Transient exposure to 2% O_2 for 0.5–1.5 min caused a rapid increase in ATP release from osteoblasts (2.5-fold). Exposure to 20% O_2 did not influence the level of ATP release at any stage. B: Treatment with 2% O_2 had no affect on the levels of LDH in the cell medium. C: Transient exposure to 2% O_2 results in reduced intracellular levels of quinacrine fluorescence compared to the 20% O_2 controls (Scale bar = 50 μm). Values are means ± SEM (n = 12), significantly different from controls: $^{***}P < 0.001$, $^{*}P < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

increase in the rate of exocytosis is also possible. ATP release from mature osteoblasts resulted in culture medium concentrations as high as 1 nmol/ml (i.e., 1 μM). Bearing in mind that osteoblasts in vivo are bathed by considerably lower volumes of extracellular fluid than is the case in vitro, our results suggest that local concentrations of ATP (or its breakdown products) in vivo could reasonably be in the range 10–100 μM. Such levels would be biologically significant, since: (1) ATP stimulates osteoclast formation and activity at concentrations as low as 0.2–1 μM (Morrison et al., 1998; Hoebertz et al., 2001); (2) ATP inhibits bone mineralization at concentrations in the range 1–100 μM (Orriss et al., 2007).

Purinergic neurotransmission is mediated by the controlled release of ATP-filled synaptic vesicles, making vesicular exocytosis the principal ATP release mechanism in neuronal cells (Bodin and Burnstock, 2001b). A number of non-neuronal cell types, including epithelial and endothelial cells (Bodin and Burnstock, 2001a; Knight et al., 2002), fibroblasts (Boudreault and Grygorczyk, 2004), astrocytes (Montana et al., 2006), and mechanically stimulated osteoblast-like cell lines (Genetos et al., 2005; Romanello et al., 2005), have also been shown to release ATP via vesicular mechanisms. We found that monensin, which blocks vesicle formation at the Golgi apparatus, inhibited ATP release from primary rat osteoblasts at concentrations ≥1 μM. The observed pattern of quinacrine staining (used to visualize intracellular ATP in osteoblasts) was granular and cytoplasmic, suggesting vesicular localization of ATP. Treatment with monensin greatly reduced intracellular quinacrine staining, suggesting that the formation of ATP-containing exocytotic vesicles had been inhibited. In contrast, *N*-ethylmaleimide, which inhibits vesicular fusion with the plasma membrane, caused only small reductions in cytoplasmic quinacrine fluorescence in osteoblasts but, as expected, blocked ATP release. Brefeldin A, which disrupts

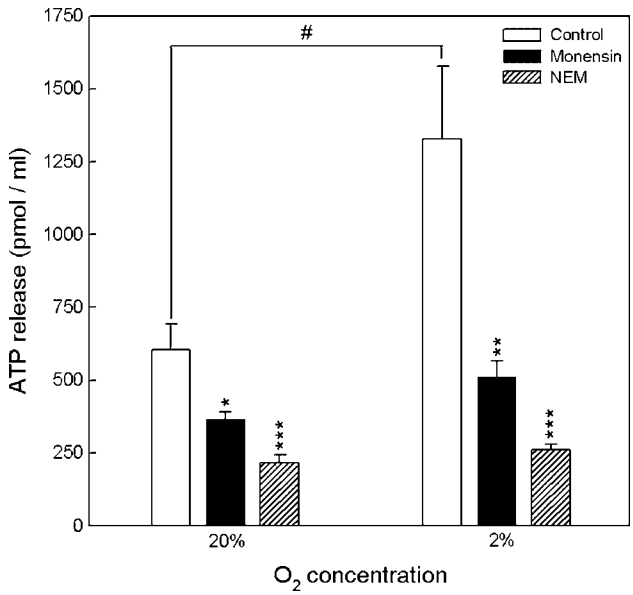

Fig. 5. Hypoxia-induced ATP release occurs via vesicular exocytosis. Osteoblasts were exposed to acute hypoxia 1 h after exchange to serum-free medium (with or without vesicular exocytosis inhibitors). Exposure to 2% O_2 for 30 sec caused a twofold increase in ATP release from osteoblasts (#$P < 0.05$). Addition of monensin and NEM (100 μM) to the culture medium inhibited both the hypoxia-induced and constitutive ATP release from osteoblasts by up to 80%. Values are means ± SEM (n = 12); significantly different from controls: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

vesicular trafficking by blocking protein transport from the endoplasmic reticulum to the Golgi apparatus, did not significantly affect ATP release from osteoblasts, in contrast with its inhibitory action in urothelial cells (Knight et al., 2002). Overall, our data are consistent with a role for vesicular exocytosis in the constitutive ATP release from primary rat osteoblasts. However, the failure of monensin and NEM to completely block ATP secretion, together with the inactivity of brefeldin A, suggests that an additional pathway may also be involved.

Although not studied here, current evidence suggests no involvement of ABC proteins, gap junctions or hemichannels in ATP release from osteoblast-like cells (Romanello et al., 2001; Genetos et al., 2005). Increased intracellular Ca^{2+} has been implicated in ATP release from many cell types including osteoblast-like cells (Knight et al., 2002; Genetos et al., 2005; Liu et al., 2008). The cation transporter, ionomycin, elevates cytosolic Ca^{2+} levels by permitting influx from the extracellular environment or release from internal stores. In agreement with a role for increased intracellular Ca^{2+} levels in ATP release, we found ionomycin stimulated ATP release from rat osteoblasts up to 50%. Mechanosensitive cation channels, including the MSCC channel present on osteoblasts (Ryder and Duncan, 2001), are blocked by gadolinium (Gd^{3+}). ATP release from osteoblasts was not affected by 1–100 μM Gd^{3+} suggesting that, under normal conditions, mechanosensitive cation channels do not mediate the increase in intracellular Ca^{2+} required for ATP secretion.

Many cell types, including endothelial cells (Bodin and Burnstock, 1995), fibroblasts (Gerasimovskaya et al., 2002), and erythrocytes (Bergfeld and Forrester, 1992; Bozzo et al., 1999) release ATP in response to hypoxia. We found that transient exposure to 2% O_2 caused a rapid increase in ATP release from osteoblasts (up to 2.3-fold) that was sustained for up to 3 min. ATP secretion was unaffected by acute exposure to 20% O_2, thus the increase in ATP release induced by acute hypoxia was not a result of the gassing process. The widespread granular fluorescence evident in quinacrine-stained osteoblasts in 20% O_2 was greatly reduced following exposure to 2% O_2. This may reflect the depletion of intracellular ATP stores following hypoxia-induced release. Since acute hypoxic exposure did not affect cell viability, the observed increase in ATP release presumably occurred via a more controlled mechanism than cell lysis or membrane damage. This is consistent with our finding that the vesicular exocytosis inhibitors, monensin, and NEM reduced hypoxia-induced ATP release by up to 80%. The ability of vesicular exocytosis inhibitors to attenuate the hypoxia-induced ATP release also suggests that 2% O_2 is acting on osteoblasts to directly stimulate ATP release rather than inhibit ATP hydrolysis. Rapid, short-term increases in ATP release have also been observed from MC3T3-E1 osteoblast-like cells exposed to fluid shear stress, with the maximum secretion occurring after 1 min; furthermore, this increased ATP release was also inhibited by monensin and NEM (Genetos et al., 2005).

Once released, ATP is rapidly hydrolyzed to ADP, AMP, and finally, adenosine; according to our observations and those of others, the half-life of ATP in vitro is between 50 sec (Bowler et al., 2001) and ~3 min (Fig. 1A). ATP and adenosine have been shown to stimulate angiogenesis in endothelial cells (Adair, 2005; Gerasimovskaya et al., 2008), whilst ADP has been implicated in endothelial cell migration (Shen and Dicorleto, 2008). Given these observations, it is possible that hypoxia-induced ATP release from osteoblasts may play a role in stimulating angiogenesis and alleviating the hypoxia. In contrast to the acute stimulatory effects of hypoxia, long-term exposure (16 days) to 2% O_2 did not result in increased constitutive ATP release from osteoblasts. Chronic hypoxia causes cells to switch from mitochondrial-based metabolism towards anaerobic glycolysis; recent work has also shown that chronic hypoxia strongly inhibits osteoblast function, inducing a state of "quiescence" (Utting et al., 2006). Thus, chronic hypoxia should result in reduced cellular ATP synthesis, which might be expected to lead to reduced ATP export. It is possible that re-oxygenation before ATP measurement in our experiments could have masked such an effect. However, the action of extracellular ATP could potentially be enhanced in chronic hypoxia, owing to reduced ecto-nucleotidase activity (and thus decreased extracellular ATP hydrolysis) (Gerasimovskaya et al., 2002).

In summary, our work indicates that primary rat osteoblasts release ATP constitutively, in biologically significant amounts, via mechanisms that could include vesicular exocytosis. The amount of ATP released is dependent on the differentiation state of osteoblasts, with mature, bone-forming cells releasing up to sevenfold more than immature, proliferating cells. Furthermore, transient hypoxia stimulates rapid, controlled ATP release from osteoblasts. Thus, osteoblast differentiation and O_2 tension in the bone microenvironment could potentially influence extracellular nucleotide concentrations and, consequently, local purinergic signaling and bone remodeling.

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