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RAPID COMMUNICATION

Hypoxia is a Major Stimulator of Osteoclast Formation and Bone Resorption

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Hypoxia is known to act as a general stimulator of cells derived from marrow precursors. We investigated the effect of oxygen tension on the formation and function of osteoclasts, the cells responsible for bore resorption, which are of promonocytic origin. Using 7- and 13-day cultures of mouse marrow cells on ivory discs, we found that reducing oxygen tension from the ambient atmospheric level of 20% by increasing the proportion of nitrogen caused progressive increases in the formation of multinucleated osteoclasts and resorption pits. Peak effects occurred in 2% oxygen, where stimulations of resorption up to 21-fold were measured. Significant stimulations of osteoclast formation and resorption were observed even in severely hypoxic cultures gassed with 0.2% oxygen. Short-term cultures of cells disaggregated from rat bones indicated that hypoxia did not alter the resorptive activity of mature osteoclasts, but reduced their survival or adherence. In 3-day organ cultures of mouse calvarial bones, exposure to 2% oxygen resulted in maximal, fivefold stimulation of osteoclast-mediated calcium release, an effect equivalent to that of prostaglandin E_2 (PGE_2), a reference osteolytic agent. Hypoxia also caused a moderate acidosis in calvarial cultures, presumably as a result of increased anaerobic metabolism; this observation is significant because osteoclast activation is dependent on extracellular acidification. Our experiments reveal a previously-overlooked mechanism of considerable potential importance for the regulation of bone destruction. These findings may help explain the bone loss associated with a wide range of pathological states involving local or systemic hypoxia, and emphasize the importance of the vasculature in bone.

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Hypoxia occurs when the blood supply to tissues is reduced or disrupted. Oxygen tension (PO_2) in arterial blood is about 95 mmHg (~12%), and in venous and capillary blood it is about 40 mmHg (~5%), approximately a quarter of that in atmospheric air. In normal tissues, interstitial PO_2 is roughly in the range 4–8%. Measurements, for example, of bone marrow aspirates from normal volunteer donors yielded mean PO_2 values of 6.2% (Ishikawa and Ito, 1988) and 6.6% (Harrison et al., 2002). In environments such as the poorly vascularized yellow fatty bone marrow of the very elderly, or in inflamed tissue, infected tissue, tumors, wounds, and fracture sites, PO_2 could be considerably lower. In some diseased tissues, interstitial PO_2 may be less than 1% (Lewis et al., 1999). Thus, conventional tissue culture using atmospheric air exposes cells to oxygen tensions that are between ~2.5 and ≥20 times higher than pathophysiological levels.

Recent work indicates that cells sense oxygen using a family of oxygen-dependent enzymes that hydroxylate a proline residue on hypoxia-induced factor (HIF), a constitutively-produced transcription factor that mediates cellular responses to hypoxia; proline hydroxylation in the presence of oxygen serves to target HIF for intracellular destruction, preventing its action (Bruick and McKnight, 2001; Jaakkola et al., 2001).

Studies to date on the role of hypoxia in the skeleton have involved tissues or cells of mesenchymal origin. There are conflicting reports as to whether chondrocytes in growth plate cartilage, which is normally avascular, are hypoxic (Shapiro et al., 1997; Schipani et al., 2001). In the case of osteocytes, which are also isolated in matrix remote from blood vessels, it has been proposed that hypoxia may represent a novel mechanotransduction pathway; numbers of hypoxic or

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HIF-1α-expressing osteocytes are increased markedly in situ in response to mechanical unloading (Dodd et al., 1999; Gross et al., 2001). Diverse responses to hypoxia have been reported for cultured osteoblasts, including: increased proliferation with decreased alkaline phosphatase (ALP) activity and collagen synthesis (Tuncay et al., 1994; Matsuda et al., 1998); decreased proliferation with increased ALP and increased synthesis of vascular endothelial growth factor (VEGF), insulin-like growth factor II (IGF-II), and transforming growth factor β1 (TGF β1) (Steinbrech et al., 1999, 2000a,b). Stimulation of osteoblastic VEGF synthesis by hypoxia, an effect mediated by HIF-2α (Akeno et al., 2001, 2002), may represent a basic homeostatic response to low PO_2, since VEGF is powerfully angiogenic. At the functional level, recent work has shown that exposure of osteoblastic precursor cells from marrow stroma to reduced oxygen causes increased osteogenesis in vitro and in vivo (Lennon et al., 2001).

Hypoxia is well known to act as a stimulator of the formation and activation of cells derived from marrow precursors, including cells of the monocyte–macrophage lineage, which are closely related to osteoclasts (Bradley et al., 1978; Broxmeyer et al., 1990; Koller et al., 1992; Lewis et al., 1999). Only one early study (Stern et al., 1966) appears to have examined the effect of oxygen tension on a bone resorption parameter: the authors found that a moderate reduction in PO_2 (10%) was associated with decreased release of ^{3}H-proline from cultured calvaria, whereas hyperoxia (30–50%) had the opposite effect.

Because of the striking effects of hypoxia on other cell types, and because active pathological bone destruction often occurs at sites where PO_2 is low, we examined the effect of oxygen tension on osteoclasts and mineralized tissue resorption.

MATERIALS AND METHODS

Reagents and materials

Culture media and buffers were purchased from Gibco (Paisley, UK). All other reagents were purchased from Sigma (Poole, UK) unless stated otherwise. Untreated elephant ivory was kindly donated by HM Customs and Excise (Heathrow Airport, London, UK). Cylinders containing custom mixtures of O_2, CO_2, and N_2 were purchased from BOC (London, UK).

Mouse osteoclast formation and resorption assay

Experiments were performed using minimum essential medium supplemented with Earle's salts, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (mixture abbreviated 'MEM'). Transverse ivory (dentine) wafers, 250 μm thick were prepared using a low speed saw (Isomet, Buehler Inc., Lake Bluff, IL); 5 mm diameter discs were cut from the wafers after soaking in water, using a standard paper hole punch. The discs were washed extensively by sonication in distilled water and stored dry at room temperature; before use, discs were sterilized by brief immersion in ethanol, allowed to dry, and then rinsed in sterile phosphate buffered saline (PBS).

Long bones were dissected from one or two 8-week-old MF1 mice, killed by cervical dislocation. The bones were cut across the epiphyses and the marrow was flushed out with PBS using a 25-gauge needle. The resulting suspension was centrifuged at 1,000*g* and resuspended at a density of 5×10^6 cells/ml in MEM supplemented with 10 nM 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), 10 nM dexamethasone, 20 ng/ml human recombinant macrophage colony stimulating factor (M-CSF), and 100 nM prostaglandin E_2 (PGE_2). This cell suspension was cultured for 20 h in a 75 cm^2 flask in a 5% CO_2/95% atmospheric air to allow attachment of stromal and other rapidly adherent cells. The non-adherent cell suspension was then removed from the flask, centrifuged, and resuspended at 4×10^5 cells/ml in MEM supplemented with 10 nM $1,25(OH)_2D_3$, 10 nM dexamethasone, 20 ng/ml M-CSF, 100 nM PGE_2, and 10 ng/ml receptor activator of nuclear factor κB (RANKL, a kind gift of Dr. Colin Dunstan, Amgen Inc., Thousand Oaks, CA). This suspension (250 μl; 10^5 cells) was added to the wells of a 96-well plate containing ivory discs. After a 24 h preincubation period in a 5% CO_2/95% atmosphere to allow initially non-adherent osteoclast precursor cells to attach to the ivory, the discs were transferred to 25 cm^2 flasks with polyethylene 'plug-seal' caps (Falcon, Becton Dickinson, Oxford, UK), containing 8 ml of the same medium (eight discs per flask). The flasks were then flushed for 2 min with gas mixtures containing 5% CO_2 and 20, 12, 5, 2, 1, or 0.2% O_2 (balance N_2) via a 21-gauge needle inserted through the loosened polyethylene cap. The cap was then tightened and the needle hub closed with a Luer plug. The sealed flasks were incubated at 37°C in a standard incubator containing 5% CO_2/95% atmospheric air and re-gassed daily. The culture medium was replaced every 2–3 days; the final replacement was with medium acidified to pH ~7.0 by the addition of 82 μl/100 ml concentrated hydrochloric acid (≅10 meq/l H^+) to activate resorption pit formation by any osteoclasts formed (Hoebertz and Arnett, 2003). Culture medium pH, PCO_2, and PO_2 were monitored at each medium change and at the end of experiments using blood gas analyzers (ABL 330; ABL 705, Radiometer, Copenhagen, Denmark). PO_2 was also monitored using a fluorescence-based oxygen probe ('FOXY', Ocean Optics, Duiven, Netherlands); this was necessary because very low O_2 levels are beyond the measurement range of clinical blood gas analyzers.

Experiments were terminated by fixing the discs in 2% glutaraldehyde, followed by staining for ~35 min to demonstrate tartrate-resistant acid phosphatase (TRAP) (Sigma Kit 387-A). A control group of ivory discs was also removed, fixed, and stained after 3 d incubation to check for the presence of any mature osteoclasts that might have been released during the initial cell preparation. The total number of TRAP-positive multinucleated osteoclasts on each ivory disc was assessed 'blind' by transmitted light microscopy. Cells were then stripped from discs by sonication for 10 min in 0.2 M NH_4OH and discs were restained in 1% toluidine blue in 1% sodium borate for 2 min to visualize resorption pits and trails. The plan surface area resorbed on each disc was quantified 'blind' using reflected light microscopy and dot-counting morphometry. Statistical comparisons were made by analysis of variance.

Rat mature osteoclast resorption assay

The effects of oxygen tension on the function of mature rat osteoclasts were studied using modifications of an assay described previously (Hoebertz et al., 2001; Hoebertz and Arnett, 2003). Briefly, mixed cell populations containing osteoclasts were obtained by mincing rapidly the pooled long bones of five 2-day-old Sprague–Dawley rat pups, killed by cervical dislocation, in 5 ml MEM, followed by vortexing for 30 sec. The resulting cell suspension was allowed to sediment for 45 min onto 5 mm ivory discs, pre-wetted with 50 μl MEM, in 96-well plates (100 μl cell suspension/disc). Discs were rinsed twice in PBS before transfer to 25 cm^2 Falcon plug-seal flasks; each flask contained 6 ml of MEM, acidified to pH 7.0, and 6 replicate ivory discs. The flasks were then gassed with mixtures containing 5% CO_2 and 20, 12, 5, 2, 1, or 0.2% O_2 (balance N_2), as described above, and incubated for 26 h in a humidified atmosphere of 5% CO_2/95% air. At the end of the experiment, medium pH, PCO_2, and PO_2 were measured as described above. Ivory discs were fixed in 2% glutaraldehyde, and then stained for TRAP. The numbers of TRAP-positive multinucleated osteoclasts (three or more nuclei) were assessed 'blind' using transmitted light microscopy; discs were subsequently stained with toluidine blue to permit assessment of total cell numbers (using three randomly selected 20× fields per disc). Cells were then stripped by sonication; discrete resorption pits were counted and plan surface area resorbed was measured 'blind' by scanning the entire surface of each disc using reflected light microscopy after restaining in toluidine blue. Statistical comparisons were made by analysis of variance.

Mouse calvarial bone resorption assay

The method, which measures bone resorption as Ca^{2+} release from organ cultures of neonatal mouse calvaria, was similar to that described in detail by Meghji et al. (2001); Ca^{2+} release in this system is almost entirely mediated by osteoclasts. Briefly, 5-day-old MF1 mice were killed by cervical dislocation. The fronto-parietal bones were removed and trimmed of any adhering connective tissue and interparietal bone, taking care not to damage the periosteum. Dissected calvaria were pooled, washed free of blood and adherent brain tissue in Hanks Balanced Salt Solution, and then divided along the sagittal suture. Half calvaria were pre-cultured individually for 24 h on 1 cm^2 stainless steel grids in 6-well plates with 1.5 ml of BGJb medium, 5% heat inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin (complete mixture abbreviated "BGJb"), at the air–liquid interface in 5% CO_2/95% humidified air. Each individual grid bearing a half calvarium was then transferred to a separate 25-cm^2 flask containing 3 ml BGJb medium (so that the bone was positioned at the air–liquid interface). The flasks were then gassed with mixtures containing 5% CO_2 and 20 or 2% O_2 (balance N_2), as described above, and incubated for 72 h without further change of culture medium. Each experimental group consisted of five individual cultures. Culture medium pH, PCO_2, and PO_2 were monitored as described above. Ca^{2+} concentrations in culture medium were measured colorimetrically, in a 'blinded' manner, as described previously. The basal calcium concentration of the BGJb medium after addition of 5% heat inactivated fetal calf serum was 2.00 mM. Statistical comparisons were made by analysis of variance.

RESULTS

Mouse marrow cultures

Osteoclasts formed from mouse marrow on ivory discs maintained in 20% O_2 for 7 days were small (generally <3 nuclei); small resorption trails were associated with these TRAP-positive cells, including cells that appeared to possess only a single nucleus (Fig. 1A). In contrast, cultures maintained in 5 or 2% O_2 showed striking increases in the size and also number of osteoclasts formed (Fig. 1B). Osteoclasts in hypoxic cultures were associated with prominent resorption trails; these cells

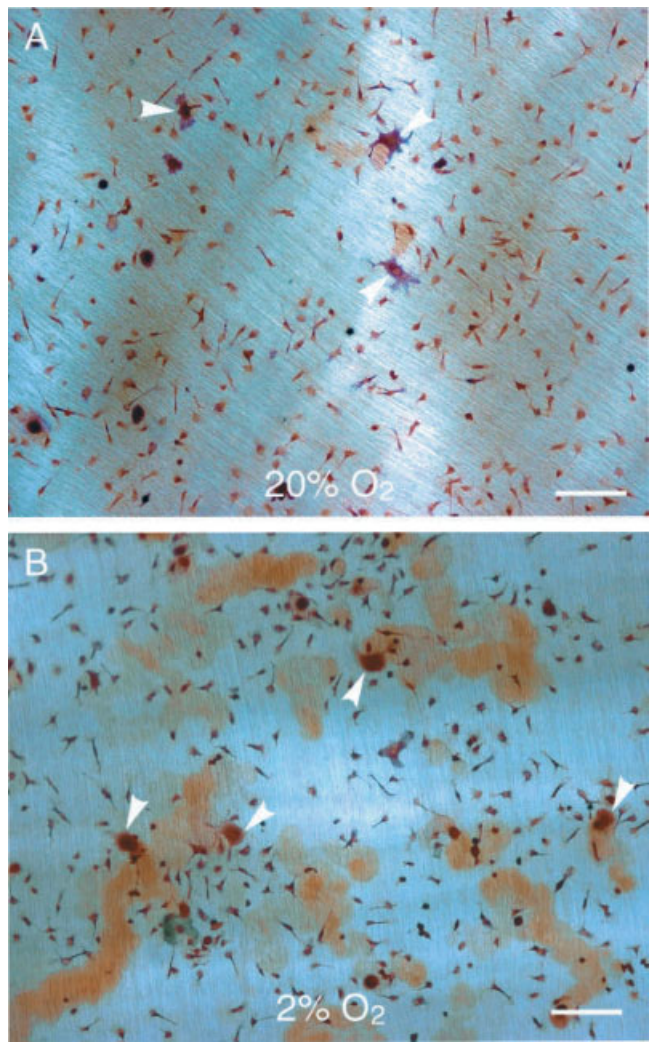

Fig. 1. Stimulation of osteoclastic resorption by hypoxia in mouse bone marrow cultures stained to demonstrate tartrate-resistant acid phosphatase (TRAP). **A**: Osteoclasts (arrowheads) formed in mouse marrow cultures on ivory discs after 7 days in normoxic (20% O_2) conditions were generally small (<3 nuclei); (**B**) in hypoxic (2% O_2) cultures, many large osteoclasts were generated; prominent, deep resorption trails (stained brown) are evident. Scale bars = 100 μm.

generally exhibited the dense, rounded morphology characteristic of activated osteoclasts, preventing quantification of nuclear number. The mean plan area of ivory resorbed was stimulated dramatically by hypoxia in 7-day cultures. In 12, 5, and 2% O_2 atmospheres, resorption increased 4, 9, and 21-fold, respectively, compared with the 20% O_2 culture (Fig. 2). Cultures medium pH was in the range 7.17–7.20 for non-acidified medium and 6.97–7.01 for the acidified medium used to activate resorption. No effects of oxygen tension on culture medium pH were noted, reflecting the relatively large volumes of culture medium used relative to the numbers of cells present.

The osteoclastogenic effects of low O_2 tension, including severe hypoxia, were also tested in longer-term (13-day) mouse marrow cultures. The peak stimulatory effects occurred in cultures exposed to 2% O_2, where a 3.5-fold increase in osteoclast formation and a 9.5-fold increase in resorption were measured. Significant stimulations of osteoclast formation (2.3-fold) and area resorbed on each disc (4.7-fold) were observed even in cultures gassed with 0.2% oxygen (Fig. 3A).

Some technical difficulties were encountered in devising suitable conditions for the maintenance of cultures over a range of reduced oxygen tensions. 'Plug-seal' tissue culture flasks (Falcon) were the most gas-tight and convenient of the systems we evaluated; nevertheless, some inward leakage of atmospheric oxygen was evident during the intervals between re-gassing. This effect was most pronounced for flasks containing low PO_2 mixtures. For example, in flasks gassed with 20, 12, 5, 2, 1, & 0.2% O_2, measured O_2 levels in culture medium after 24 h were 20.0, 13.0, 6.5, 3.8, 3.8, & 2.6%, respectively.

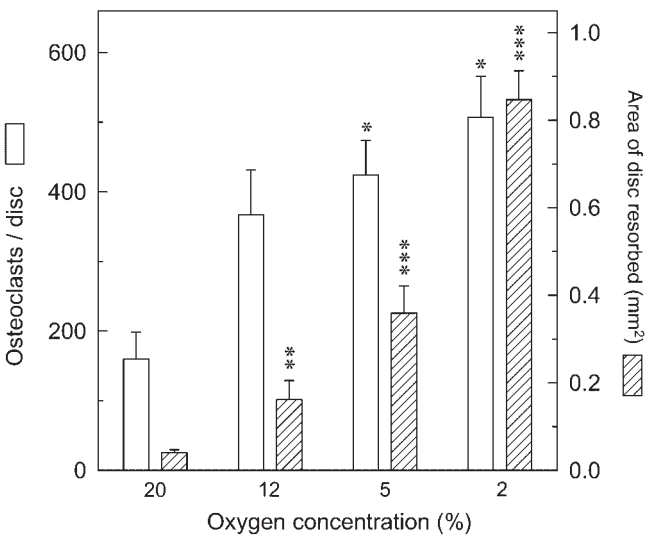

Fig. 2. Stimulation of TRAP-positive osteoclast formation and resorption in 7-day cultures of mouse bone marrow on ivory discs with progressive reduction in ambient oxygen tension. Mean pH at day 5 (pre-acidification) and day 7 (post-acidification) of cultures was 7.18 ± 0.01 and 7.00 ± 0.01, respectively; pH was unaffected by PO_2. Values are means $\pm$ SEM (n = 8); $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to 20% O_2.

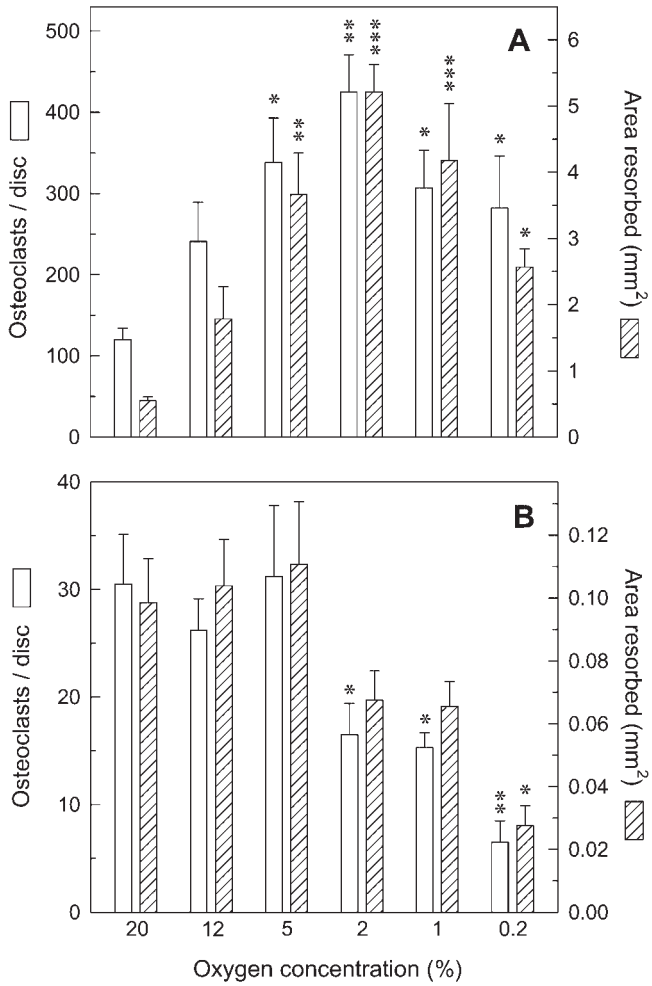

Fig. 3. **A**, **B**: Comparison of the effects of severe hypoxia on longer-term mouse marrow cultures with effects on short-term cultures of mature rat osteoclasts. A: In 13 d cultures of mouse marrow cells, peak stimulation of osteoclast formation and resorption was observed in 2% O_2, but significant increases were still evident even in 0.2% O_2. Cultures were maintained at pH 7.35 for the first 10 d, then acidified to pH 6.92 for the final 3 d to activate resorption; pH was unaffected by PO_2. Values are means $\pm$ SEM (n = 8); $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to 20% O_2. B: In 26 h cultures of cells disaggregated from neonatal rat long bones, decreasing O_2 concentration below 5% caused progressive reduction in the numbers of TRAP-positive multinucleated osteoclasts present on ivory discs. The area of each disc resorbed reduced in similar proportion as O_2 concentration decreased; the corresponding numbers of resorption pits in 20, 12, 5, 2, 1, & 0.2% O_2 were 87.2 ± 13.5, 93.8 ± 18.6, 113.3 ± 24.4, 53.0 ± 8.6, 68.3 ± 8.9, & 22.3 ± 3.4, respectively; these data indicate that hypoxia did not significantly affect the resorptive activity of osteoclasts. Culture medium pH was 7.09 ± 0.01, and was not altered by O_2 tension. Values are means $\pm$ SEM (n = 6); $^*P < 0.05$, $^{**}P < 0.01$, compared to 20% O_2.

Rat osteoclast cultures

Oxygen tensions of 2% or below caused progressive reductions in the number of osteoclasts present on ivory discs after culture for 26 h; resorption pit formation was reduced equally in low O_2 (Fig. 3B). Thus, hypoxia did not significantly alter the resorptive activity of mature osteoclasts, but reduced their survival or adherence. Hypoxia was also associated with smaller,

non-significant reductions (≤23%) in total cell numbers present on ivory discs.

Mouse calvarial cultures

Osteoclast-mediated Ca^{2+} release into the culture medium was fivefold greater from calvaria gassed with 2% O_2 than from bones in 20% O_2. The osteolytic effect of 2% O_2 was equivalent to the maximum stimulation observed in bones treated with the reference pro-resorptive agent, PGE_2 (1 μM). Hypoxia-stimulated resorption was completely blocked by the prostaglandin synthesis inhibitor, indomethacin (0.1 μM) (Fig. 4).

In the calvarial cultures, hypoxia consistently reduced the culture medium pH by ~0.1 U. Such acidification is consistent with increased anaerobic metabolism and presumably reflects the large number of cells present in the whole bone explants relative to the volume and buffering capacity of the culture medium.

DISCUSSION

Despite the obvious importance of oxygen tension and the vasculature in skeletal homeostasis, the subject has been somewhat neglected until recently. We show here that oxygen tension acts as a key regulator of osteoclastic bone resorption. Our results indicate that at constant pH, hypoxia stimulates bone resorption by accelerating the formation of large osteoclasts.

As expected, in the 7-day mouse marrow cultures maintained in 20% O_2 with a permissive but sub-optimal concentration of RANKL (10 ng/ml), relatively few osteoclasts formed; typically, these were small cells, with <3 nuclei. Absolute numbers of osteoclasts were increased 3.2-fold in 2% O_2 after 7 days, but resorption was stimulated 21-fold, suggesting an important contribution of increased osteoclast size in hypoxic cultures. Similar stimulatory effects of hypoxia on osteoclast formation and resorption were also evident in longer-term (13-day) mouse marrow cultures. Peak stimulatory effects were observed in 2% O_2, but it is noteworthy that osteolysis was markedly enhanced even in extreme hypoxia, where O_2 concentrations were as low as 1/100 of atmospheric levels. The dense, rounded morphology of TRAP-stained, activated osteoclasts in hypoxic marrow cultures prevented quantification of the numbers of nuclei per cell by conventional light microscopy. Investigation of this question in a future study will require confocal microscopy.

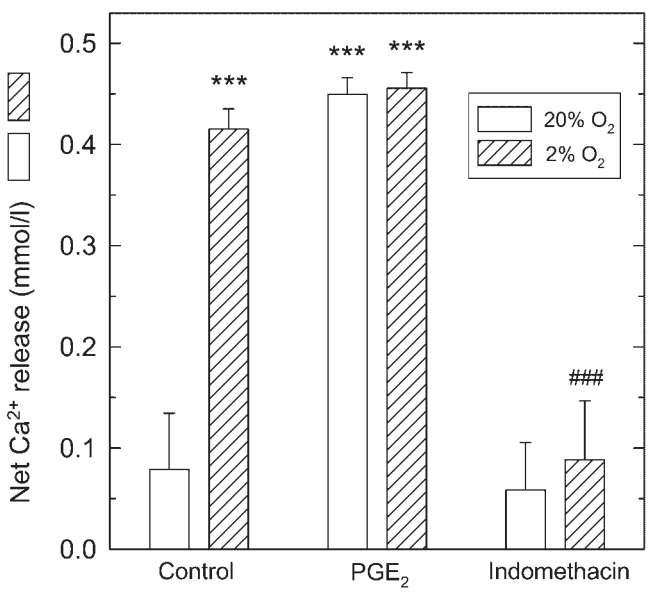

Fig. 4. Stimulation of osteoclast-mediated calcium release by hypoxia in 3-day calvarial explant cultures. The effect of hypoxia is equivalent to that of a maximal dose of prostaglandin E_2 (PGE_2, 1 μM). The effect of 2% O_2 was blocked by the prostaglandin synthesis inhibitor, indomethacin (0.1 μM). The 2% O_2 cultures were markedly acidified (pH 7.06 ± 0.02) compared with 20% O_2 cultures (pH 7.18 ± 0.02). Values are means ± SEM (n = 5); $^{***}P < 0.001$, compared with 20% O_2 control value; $^{###}P < 0.001$, with respect to 2% O_2 control value.

Short-term cultures with mature rat osteoclasts showed that hypoxia did not increase resorptive activity, but caused moderate, selective reductions in osteoclast numbers over 26 h. Thus, the increases in osteoclast formation and resorption observed in the hypoxic marrow cultures may have been taking place against a background of reduced osteoclast life span. These results provide additional evidence that the increased resorption per osteoclast at low PO_2 in the marrow cultures was due to increases in osteoclast size, rather than activity. The cell culture experiments used excess growth medium, so that surplus H^+ production due to increased anaerobic metabolism in hypoxia was effectively buffered and pH did not vary between treatment groups. Constant pH was needed in order to distinguish the effects of hypoxia because resorption pit formation by mature osteoclasts is dependent on extracellular acidification (Arnett and Dempster, 1986, 1987; Arnett and Spowage, 1996; Hoebertz and Arnett, 2003). The method we used enabled comparison of the effects of several different O_2 tensions within one experiment at the same temperature (i.e., within a single incubator). However, because it was not possible to devise hypoxic culture vessels that eliminated entirely the inward leakage of atmospheric O_2, it is not clear whether the effective PO_2 levels experienced by cells could be approximated roughly as the mean of the initial and 24 h PO_2 values, or whether the initial exposure to the lower PO_2 levels was of particular importance.

Exposure to 2% O_2 also caused maximal stimulation of osteoclast-mediated calcium release from cultures of mouse calvarial bones, together with a marked reduction in culture medium pH. Bone resorption in calvarial cultures is also highly sensitive to stimulation by small pH reductions (Meghji et al., 2001), and we estimate that about half of the hypoxia-stimulated calcium release observed in Figure 4 could be accounted for by acidosis. In vivo, the enhanced acid production in hypoxic tissues is likely to be compounded by the reduced vascular perfusion responsible for the hypoxia, resulting in complex local pH (and PO_2) gradients. Thus, the effects of hypoxia and acidosis are closely linked, causing bone destruction by recruiting and then stimulating osteoclasts, respectively. Normal extracellular pH in bone has not been determined but it is likely to be somewhat less than blood pH; in normal skin, for example interstitial pH has been measured at ~7.1 (Martin and Jain, 1994), which approximates to the half-maximal

activation pH of dissociated rat osteoclasts (Arnett and Spowage, 1996).

Further studies will be needed to determine whether the stimulatory action of hypoxia on osteoclast formation is mediated intracellularly via prolyl hydroxylases and HIF, and if so, by which subtype(s). Extracellularly, there are a number of strong candidates for paracrine or autocrine mediators. Firstly, our experiments showed that hypoxia-stimulated resorption in calvaria appeared to be dependent on prostaglandin synthesis, since the effect was abolished in the presence of a relatively low concentration (0.1 μM) of the cyclooxygenase inhibitor, indomethacin. Responses to many osteolytic agents, including acidosis, are similarly prostaglandin-dependent in calvarial cultures (Meghji et al., 2001). Prostaglandins also stimulate osteoclast formation (Collins and Chambers, 1992; Lader and Flanagan, 1998), and commonly mediate hypoxic responses in other cell types, such as macrophages (Lewis et al., 1999). Secondly, hypoxia stimulates purine nucleotide release from endothelial cells (Bodin and Burnstock, 1995), and we have previously shown that ATP and ADP are powerful osteolytic agents, acting through P2 receptors on bone cells (Morrison et al., 1998; Hoebertz et al., 2000, 2001); this mechanism could account for some of the resorptive action of hypoxia in intact bone but it remains to be investigated whether nucleotide release from other cell types in bone might also be enhanced by low PO_2. Thirdly, one of the major effects of hypoxia on cells is to stimulate the production of potent angiogenic factors such as VEGF, tumor necrosis factor-α, and fibroblast growth factors (Lewis et al., 1999); these factors are also stimulators of the formation and/or function of osteoclasts (Simmons and Raisz, 1991; Nakagawa et al., 1999, 2000; Suda et al., 2001). It is already well documented that hypoxia increases VEGF production by osteoblasts (Steinbrech et al., 1999, 2000a; Akeno et al., 2001), and VEGF production by human peripheral blood-derived macrophages is also strongly upregulated by hypoxia (Lewis et al., 1999). Similarly, IGF-1 and TGFβ, produced by osteoblasts in response to low oxygen (Steinbrech et al., 2000b; Warren et al., 2001) are also stimulators of osteoclast formation (Hill et al., 1995; Fuller et al., 2000; Massey et al., 2001).

Our results highlight the fact that conventional tissue culture conditions expose bone cells to non-physiological, atmospheric levels of oxygen (20%). With the exception of respiratory epithelia, most cells are not normally exposed to such high PO_2 in vivo. Measurements of PO_2 in marrow aspirates (Ishikawa and Ito, 1988; Harrison et al., 2002) may yield misleadingly high average values (6.2–6.6%) because haematopoietic cells in vivo are arranged in clusters around sinusoids, such that some cells may be located as many as 10 cell diameters away from the nearest vascular channel. Modeling studies of the intact marrow indicate that many haematopoietic cells, particularly the most primitive progenitors, are probably located in a very low PO_2 environment (Chow et al., 2001). Therefore, atmospheric oxygen levels are likely to be inhibitory or perhaps even somewhat toxic for osteoclast marrow precursor cells. Osteoclasts can additionally form from mononuclear precursors present in circulating human blood (Massey et al., 2001), a process which is also stimulated dramatically by culture in 2% O_2 (Arnett et al., 2003). Thus, it seems reasonable to suppose that when promonocytic cells extravasate from blood, where PO_2 levels are normally in the range 5–12%, into the interstitial microenvironments of bone, where PO_2 may be lower, osteoclast formation will be favored.

The present findings may have wide-ranging implications for understanding pathophysiological bone loss. Osteoclasts are unusual cells in that their formation is increased by low oxygen levels and their activity is dependent upon low extracellular pH. Bone hypoxia, with resultant acidosis could result from a number of local or systemic causes, including: (1) fractures, which entail local disruption of blood supply; (2) inflammation, including arthritis; (3) infection; (4) tumors; (5) diabetic ischaemia; (6) microvascular damage due to long-term smoking; (7) aging, where there is a progressive shift from endosteal to periosteal blood supply (Bridgeman and Brookes, 1996; Brookes, 1998); (8) chronic respiratory failure or breathing air with a depleted O_2 content (Fujimoto et al., 1999); (9) excessive exercise, e.g., in certain endurance sports such as cycling or running.

In conclusion, these experiments reveal a previously overlooked mechanism of major importance for the regulation of bone destruction. Our observations point to the critical role of the vasculature in maintaining bone health.

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