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Acid–base regulation of bone metabolism

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Abstract. It has been known for almost a century that systemic acidosis causes depletion of the skeleton—an effect assumed to result from physicochemical dissolution of bone mineral. However, our work has shown that resorption pit formation by cultured osteoclasts is dependent on extracellular acidification. Osteoclasts are almost inactive above pH 7.4 and show maximum acid-activation at about pH 6.9. Within this pH range, small shifts in H^+ concentration can cause large changes in resorption. Bone mineralisation by cultured osteoblasts is inhibited in a reciprocal manner by acidosis. *In vivo*, acidosis can occur systemically as a result of renal, bronchial or gastrointestinal disease, diabetes, severe (anaerobic) exercise, excessive protein intake, ageing, or the menopause. Acidosis can also occur locally as a result of inflammation, infection, wounds, tumours or ischaemia (due to increased anaerobic metabolism and reduced perfusion). The robust functional responses of bone cells to extracellular pH changes probably represent a primitive 'failsafe' to correct systemic acidosis by releasing alkaline bone mineral when the lungs and kidneys are unable to remove sufficient H^+ equivalent. The association of acidosis with hypoxia led us to investigate the effects of oxygen tension on bone cell function. We found that hypoxia causes impressive stimulation of osteoclast formation, independent of pH changes, whereas osteoblast growth and differentiation are blocked. Our results provide strong evidence for the critical role of the vasculature in the maintenance of bone health.

Keywords: Osteoclasts; Osteoblasts; Bone; Acidosis; pH; Hypoxia; Oxygen; Vasculature

1. Introduction

A fundamental problem faced by all multicellular organisms is the buffering and elimination of the acid produced as a result of metabolism. The most basic function of the vasculature is to deliver nutrients and O_2 to cells and to remove waste products, including H^+ and CO_2 which, in land vertebrates, are excreted via urine and expired air, respectively. The skeletons of land vertebrates contain a massive reserve of base, which is ultimately

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available as a "failsafe" mechanism to buffer H^+ if the kidneys and lungs are unable to maintain acid–base balance within narrow limits. Systemic or local acidosis can result from many causes and there is longstanding evidence of the association of acidosis with bone loss. This review will focus on advances in our understanding of the responses of bone cells to extracellular pH changes, with consideration of the potential role of the vasculature in regulating osteoclast and osteoblast function.

2. Acid–base balance and bone

2.1. General considerations

Precise maintenance of pH in the blood and extracellular fluid is needed because the machinery of cells is very sensitive to changes in H^+ concentration. Blood pH is principally buffered via the CO_2/HCO_3^- system but also by the numerous histidine residues of haemoglobin and by plasma proteins. Addition of CO_2 to the system as a result of respiration causes an increase in H^+ concentration (i.e., pH reduction) leaving the HCO_3^- concentration relatively unaltered. If insufficient CO_2 is expelled via the lungs, a 'respiratory acidosis' results. Conversely, addition of H^+ to the system, for example as a result of the metabolism of sulphur, nitrogen and phosphorus-containing molecules will result in a pH decrease with a reduction of HCO_3^- levels without altering the CO_2 concentration much; this is termed a 'metabolic acidosis'. Protons generated in this way, together with associated waste anions, must be excreted via the kidneys to produce an acidified urine. It is important to bear in mind that although the pH of arterial blood is normally close to 7.40, and that of venous blood $\approx$7.36, the pH of the interstitial fluid film bathing cells in tissues will generally be lower, and subject to complex, dynamic gradients, depending on the metabolic activity of the cells, their distance from the nearest capillary and the quality of the microvasculature. Because of obvious technical difficulties, this is not a well-investigated area. Data are not available for bone, but in normal skin, interstitial pH is around 7.1 [1].

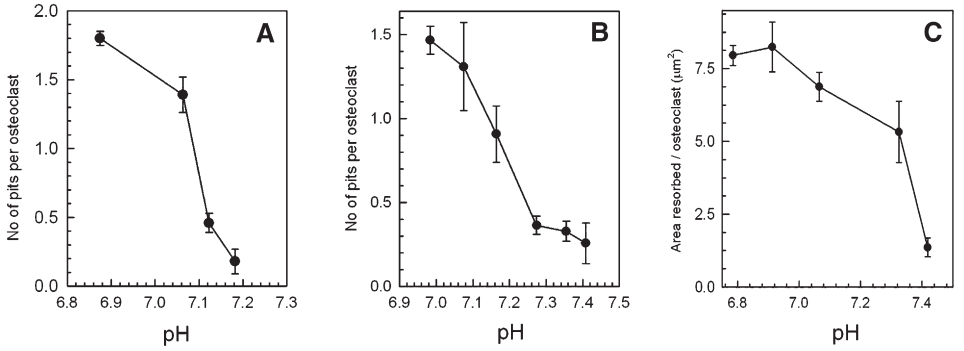

Fig. 1. Acid-activation of normal, mature osteoclasts derived from neonatal rats (A), pre-hatch chicks (B), and human peripheral blood (C), cultured on dentine discs for 24 h. Culture medium pH was adjusted by addition of HCl or NaOH; pH measurements were made by blood gas analyser. Rat osteoclasts are essentially 'switched off' above pH 7.2, whereas chick and human osteoclasts retain limited resorptive activity at blood pH (7.4). Values are means±S.E.M. (n=5).

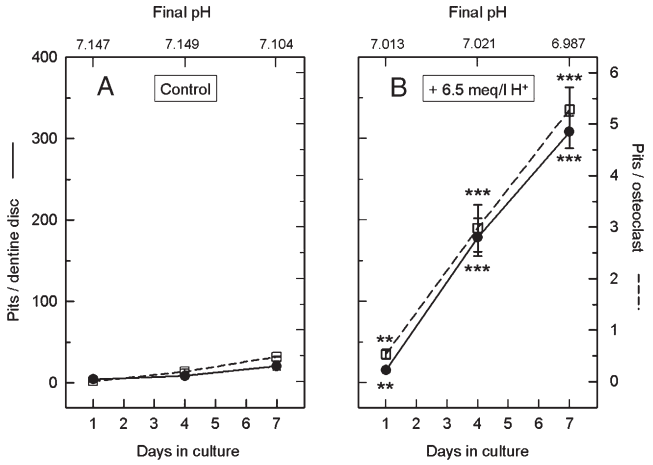

Fig. 2. Long-term stimulation of resorption pit formation by mature rat osteoclasts cultured on dentine discs in slightly acidified medium (B) for up to 7 days, compared with control (A). A 15-fold increase in resorption is associated with a mean pH difference of only 0.13 unit. $^{**}p<0.01$, $^{***}p<0.001$, compared with respective values in non-acidified control (A); values are means ± S.E.M. ($n=5$).

2.2. Dietary considerations

Metabolic oxidation of proteins containing sulphur and phosphorus ultimately yields H^+ residues corresponding to sulphuric and phosphoric acids ('fixed acids') which must be excreted via the kidneys. The average American diet has been estimated to generate an inorganic H^+ residue of about 0.1 mol/day [2], which is equivalent to about 8 ml of concentrated hydrochloric acid. Recent data indicate that excess acid generated from high protein intakes increases calcium excretion and bone resorption, assessed as urinary pyridinoline and deoxypyridinoline; it was suggested that fruit and vegetable intake could balance this excess acidity by providing alkaline salts of potassium [3]. In middle-aged women, estimated dietary non-carbonic acid production is associated with lower bone mineral density [4]. Another study with human volunteers has shown that increasing dietary acid load without altering overall protein intake results in increases in urinary Ca^{2+} and collagen C-telopeptide excretion, suggesting increased bone resorption [5]. A significant contribution to dietary acid intake may be made by cola drinks in some individuals. Phosphoric acid, H_3PO_4, is added to such drinks to yield a pH of ~2.6; simple titration against sodium hydroxide shows that 1 litre of the common cola drinks contains an acid load equal to ~36 ml of 1 M HCl, corresponding to about 40% of the fixed H^+ generated daily by the diet. For comparison, this amount of acid would be neutralised by approximately four 500 mg $CaCO_3$ antacid tablets (Arnett, unpublished). However, any deleterious effect of the acid load from H_3PO_4 in cola drinks may be offset, at least for bone, because PO_4^{3-} is a powerful, reversible inhibitor of the formation and activity of osteoclasts. In this regard, it is also noteworthy that blood PO_4^{3-}, unlike Ca^{2+} levels, are not tightly regulated, and may fluctuate markedly in normal mammals within the range which also regulates osteoclast function (~2–4 mM) [6].

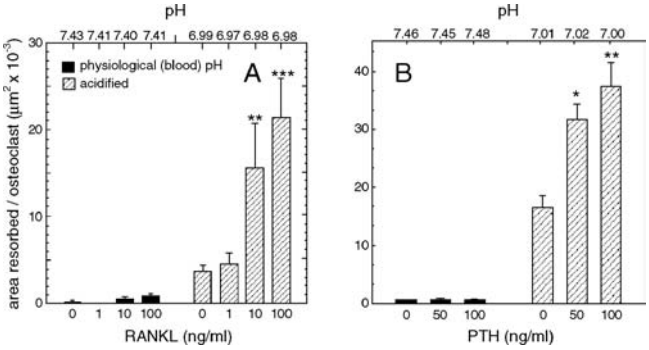

Fig. 3. Osteoclast stimulation is a 2-step process, with acid-activation as the key initial requirement. (A) RANK ligand (RANKL) fails to stimulate resorption pit formation by mature rat osteoclasts cultured for 24 h at physiological pH (~7.4) on dentine discs; however when osteoclasts are acid-activated (pH ~7.0), RANKL causes striking additional stimulation of resorption. (B) Similarly, parathyroid hormone (PTH) stimulates mature human peripheral blood-derived osteoclasts only at low pH (~7.0); note that PTH stimulation of human blood-derived osteoclasts occurs in cultures that are essentially free of osteoblasts or stromal cells, indicating a direct action of PTH on osteoclasts. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, compared with non-acidified control in the same pH group; values are means±S.E.M. ($n=5$).

In postmenopausal women, dietary supplementation with $KHCO_3$ caused marked improvements in mineral balance and biochemical indices of resorption, as well as small increases in blood pH (0.02 unit) and HCO_3−(1.8 mmol/l) [7]. Regular consumption of alkaline HCO_3^- rich mineral waters may also be of anti-resorptive benefit [8]. The bone-sparing effect of dietary supplementation with alkaline Ca^{2+} salts is now well-established, particularly for elderly women [9,10]. Blood Ca^{2+} levels, which are tightly regulated in normal subjects, are not significantly altered by ingestion of large quantities of Ca^{2+} salts. It

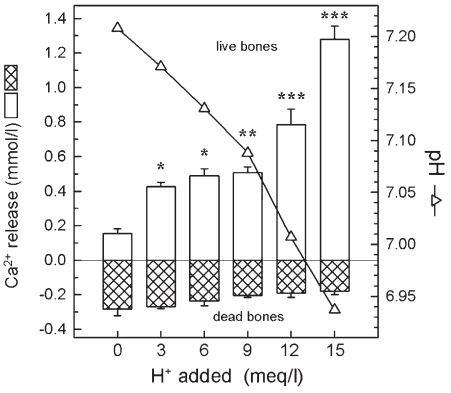

Fig. 4. Stimulatory effect of small decreases in medium pH on Ca^{2+} release from live mouse half-calvaria cultured for 3 days (open bars). In dead bones, killed by freeze thawing (hatched bars), a net Ca^{2+} influx occurred which was slightly reduced as pH decreased. Values are means±S.E.M. ($n=5$). Significantly different from non-acidified control: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

remains to be determined whether a component of the osteoprotective action of calcium salts is due also to their alkaline nature. However, this idea is certainly consistent with the observations that the anti-osteoporotic effect of Ca^{2+} supplementation is due to an inhibition of osteoclastic bone resorption [11,12], and that alkaline Ca^{2+} salts are most effective in the individuals who are likely to be acidotic, i.e., the elderly [13,14]. It will also be of interest to determine whether the anti-resorptive effects reported for strontium salts [15] could be related, at least in part, to their alkaline nature.

2.3. Other causes of acidosis

A multitude of potential causes of systemic or local acidosis exist, in addition to renal excessive dietary acid intake including and respiratory disease, ageing, menopause, gastroenteritis, excessive/anaerobic exercise, diabetes, growth factor/cytokine stimulation of cell metabolism, hypoxia (via reduced perfusion and increased anaerobic metabolism), inflammation, infection, tumours and fractures/wounds. A few selected examples will be considered here. A commonly-overlooked cause of severe, acute systemic acidosis in healthy individuals is vigorous exercise: cycling and running can both reduce arterial blood pH to ~7.2 within minutes [16,17]. There is abundant evidence of reduced bone mineral density in elite endurance athletes [18], but the contribution, if any made by acidosis is difficult to separate from other systemic factors such as hypoxia (see below) or hypogonadism. Acute, severe systemic acidosis also occurs commonly in gastroenteritis (mainly because of HCO_3^- loss), where it is associated with increases in bone resorption indices [19]. Acidosis can arise locally (i.e., at tissue level) as a result of reduced vascular

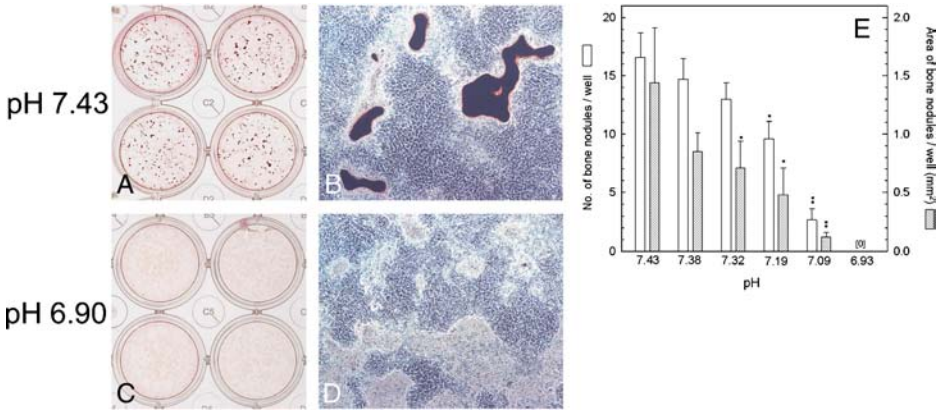

Fig. 5. Inhibitory effect of acidosis on bone nodule mineralisation. Rat primary calvarial osteoblasts were cultured in 1.5 cm diameter plastic wells for 16 days in control (pH 7.43) or acidified (pH 6.90) medium, with 2 mM β-glycerophosphate, 10^{-8} M dexamethasone and 50 μg/ml ascorbate. (A, C) Mineralised bone nodules, visualised by alizarin red staining, are evident only in control wells. (B, D) Appearance of control and acidified cultures at higher magnification (phase contrast microscopy, 100×); the failure of matrix to mineralise at pH 6.90 is clearly evidenced by the lack of alizarin red staining (although cell proliferation and matrix formation are not reduced). (E) Acidification progressively reduces bone nodule mineralisation, with complete abolition at pH 6.93. Significantly different from pH 7.43 control: $*p<0.05$; $**p<0.01$.

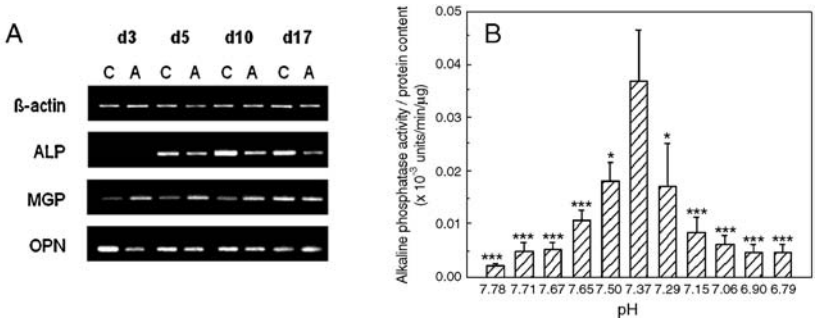

Fig. 6. (A) RT-PCR showing the effect of extracellular pH on expression of mRNAs for alkaline phosphatase (ALP), matrix Gla protein (MGP) and osteopontin (OPN) by differentiating rat primary osteoblasts. Osteoblasts were cultured for 3, 5, 10 or 17 days in bone nodule-forming medium at pH 7.42 (control, C) or pH 6.92 (acid, A). ALP, which is required for mineralisation, was inhibited by acidosis, whereas MGP, an inhibitor of mineralisation was slightly upregulated. (B) Alkaline phosphatase (ALP) enzyme activity in primary osteoblasts, cultured for 6 days at pH values spanning the pathophysiological range, peaks at 'physiological' pH and is strongly inhibited by acidosis. Values are means ± S.E.M. ($n=6$); significantly different from control (pH 7.37) value, *$p<0.05$, ***$p<0.001$.

supply; this is discussed further below. At the cellular level, a fundamental action of many growth factors and cytokines is to stimulate rapid proton efflux from cells, most simply as a result of increased cellular metabolism (this phenomenon is often exploited to provide a convenient means of monitoring the bioactivity of growth factors). Parathyroid hormone (PTH), which may be considered a mitogenic growth factor, causes acidification of bone organ cultures [20]. Moreover, both PTH and insulin-like growth factor 1 stimulate extracellular acidification by cultured osteoblasts within minutes [21,22].

3. Acidosis and osteoclast function

The deleterious action of systemic acidosis on the skeleton has long been known [2,23–30] but was generally thought to result simply from physicochemical dissolution of bone mineral—i.e., the skeleton acted as a 'giant ion exchange column' to buffer systemic acidosis in a passive manner [2,31–33]. However, cell culture experiments showed that protons exerted a direct stimulatory effect on bone resorption by cultured rat osteoclasts [34,35]. Mature rat osteoclasts were observed to be almost inactive at pH 7.4, which corresponds to 'physiological' or blood pH, but resorption pit formation increased steeply as pH was reduced, reaching a plateau at about pH 6.8. Subsequent studies showed that avian [36] and human [37] osteoclasts show similar acid-activation responses (Fig. 1).

The sensitivity of OC to extracellular H^+ is such that pH reductions of only a few hundredths of a unit cause a doubling of resorptive activity [35,38]. This effect is not subject to tachyphylaxis (or 'escape') in longer-term cultures: acid-activated osteoclasts continue to form resorption pits over periods of 7 days or more, amplifying the effects of modest pH differences (Fig. 2). Acidosis is required for the initiation of resorption; once activated, OC can be further stimulated by factors such as RANKL, $1,25(OH)_2$ vitamin D, PTH and ATP (e.g., [38,39]); note that pro-resorptive agents such as RANKL and PTH are

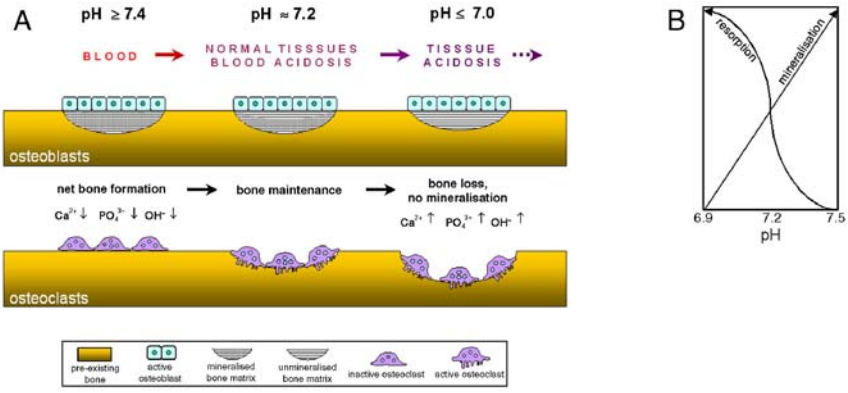

Fig. 7. Schematics summarising the effects of extracellular pH on bone formation and resorption. (A) Approximate ranges for blood and extracellular tissue pH are shown. There is little or no effect of extracellular pH between 7.4 and 7.0 on collagenous matrix production by osteoblasts, nor on osteoblast proliferation; however, mineralisation (grey shading) is strongly inhibited by acidosis, and fails to occur at pH 7.0 or below. Conversely, acidosis is the key initial requirement for osteoclast activation to occur: osteoclasts show little or no activity at pH 7.4, and are strongly activated to form resorption pits as pH is reduced to 7.0; osteoclast recruitment and survival are not sensitive to pH in the range 7.4–7.0. Thus, at pH $\geq$ 7.2, Ca^{2+}, PO_4^{3-} and OH^- ions are deposited in bone, whereas at lower pH systemic availability of these ions in solution is favoured. (B) Reciprocal relationship between resorption and formation over the pathophysiological pH range.

inactive on osteoclasts at pH 7.4 or above (Fig. 3). Thus, osteoclast stimulation is a 2-step process, with acid-activation as the key initial requirement—and extracellular H^+ may be regarded as the long-sought 'OC activation factor' (OAF).

Acidosis stimulates resorption in calvarial bone organ cultures similarly. Furthermore, H^+-stimulated Ca^{2+} release from calvaria is almost entirely osteoclast-mediated, with a negligible physicochemical component [40,41] (Fig. 4). This finding is consistent with the fact that mineralised bone surfaces are normally covered by living cells, and are thus not directly exposed to ion-exchange phenomena. These observations suggest that the effects of acidosis on bone loss *in vivo* are likely to be mostly cell-mediated.

Some progress has been made towards understanding the mechanisms by which osteoclasts might detect and respond to changes in extracellular pH in such a sensitive manner. Several classes of proteins could function as extracellular pH sensors within the relevant pH range. These include the H^+-sensing G-protein-coupled receptors (H^+-GPCRs), two of which (TDAG8 and OGR1) are expressed by osteoclasts and osteoblasts [42,43], the acid-sensing ion channels (ASICS), several of which are expressed in bone [44], the $P2X_2$ receptor for extracellular ATP [39], and the TRP cation channels [45,46]. The TRP channels appear to be of particular interest. TRPV1, also known as the vanilloid receptor (VR1), responds not only to low pH but also to the irritant alkaloid capsaicin (the 'active ingredient' of hot chili peppers) and to heat. TRPV1 is expressed in peripheral sensory nerves and appears to play an important role in mediating pain due to hot temperatures, acidosis and exposure to capsaicin [45,46]. Increasing attention is being paid to the role played by TRPV1 in mediating bone cancer pain [47,48], which probably involves local acidosis. We recently found that that TRPV1 is expressed by normal human osteoclasts and

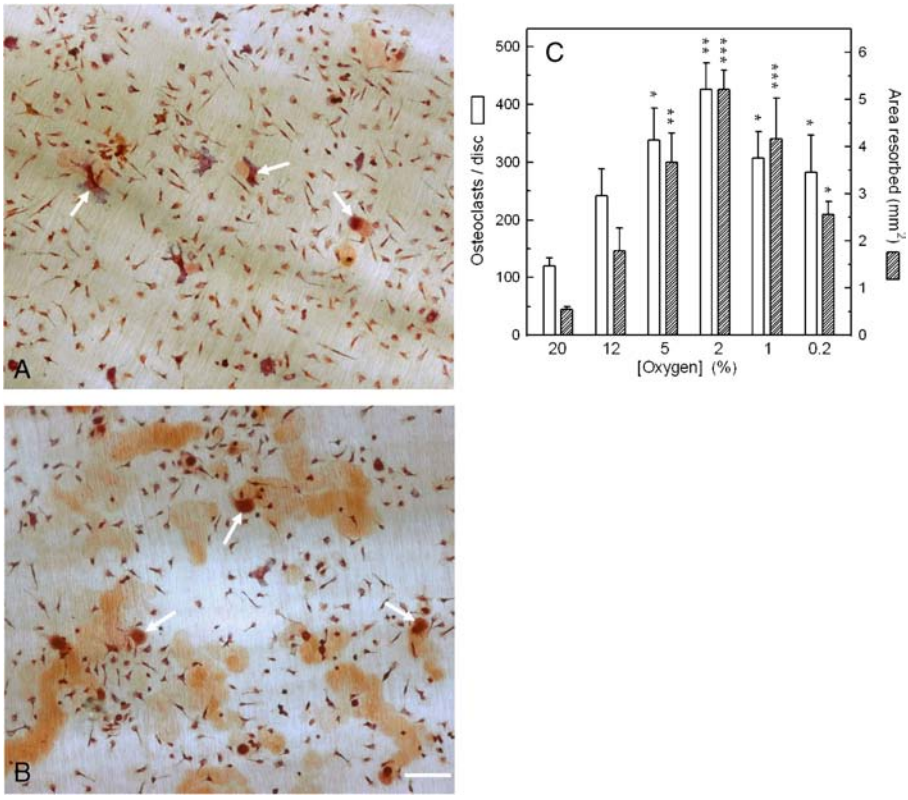

Fig. 8. Stimulation of osteoclast formation (and thus resorption) by hypoxia in mouse bone marrow cultures stained to demonstrate tartrate-resistant acid phosphatase (TRAP). (A) Osteoclasts (*arrows*) formed in mouse marrow cultures on ivory discs after 7 days in normoxic (20% O_2) conditions were generally small (<3 nuclei). (B) In hypoxic (2% O_2) cultures, many large osteoclasts were generated; prominent, deep resorption trails (stained brown) are evident; scale bar=100 μm. (C) Effect of hypoxia on longer term (13 day) mouse marrow cultures. Peak stimulation of osteoclast formation and resorption occurs in 2% O_2 but significant increases are evident even in severe hypoxia (0.2% O_2). Additional buffering was used to ensure that pH was unaffected by PO_2. Values are means±S.E.M. (n=8); *p<0.05, **p<0.01, ***p<0.001, compared to 20% O_2.

that low concentrations of capsaicin strongly activate resorption pit formation in non-acidified conditions [49]. A recent report has also described marked loss of trabecular bone 4 weeks after intravenous injection of capsaicin into rats, although the effect was ascribed to partial destruction of unmyelinated sensory neurons [50]. Dietary chili pepper intake is high in some parts of the world (e.g., Thailand, Mexico, Indian sub-continent); it is unclear whether significant quantities of ingested capsaicin in humans could reach bone. However, in rats it has been reported that capsaicin absorbed after ingestion is almost completely metabolised before reaching the general circulation [51]. Also of potential interest is the TRPM8 'cold' channel, which responds to menthol in a manner that is modulated by pH over the pathophysiological range [52]; menthol is reported to be a powerful anti-resorptive in rats [53].

A number of reports have described upregulation of the key resorptive machinery in osteoclasts following exposure to acidosis. Expression of the vacuolar-type H^+-ATPase and carbonic anhydrase II mRNA are increased rapidly in osteoclasts following acidification [54,55]. Acidosis has been reported to prevent inactivation of the transcription factor NFAT2 in osteoclasts, resulting in its nuclear accumulation [56]. Our own work shows that mouse bone and human osteoclasts exhibit striking upregulation of cathepsin K, tartrate-resistant acid phosphatase (TRAP-5) and TNF receptor associated factor 6 (TRAF-6) in low pH conditions [57].

4. Acidosis and osteoblast function

Bushinsky and colleagues reported that acidosis inhibited osteoblast function by decreasing expression of extracellular matrix genes, including collagen [58,59]. We investigated the effects of pH on osteoblast function in our laboratory using bone nodule-forming primary rat osteoblast cultures [60]. We found that abundant, matrix-containing mineralised nodules formed at pH 7.4, but acidification progressively reduced mineralisation of bone nodules, with complete abolition at pH 6.9 (Fig. 5). We also found that osteoblast proliferation and collagen synthesis were unaffected by pH in the range 7.4 to 6.9; moreover, no effect of acidification on collagen ultrastructure and organisation was evident. However, osteoblast alkaline phosphatase activity, which peaked strongly near pH 7.4, was reduced 8-fold at pH 6.9. Reducing pH to 6.9 also downregulated mRNA for alkaline phosphatase, but upregulated mRNA for matrix Gla protein, an inhibitor of mineralisation (Fig. 6). The same pH reduction is associated with 2- and 4-fold increases in Ca^{2+} and PO_4^{3-} solubility for hydroxyapatite, respectively [60].

Our results show that acidosis exerts a selective, inhibitory action on matrix mineralisation that is reciprocal with the OC activation response. Thus, in uncorrected acidosis, the deposition of alkaline mineral in bone by OB is reduced, and OC resorptive activity is increased in order to maximise the availability of hydroxyl ions in solution to buffer protons (Fig. 7). It is possible that these results could help to account for the osteomalacia that sometimes accompanies acidosis in renal disease [28,29,61].

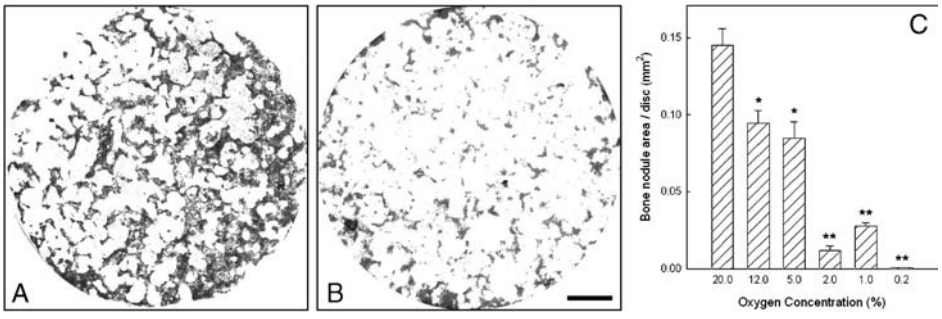

Fig. 9. Hypoxia inhibits osteoblast function. (A, B) Low power scans showing 'trabecular' bone nodule formation in unstained long term (21 day) osteoblast cultures in 20% and 2% O_2, respectively; scale bar=2 mm. (C) Bone nodule formation is progressively inhibited by decreasing O_2 and is completely blocked in severe hypoxia (0.2% O_2); *$p<0.05$, **$p<0.01$, compared to 20% O_2.

5. Hypoxia and the role of the vasculature in bone homeostasis

With advancing age, there is a slight but significant decrease in blood pH and HCO_3^-, i.e., a progressive, slight metabolic acidosis; this acidosis, which is probably of dietary origin, is ultimately due to the normal, age-related decline in renal function [13,14]. The general quality of the vascular supply around the body also tends to decline with age. In bone, which is highly vascular in young animals, ageing results in a progressive loss of the medullary blood supply which is only partly compensated by an increase in the periosteal blood supply, leading to marrow ischaemia [62] and hypoxia. This trend is also evidenced by the increase in yellow fatty marrow (at the expense of red marrow) with age.

Hypoxia has long been known to act as a stimulator of the formation or activation of cells derived from marrow precursors, including cells of the monocyte–macrophage lineage. Measurements of bone marrow aspirates from normal volunteer donors yield PO_2 values of about 6.5% [63,64]. These observations led us to investigate the effect of oxygen tension on the formation and function of osteoclasts, which are derived from myeloid precursors. We found that hypoxia causes a profound stimulation of osteoclast formation from mouse marrow and human peripheral blood cells, resulting in large stimulations of bone resorption. Peak effects occur in 2% oxygen, although osteoclast formation is stimulated strongly even in extreme hypoxia, where PO_2 is as low as 1/100 of atmospheric levels (Fig. 8). Hypoxia *per se* does not alter the resorptive activity of mature OC [65,66].

We also found that hypoxia strongly inhibits bone formation by osteoblasts, by reducing proliferation and collagen production/quality (Fig. 9); hypoxia does not increase osteoblast apoptosis but induces a state of reversible quiescence or 'suspended animation' [67]. Thus, oxygen tension exerts reciprocal effects on bone resorption and bone formation in a manner analogous to the effects of pH.

6. Conclusions

Our work shows that osteoclasts and osteoblasts display sensitive, reciprocal responses to acidosis. These effects may represent a primitive 'failsafe' mechanism that evolved with terrestrial vertebrates to correct systemic acidosis by ensuring release of alkaline bone mineral when the lungs and kidneys are unable to remove sufficient H^+ equivalent. Thus, the earlier concept that the skeleton functions as a passive 'ion-exchange column' with respect to acid–base balance must now be revised; the 'last defence' of systemic pH is perhaps too important to be left to physicochemical processes.

Osteoclast and osteoblast function also shows an impressive, reciprocal modulation by hypoxia. Since tissue hypoxia causes acidosis due to increased anaerobic metabolism and reduced vascular perfusion, the regulatory actions of oxygen tension and pH on bone cell function will occur in tandem in bone. These fundamental responses are indicative of the key role of the vasculature in bone. Consideration of the influence of the vasculature could yield potentially useful new insights into bone pathophysiology in a wide range of conditions, including fractures, tooth movement, inflammation/infection, arthritis, tumours, diabetes, anaemias, smoking, ageing, chronic respiratory failure, and excessive/anaerobic exercise.

A number of existing drug therapies, in addition to dietary alkali supplementation may exert beneficial effects on bone via alterations in systemic acid–base balance or improved

blood flow. Hormone replacement therapy with oestrogen and progestin is reported to cause a slight respiratory alkalosis, possibly via a stimulatory action of progestins on respiration [68]. Oestrogen, on the other hand, acts to increase blood flow via a vasodilatory action [69]. In rats, testosterone deficiency due to orchiectomy results in mild metabolic acidosis and osteoporosis which is alleviated by supplementation with alkaline salts [70]. The thiazide diuretics, which induce alkalosis are also osteoprotective [71,72]. Future drug therapies could target H^+-sensing receptors to reduce osteoclast activity or boost blood flow to bone. The present work also provides further rationale for public health measures that promote vascular function, including exercise, avoidance of smoking and good diet.

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