pH dependence of bone resorption: mouse calvarial osteoclasts are activated by acidosis

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Meghji, Sajeda, Matthew S. Morrison, Brian Henderson, and Timothy R. Arnett. pH dependence of bone resorption: mouse calvarial osteoclasts are activated by acidosis. Am J Physiol Endocrinol Metab 280: E112–E119, 2001—We examined the effects of HCO3− and CO2 acidosis on osteoclast-mediated Ca2+ release from 3-day cultures of neonatal mouse calvaria. Ca2+ release was minimal above pH 7.2 in control cultures but was stimulated strongly by the addition of small amounts of H+ to culture medium (HCO3− acidosis). For example, addition of 4 meq/l H+ reduced pH from 7.12 to 7.03 and increased Ca2+ release 3.8-fold. The largest stimulatory effects (8- to 11-fold), observed with 15–16 meq/l added H+, were comparable to the maximal Ca2+ release elicited by 1,25-dihydroxyvitamin D3 [1,25(OH)2D3; 10 nM], parathyroid hormone (10 nM), or prostaglandin E2 (1 μM); the action of these osteolytic agents was attenuated strongly when ambient pH was increased from ~7.1 to ~7.3. CO2 acidosis was a less effective stimulator of Ca2+ release than HCO3− acidosis over a similar pH range. Ca2+ release stimulated by HCO3− acidosis was almost completely blocked by salmon calcitonin (20 ng/ml), implying osteoclast involvement. In whole mount preparations of control half-calvaria, ~400 inactive osteoclast-like multinucleate cells were present; in calvaria exposed to HCO3− acidosis and to the other osteolytic agents studied, extensive osteoclastic resorption, with perforation of bones, was visible. HCO3− acidosis, however, reduced numbers of osteoclast-like cells by ~50%, whereas 1,25(OH)2D3 treatment caused increases of ~75%. The results suggest that HCO3− acidosis stimulates resorption by activating mature osteoclasts already present in calvarial bones, rather than by inducing formation of new osteoclasts, and provide further support for the critical role of acid-base balance in controlling osteoclast function.

THE SKELETON CONTAINS a massive reserve of base that is available as a “fail-safe” mechanism to buffer protons if the kidney and lungs are unable to maintain acid-base balance within narrow physiological limits (8, 15). The deleterious effects of systemic acidosis on the skeleton have been recognized at least since the early part of this century (5, 18). More recent in vivo studies have suggested that the bone loss associated with acidosis is not due to passive physicochemical processes but involves enhanced osteoclastic resorption (9, 22).

Experiments with disaggregated rat osteoclasts cultured on cortical bone or dentine wafers provided the first direct evidence for the stimulatory action of extracellular protons on cell-mediated bone resorption. Resorption pit formation by rat osteoclasts in culture media buffered nonphysiologically, with the use of HEPES only, was activated by progressive acidification from pH 7.4 to 6.8 (3). Cultured osteoclasts are also stimulated to resorb in physiologically buffered media when pH is reduced either by decreasing HCO3− concentration or by increasing PCO2 (2). Recent work has shown that rat osteoclasts are particularly sensitive to extracellular pH in the range 7.2–7.0, such that shifts of <0.1 unit are sufficient to cause changes of several-fold in pit formation (6).

Experiments with cultured mouse calvaria showed that calcium efflux from bones was stimulated much more strongly when extracellular pH was reduced by decreasing HCO3− concentration (metabolic acidosis) than by increasing PCO2 (respiratory acidosis) (11, 12). The aims of the present study were 1) to investigate the effects of small extracellular pH changes resulting from HCO3− and CO2 acidosis on osteoclastic resorption in cultured calvaria; 2) to compare the effects of acidosis on calvarial resorption with those of “classical” osteolytic agents and to determine whether the action of these agents was dependent on ambient acidification; and 3) to investigate whether acidosis stimulates resorption by activating mature osteoclasts already present in calvarial bones or by inducing formation of new osteoclasts.

MATERIALS AND METHODS

Reagents. 1,25-Dihydroxyvitamin D3 [1,25(OH)2D3] was kindly provided by Dr K. W. Colston (St. George’s Hospital Medical School, London, UK). BWA 70C, a 5-lipoxygenase inhibitor of the iron ligand class, containing the hydroxamic acid release; carbon dioxide; bicarbonate ion; acid-base balance in controlling osteoclast function.

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acid chelating group, was a gift of the Wellcome Foundation (Beckenham, Kent, UK). MK 886, a selective inhibitor of the 5-lipoxygenase-activating protein (FLAP), was donated by Merck Frosst (Kirkland, QC, Canada). Other reagents, unless specified, were purchased from Sigma (Poole, Dorset, UK).

**Mouse calvarial bone resorption assay.** The method, which measures bone resorption as Ca\(^{2+}\) release from neonatal mouse calvaria, was similar to that described in detail by Meghji et al. (24). Briefly, 5-day-old MF1 mice were killed by cervical dislocation. The frontaliparietal bones were removed and trimmed of any adhering connective tissue and interparietal bone, with care being taken 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 cultured individually on 1-cm\(^2\) stainless steel grids (Minmesh, FDP quality, Expanded Metal, West Hartlepool, UK) in 6-well plates with 1.5 ml of BGJ medium, 5% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 \(\mu\)g streptomycin (ICN Biomedicals, Basingstoke, Hants, UK) in 6-well plates with 1.5 ml of BGJ medium after addition of 5% heat-inactivated fetal calf serum was 2.00 mM. All measurements were performed blind on coded samples.

**Whole mount histology.** After fixation/decalcification with 95% ethanol-5% glacial acetic acid, calvaria were stained for tartrate-resistant acid phosphatase (TRAP) (20, 23) by means of a Sigma kit 387-A and were mounted in melted glycerol jelly. The numbers of TRAP-positive multinucleated osteoclasts (two or more nuclei) were assessed blind on coded samples by means of transmitted light microscopy.

**Statistics.** Statistical comparisons, where appropriate, were made by one-way analysis of variance, with the use of Bonferroni’s correction for multiple comparisons. Representative data are presented as means \(\pm\) SE for 5 replicates. Results are shown for representative experiments that were each repeated at least three times.

### RESULTS

**Effect of \(\text{HCO}_3^-\) acidosis on calvarial resorption.** Basal levels of \(\text{Ca}^{2+}\) release in nonacidified control bones (pH 7.20–7.25) were very low after 3 days of culture. Acidification of culture medium by addition of small amounts of \(\text{H}^+\) as \(\text{HCl}\) resulted in a steep increase in \(\text{Ca}^{2+}\) release from live bones. For example, decreasing the pH of the culture medium from 7.21 (control) to 7.17 by addition of 3 meq/l \(\text{H}^+\) reduced \(\text{HCO}_3^-\) concentration from 13.2 to 12.2 mmol/l, resulting in a 2.8-fold increase in \(\text{Ca}^{2+}\) release, and decreasing the pH to 6.94 by addition of 15 meq/l \(\text{H}^+\) reduced \(\text{HCO}_3^-\) concentration to 7.1 mmol/l, causing an 8.3-fold stimulation of \(\text{Ca}^{2+}\) release. In contrast, in bones killed by freeze-thawing, there was a marked influx of \(\text{Ca}^{2+}\), evidenced by a decrease in culture medium \(\text{Ca}^{2+}\) concentration; the magnitude of this influx was slightly reduced with progressive acidification (Fig. 1).

**Effect of \(\text{CO}_2\) acidosis.** When culture medium was acidified from 7.12 to 6.90 by increasing incubator \(\text{PCO}_{2}\) from 54.5 to 87.2 mmHg, only a small, nonsignificant increase in \(\text{Ca}^{2+}\) release from calvaria was observed after 3 days of culture. The stimulation of \(\text{Ca}^{2+}\) release resulting from \(\text{CO}_2\) acidosis was markedly lower than that elicited by \(\text{HCO}_3^-\) acidosis at comparable \(\text{pH}\) values. However, further acidification to pH 6.81 by increasing \(\text{PCO}_{2}\) to 108.5 mmHg resulted in a 5.3-fold stimulation of \(\text{Ca}^{2+}\) release compared with control. This stimulation occurred in the face of an increase in \(\text{HCO}_3^-\) concentration from 15.8 to 22.5 mM (Fig. 2).

**Effects of inhibitors.** \(\text{HCO}_3^-\) acidosis-stimulated \(\text{Ca}^{2+}\) release (resulting from addition of 15 meq/l \(\text{H}^+\)) was completely blocked by salmon calcitonin (20 ng/ml; Fig. 3). Thus \(\text{Ca}^{2+}\) release stimulated by \(\text{HCO}_3^-\) acidosis was due to osteoclastic resorption. \(\text{Ca}^{2+}\) release resulting from severe \(\text{CO}_2\) acidosis was also blocked by salmon calcitonin (20 ng/ml; data not shown). \(\text{HCO}_3^-\) acidosis-stimulated \(\text{Ca}^{2+}\) release was additionally completely blocked by the cyclooxygenase inhibitors indomethacin (Fig. 4) and ibuprofen (data not shown) and was inhibited by the 5-lipoxygenase inhibitors, BWA 70C (Fig. 5A) and MK 886 (Fig. 5B), suggesting that the effect may be mediated by both prostaglandins and leukotrienes.

Effects of “classical” resorption stimulators. The maximal effects of the osteolytic agents 1,25(OH)\(_2\)D\(_3\)
(10 nM), bovine parathyroid hormone-(1–34) (PTH, 20 ng/ml) and PGE2 (1 μM) on Ca2+ release from 3-day calvarial cultures were similar and were equivalent in magnitude to the effect of HCO3− acidosis at pH 6.94 (15 meq/l added HCl; Fig. 6). In most experiments, PTH, 1,25(OH)2D3, and PGE2 treatments themselves resulted in slight acidification of culture medium.

In an additional series of experiments, we examined the dependence of the osteolytic action of PGE2, 1,25(OH)2D3, and PTH on ambient acidification in 3-day cultures of mouse half-calvaria. Addition of 15 meq/l OH− as NaOH increased final medium pH from ~7.1 in control cultures to ~7.3, resulting in marked attenuation of Ca2+ release in all treatment groups (Fig. 7). Addition of equivalent amounts of NaCl to culture medium was without effect on bone resorption. In the case of PGE2-stimulated bones, increasing pH from 7.12 to 7.33 reduced resorption by 80%.

**Histology (TRAP staining) and cell counting.** In whole mount preparations of half-calvaria cultured in control medium for 3 days and then stained to demonstrate tartrate-resistant acid phosphatase, ~200–400 inactive TRAP-positive osteoclast-like multinucleate cells were typically present; resorption cavities visualized by the TRAP staining were small and relatively inactive (Figs. 8A and 9). The appearance of freshly isolated bones (i.e., not cultured) was similar. In calvaria exposed to HCO3− acidosis (~12 meq/l H+), extensive osteoclastic resorption cavities, with characteristic scalloped edges, were evident (Fig. 8B); resorption was sometimes sufficiently aggressive to cause complete perforation of bones. In bones exposed to severe CO2 acidosis (108.5 mmHg) at pH 6.82, osteoclasts in scalloped resorption bays were also clearly evident (Fig. 8C). Extensive resorption cavities were also ob-
served in bones treated with 10 nM 1,25(OH)2D3 (Fig. 8D), 20 ng/ml PTH-(1–34), or 1 μM PGE2. However, cell counts revealed that HCO3− acidosis reduced the numbers of osteoclast-like cells by ~50% compared with controls, whereas 1,25(OH)2D3 treatment caused increases of ~75% (Fig. 9).

**DISCUSSION**

The present study correlates Ca2+ release data with histological evidence to demonstrate that osteoclastic resorption in cultured mouse calvarial bones is extremely sensitive to activation by HCO3− acidosis, an effect which can cause bone destruction equivalent to the maximal osteolysis produced by agents such as 1,25(OH)2D3, PTH, or PGE2. Furthermore, our results show that the action of these classical osteolytic agents is attenuated markedly by slight alkalinization. We also found that severe CO2 acidosis resulted in increased osteoclastic resorption.

Three separate lines of evidence indicate that net Ca2+ release into the culture medium from mouse calvarial bones stimulated by HCO3− acidosis is almost

![Fig. 4](http://example.com/fig4.png)

**Fig. 4.** Complete blockage of HCO3− acidosis-stimulated Ca2+ release from 3-day cultures of mouse half-calvaria by the cyclooxygenase inhibitor indomethacin. Hatched bar shows Ca2+ release in nonacidified control cultures. PCO2 was 64.0 mmHg. Values are means ± SE (n = 5). Significantly different from cultures treated with 12 meq/l H+ alone: *P < 0.05; **P < 0.01.

![Fig. 5](http://example.com/fig5.png)

**Fig. 5.** Inhibition of HCO3− acidosis-stimulated Ca2+ release from 3-day cultures of mouse half-calvaria by the 5-lipoxygenase inhibitors BWA 70C (A) and MK 886 (B). Hatched bars show Ca2+ release in nonacidified control cultures. PCO2 was 64.0 mmHg. Values are means ± SE (n = 5). Significantly different from cultures treated with 12 meq/l H+ alone: *P < 0.05.

![Fig. 6](http://example.com/fig6.png)

**Fig. 6.** Equivalence of the stimulatory action of HCO3− acidosis (15 meq/l H+; pH 6.94) on Ca2+ release from mouse half-calvaria to the maximal effects of the classical osteolytic agents prostaglandin E2 (PGE2), bovine parathyroid hormone-(1–34) (PTH), or 1,25-dihydroxyvitamin D3 (1,25 D3). PTH and 1,25(OH)2D3 treatment resulted consistently in slight acidification of culture medium. PCO2 was 54.5 mmHg. Values are means ± SE (n = 5). Significantly different from pH 7.21 control: ***P < 0.001.
marrow cultures (29) show remarkably similar responses to extracellular pH changes. Taken together, these observations suggest that the local pH in the microenvironment of osteoclasts in cultured mouse parietal bones is close to that of the tissue culture medium and thus considerably lower than that of blood pH (~7.40).

We also demonstrated that CO$_2$ acidosis is a less effective stimulator of Ca$^{2+}$ release from calvarial bones than HCO$_3^-$ acidosis over a similar pH range, a result that is in good agreement with data from the $^{45}$Ca$^{2+}$ flux experiments of Bushinsky and colleagues (11, 12). One partial explanation for the smaller stimulatory effect of CO$_2$ acidosis may be that hypercapnia promotes deposition of Ca$^{2+}$, as carbonates, on bone surfaces (11). Nevertheless, our own results show that resorption is activated quite strongly by severe CO$_2$ acidosis at low pH (Pco$_2$ = 108.5 mmHg; pH = 6.81). It may be that, when Pco$_2$ is increased, the "set point" at which resorption begins to be acid activated is shifted to lower pH values. It is also noteworthy that the striking stimulation of bone resorption caused by severe CO$_2$ acidosis occurred despite an increase in HCO$_3^-$ concentration (22.5 mM compared with control value of 15.8 mM). This result suggests that, in the main, it is the increased H$^+$ concentration that is ultimately responsible for acidosis-stimulated osteoclastic bone resorption. Experiments with cultured osteoclasts have not shown clear differences between the effects of CO$_2$ and HCO$_3^-$ acidosis in stimulating pit formation (Ref. 2; M. Morrison and T. R. Arnett, unpublished data). The reasons for this discrepancy are unknown. In vivo, HCO$_3^-$ acidosis is associated with bone loss (reviewed in Ref. 5). In thyroparathyroidectomized rats, acute HCO$_3^-$ acidosis induced by acid feeding results in a striking hypercalcemia that is prevented by calcitonin or colchicine, implying osteoclast involvement (22). The effects of CO$_2$ acidosis in vivo are less well investigated; however, a recent study (32) reported that hormone replacement therapy causes a respiratory alkalosis in normal postmenopausal women and that changes in blood pH were inversely correlated with those in urinary excretion of hydroxyproline, an index of bone resorption.

The behavior of the calvarial system in response to acid stimulation also differs from that of cultured osteoclasts in another key respect. We found that resorption stimulated by HCO$_3^-$ acidosis could be blocked by the cyclooxygenase inhibitors indomethacin and ibuprofen, in line with earlier findings (17, 33) and suggesting a requirement for endogenous prostaglandin synthesis. In contrast, resorption pit formation by cultured osteoclasts is stimulated by cyclooxygenase inhibitors (26, 27) and is inhibited by prostaglandins (4, 13). Our observation that HCO$_3^-$ acidosis-activated resorption was additionally attenuated by two mechanistically distinct inhibitors of the 5-lipoxygenase pathway suggests that leukotrienes, which stimulate bone resorption in a number of systems (16, 25), are also involved in mediating the effect.

Our findings show that bone resorption in cultured mouse calvaria may be more sensitive to small pH changes than was previously appreciated. Ca$^{2+}$ release was minimal above pH 7.2 in control cultures but was stimulated strongly by the addition of small amounts of H$^+$ (i.e., HCO$_3^-$ acidosis) to culture medium. Figure 2 shows that addition of 4 meq/l H$^+$ reduced pH from 7.12 to 7.03 but increased Ca$^{2+}$ release 3.8-fold; an 11-fold increase was observed with 16 meq/l added H$^+$. The steep responses to HCO$_3^-$ acidosis evident in Figs. 1 and 2 resemble closely the acid-activation curve for resorption pit formation by cultured rat osteoclasts in which resorption is essentially "switched off" above pH 7.2 and stimulated maximally at pH ~6.9 (6, 31). Osteoclasts derived from chick long bones (4, 28), human osteoclastoma tissue (19), or long-term mouse

![Fig. 7. Dependence of the osteolytic action of PGE$_2$, 1,25(OH)$_2$D$_3$, and PTH on ambient acidification in 3-day cultures of mouse half-calvaria. Addition of 15 meq/l OH$^-$ as NaOH increased final medium pH from ~7.1 in control cultures to ~7.3, resulting in marked attenuation of Ca$^{2+}$ release in all treatment groups. PCO$_2$ was 57.2 mmHg. Values are means ± SE (n = 5). Significantly different from control group (C): *P < 0.05, ***P < 0.001; significantly different from respective, nonalkalinized treatment group: #P < 0.05 (PTH), ###P < 0.01 (PGE$_2$).]
In this study, we correlated a biochemical index of calvarial bone resorption (Ca\(^{2+}\) release) with quantitative histological analysis of the same bones. Surprisingly, perhaps, this approach has not been used before. The simple whole mount histological technique we used was first described by Marshall and colleagues (20, 23), who reported that ~200 multinucleate, TRAP-positive osteoclasts were visible in single parietal bones of 4-day-old mice before being placed in culture; our own data for control bones agree well with these values. In calvaria exposed to HCO\(_3\) acidosis, extensive osteoclastic resorption cavities were evident, but TRAP-positive osteoclast numbers were reduced consistently. Whether this change in osteoclast numbers reflects reductions in osteoclast survival, formation, or both, is uncertain. Cell culture experiments have shown that osteoclast formation in 10-day mouse marrow cultures is inhibited at pH 6.9–7.0 (29); however, survival of mature osteoclasts in this pH range appears unimpaired (3, 6, 31). Thus the impressive resorption

![Fig. 8. Whole mount mouse half-calvaria stained to demonstrate tartrate-resistant acid phosphatase (TRAP) after 72-h culture, viewed by transmitted light microscopy. A: nonacidified control, pH 7.208, showing small resorption cavities (arrowheads); scale bar = 500 μm. B: bone treated with 12 meq/l H\(^+\), pH 7.01 (HCO\(_3\) acidosis); scale bar = 500 μm. C: higher power detail of bone exposed to severe CO\(_2\) acidosis (108.5 mmHg) at pH 6.82; osteoclasts in scalloped resorption bays (arrowheads) are clearly evident; scale bar = 100 μm. D: bone treated with 10 nM 1,25(OH)\(_2\)D\(_3\), pH 7.09; scale bar = 500 μm. Large areas of osteolysis, demonstrated by red-black TRAP staining of scalloped resorption fronts and osteoclasts (arrowheads), are visible in acidosis and 1,25(OH)\(_2\)D\(_3\)-treated bones.](http://ajpendo.physiology.org/)

![Fig. 9. Stimulation of TRAP-positive osteoclast (OC)-like cell numbers in whole mount mouse half-calvaria by 10 nM 1,25(OH)\(_2\)D\(_3\) (1,25 D\(_3\)), but not 1 μM PGE\(_2\) or HCO\(_3\) acidosis resulting from addition of 15 meq/l H\(^+\). Significantly different from control (C): *P < 0.05.](http://ajpendo.physiology.org/)
cavities seen in calvaria exposed to acidosis may reflect mainly the activation of preexisting quiescent osteoclasts rather than the formation of new osteoclasts. However, the possibility cannot be excluded that acidosis rapidly stimulates the formation of new osteoclasts, which are then immediately activated, alongside increased apoptosis of preexisting quiescent osteoclasts.

In contrast, the osteolytic effects of 1,25(OH)2D3 appeared to be related, at least in part, to increases in TRAP-positive osteoclast numbers, reflecting the stimulatory action of this hormone on osteoclast formation in long-term marrow cultures. Ca2+ release in 1,25(OH)2D3-treated cultures was similar to that in cultures exposed to HCO3– acidosis, but resorption on a per cell basis was ~3.5-fold lower, based on the cell counts shown in Fig. 9. Interestingly, cultures treated with 1,25(OH)2D3, PTH, or PGE2 generally exhibited acidification by cultured primary osteoblast-like cells derived from rodent calvaria (34). Other bone-resorbing agents reported to stimulate H+ secretion for the role of acid-base imbalance in the genesis of osteoporosis. J Bone Miner Res 10: 1431–1436, 1995.


REFERENCES


