The effects of acid on bone

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Metabolic acidosis induces calcium efflux from bone and in the process buffers the additional hydrogen ions. Initially metabolic acidosis stimulates physicochemical mineral dissolution and then cell-mediated bone resorption. Acidosis increases activity of the bone resorbing cells, the osteoclasts, and decreases activity of the bone forming cells, the osteoblasts. Osteoblastic immediate early response genes are inhibited as are genes controlling matrix formation. Curr Opin Nephrol Hypertens 9:369–379.

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Introduction

The maintenance of a stable physiologic systemic pH is of critical importance to the survival of mammals [1]. However, there are relatively common clinical disorders, including chronic diarrhea, renal failure and renal tubular acidosis, in which a gain of acid or loss of base results in decreased systemic pH (acidosis) [1–3]. While only net loss of hydrogen ions can ultimately correct acidosis [1], bone appears to be instrumental in the maintenance of a stable physiologic systemic pH during metabolic acidosis. However, this homeostatic function is often at the expense of bone mineral content [1–28, 29••,30,31••,32–34].

In-vivo observations

During in-vivo acute metabolic acidosis (a primary decrease in bicarbonate ion concentration), approximately sixty percent of the administered protons are buffered outside of the extracellular fluid [35] by soft tissues [36–38] and by bone [2–4,7–9,16,26,39–41]. The in-vivo evidence that bone acutely buffers hydrogen ions, and in the process releases calcium ions, derives principally from the loss of bone sodium and/or potassium [6,15,23,24,42–44], carbonate [8,16,44,45], and the increase in serum calcium [46] observed during acidosis. Bone sodium (or potassium) loss implies protons for sodium (or potassium) exchange and carbonate loss suggests consumption of this buffer by the administered protons. At least 98% of body calcium is contained within bone [47,48] therefore the increase in serum calcium is likely to derive from mineral stores. Chronic metabolic acidosis, found in patients with renal insufficiency [1,3,49] and renal tubular acidosis [1,50], increases urinary calcium excretion [51–53] without an increase in intestinal calcium absorption [54,55]. This results in a negative calcium balance [56,57] that appears to reflect proton-mediated dissolution of bone mineral [1,3,39,51,58,59]. Indeed, in most in-vivo studies chronic metabolic acidosis appears to decrease mineral content [56–58,60].

On a daily basis, metabolism of dietary protein generates approximately 1 meq/kg of protons in adults [1,3]. The coupling of this chronic acid ingestion, induced by the common North American high protein diet, with the known effects of acid on bone has led to the suggestion that it may play a role in the etiology of osteoporosis [58,61–63]. Supporting this hypothesis is the observation that administration of base appears to decrease the negative calcium balance induced by a high protein diet [64–66].

In addition to its effects on existing bone, metabolic acidosis also has the potential to affect de-novo bone formation. Normally, osteoblasts synthesize an extracellular matrix consisting largely of type 1 collagen fibrils but also including noncollagenous proteins such as osteopontin, osteonectin, osteocalcin, matrix Gla protein, and bone sialoprotein [67]. As this matrix matures it sequesters phosphate from the environment [68]. The independent effect of acidosis to suppress bone formation was elegantly demonstrated in children with renal tubular acidosis [69–71]. In a radiographic study the majority of patients with proximal renal tubular acidosis had rickets or osteopenia [72]. There is ample clinical evidence that acidosis adversely affects bone during renal failure [73–76]. Bone carbonate is decreased in acidic uremic patients [77–79]. This decrease may represent dissolution of bone carbonate stores or replacement by phosphate resulting in the incorporation of protons into the mineral [8,16,45]. Bone mineral loss may be corrected by bicarbonate ion administration [80–82].

In-vitro observations

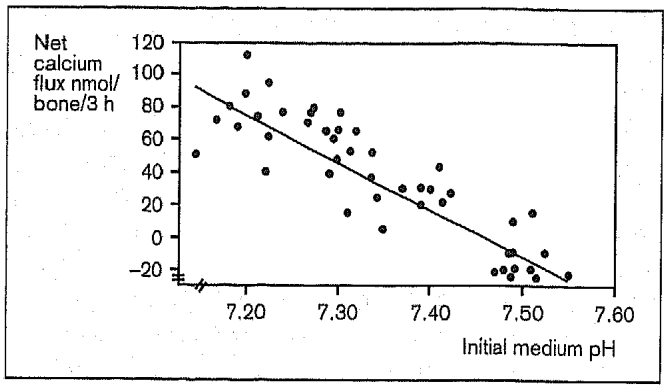
Although in-vivo evidence strongly suggests that bone is involved in the systemic response to acid-base disorders, until recently there was little direct in-vitro confirmation [2]. Neuman *et al.* found that a reduction of medium pH produced a marked increase in hydroxyapatite solubility [83]. Dominguez and Raisz [84] determined that an acid medium induced movement of ^{45}Ca from prelabelled bone.

We undertook a series of studies to test the hypotheses that cultured bone exposed to a physiologically acidic medium would release calcium into the medium and buffer the increased medium protons. We utilized the model of cultured neonatal mouse calvariae (frontal and parietal bones of the skull), which, like bones *in vivo*, have functioning osteoclasts and osteoblasts [85,86], respond to hormones, and synthesize DNA and proteins [87]. We found that calvariae can be cultured in the physiologic CO_2-HCO_3^- buffer system [4]. Medium pH can be regulated precisely by independently altering the partial pressure of carbon dioxide or bicarbonate ion concentration, simulating either respiratory or metabolic acid-base disorders respectively [1,4].

Acute acid-induced calcium release

We found that cultured calvariae exhibit a proton-dependent net calcium efflux during both acute (3 h) and more chronic (>24–99 h) incubations [1–27]. During acute incubations there was a net calcium efflux from the calvariae when medium pH was decreased to less than the physiologic normal of 7.40 by decreasing the bicarbonate ion concentration. There was no net flux at pH = 7.40 and an influx of calcium into bone when pH was greater than 7.40 [4] (Fig. 1).

Figure 1. Effect of initial medium pH on net calcium flux in calvariae cultured for three hours

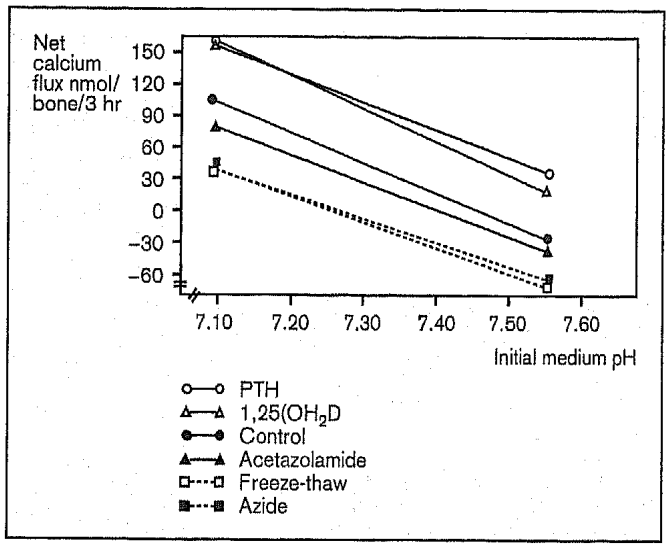


A positive flux indicates net calcium movement from the calvariae into the medium. pH was adjusted for the 3 h incubation with concentrated HCl or NaOH at a PCO_2 of 40 mmHg. Calvariae were preincubated in control medium for 24 h prior to this 3 h incubation. $r = 0.890$, $n = 46$, $P < 0.001$. Reproduced with permission from [5].

We next tested the hypothesis that the mechanism of proton-mediated calcium efflux from bone during these acute incubations was direct physicochemical (non-cell-mediated) calcium release. We cultured calvariae with agents that would stimulate or suppress bone cell activity but not affect the mineral directly [5]. We found that the contribution of bone cells to calcium efflux from the mineral during these acute (3 h) experiments was constant and pH-independent, thus acute proton-mediated calcium release was due to physicochemical and not cell-mediated mechanisms [5] (Fig. 2). To confirm that protons could alter physicochemical forces and promote dissolution of the bone mineral, synthetic carbonated apatite disks were cultured in physiologically acid medium [17]. The carbonated apatite disks are an accurate cell-free model of bone mineral [88–94]. We demonstrated calcium efflux from cultured carbonated apatite disks similar to those from cultured calvariae in response to a physiologic acidosis, supporting the hypothesis that protons can induce physicochemical calcium release from bone [17].

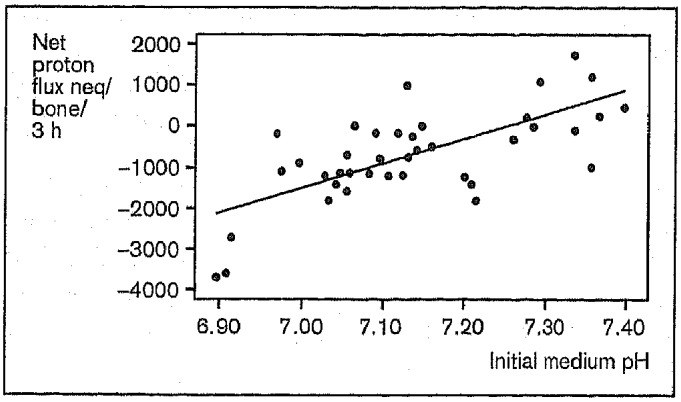
The type of bone mineral in equilibrium with the medium, and thus altered by the physicochemical forces, could be either carbonate or phosphate in association with calcium. We tested the effect of altering the driving forces for crystallization with respect to the solid phase of the bone mineral by measuring the calcium efflux after an alteration in phosphate or carbonate concentration at either neutral or acid medium pH in calvarial cultures [8]. With respect to calcium and carbonate, but not calcium and phosphate, there was bone formation in a supersaturated medium, no change in the bone mineral when cultured in a saturated medium and bone dissolution into an undersaturated medium. Thus bone carbonate appears to be selectively solubilized during an

Figure 2. Comparison of regressions of initial medium pH on net calcium flux for six separate groups of calvariae



Calvariae were incubated for 24 h in similar medium prior to the 3 h reincubation. Acetazolamide, calvariae incubated in control medium with acetazolamide 4×10^{-4} M; azide, calvariae incubated in control medium with azide 0.1% final concentration; control, calvariae incubated in control medium (pH ~ 7.40); freeze-thaw, calvariae incubated in control medium after three successive freeze-thaw cycles; parathyroid, calvariae incubated in control medium with parathyroid hormone at a concentration of 1×10^{-8} M; $1,25(OH)_2D_3$, calvariae incubated in control medium with 1,25 dihydroxyvitamin D_3 at a concentration of 1×10^{-8} M. Regressions are different due to a variation in intercepts of all groups except parathyroid hormone and $1,25(OH)_2D_3$, which are similar, and azide and freeze-thaw, which are similar. Slopes are similar in all six groups. Reproduced with permission from [5].

Figure 3. Effect of initial medium pH on net proton flux in calvariae cultured for three hours



A positive flux indicates net proton movement from the calvariae into the medium, a negative flux the opposite. pH was adjusted for the 3 h incubations with concentrated HCl or NaOH at a partial pressure of carbon dioxide of 40 mmHg. $r = 0.735$, $n = 41$, $P < 0.001$. Reproduced with permission from [4].

acute reduction in pH leading to a release of calcium. When we cultured calvariae in acidic medium there was a progressive loss of total bone carbonate during a model of metabolic acidosis [16]. Further support for the role of carbonate in acid-mediated bone mineral dissolution comes from studies in which we demonstrated that at a constant pH, whether physiologically neutral or acidic, bone calcium efflux is dependent on the medium bicarbonate concentration. A lower bicarbonate concentration leads to greater calcium efflux [14]. Bone carbonate appears to be in the form of carbonated apatite [91,95,96].

Hydrogen ion buffering

The in-vitro evidence for proton buffering by bone is derived from studies of acidosis-induced proton flux into bone [4,7–9] and microprobe evidence for a depletion of bone sodium and potassium during acidosis [6,11,15,23,24]. We have shown that when calvariae are cultured in medium acidified by a decrease in carbonate concentration, there is a net influx of hydrogen ions into the bone, decreasing the medium hydrogen ion concentration and indicating that the additional ions are being buffered by bone [4,7–9] (Fig. 3). This influx of protons into bone leads to an increase in the pH of the culture medium.

Proton for sodium/potassium exchange

Bone is a reservoir for sodium and potassium and its surface has fixed negative sites that normally complex with sodium, potassium and hydrogen ions. The sodium and potassium appear to exchange freely with the surrounding fluid [47,48]. Using a high resolution scanning ion microprobe with secondary ion mass spectroscopy we found that the surface of the bone is rich in sodium and potassium relative to calcium [6,11,15,97–99]. After incubation in acidic medium there is loss of surface sodium and potassium relative to calcium [6,11,15,23,24] in conjunction with proton buffering, suggesting that sodium and potassium exchange with hydrogen ions on the bone surface resulting in a decrease in medium acidity [6,42,43]. When osteoclastic function is inhibited with calcitonin, microprobe analysis indicates that physicochemical proton buffering by bone causes relatively equal calcium and sodium loss [15]. In acidic medium, osteoclastic function is necessary to support the enriched levels of bone potassium [24].

Fall in bone carbonate

Bone contains approximately eighty percent of the total carbon dioxide (including CO_3^{2-}, HCO_3^- and CO_2) in the body [100]. Approximately two-thirds of this is in the form of carbonate complexed with hydrogen ions as bicarbonate ions (HCO_3^-) or with calcium, potassium and sodium and other cations. It is located in the lattice of the bone crystals where it is relatively inaccessible to the systemic circulation. The other third is located in the hydration shell of hydroxyapatite where it is readily available to the systemic circulation. Acute metabolic acidosis decreases

bone total carbon dioxide [45]. We have shown that acidosis induces the release of calcium and carbonate from bone [8] leading to a progressive loss of bone carbonate during metabolic acidosis [16].

When both the in-vitro and in-vivo studies are considered together there is strong evidence that bone is a proton buffer capable of maintaining the extracellular fluid pH near the physiologic normal. The loss of both bone sodium and carbonate suggests that in addition to sodium for proton exchange, there is a progressive loss of carbonate in response to acidosis.

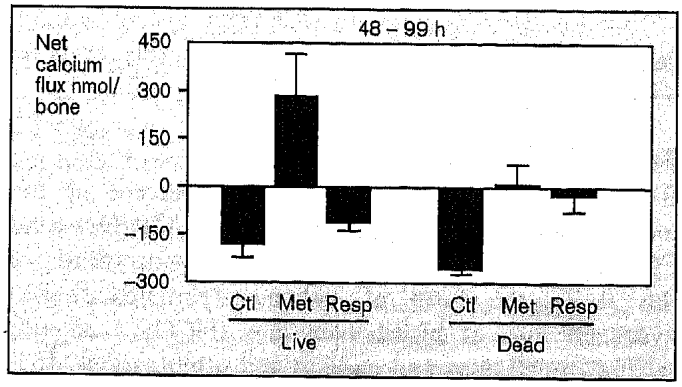
Chronic acid-induced calcium release

Chronic metabolic acidosis induces the release of bone calcium, predominantly by enhanced cell-mediated bone resorption and decreased bone formation [10,13,18,19,21,22,28,101]. However, there is a component of direct physicochemical acid-induced dissolution, as in acute metabolic acidosis [4,5,8,9,15,17]. In-vivo rat studies have shown stimulation of cell-mediated bone calcium resorption during prolonged acidosis [46,102].

We demonstrated increased cell-mediated bone calcium resorption after 99 h of culture in acidic medium produced by a decrease in bicarbonate ion concentration [10] (Fig. 4). We have also shown that acidosis increases osteoclastic and inhibits osteoblastic activity [13]. Release of the osteoclastic enzyme β-glucuronidase was stimulated (Fig. 5) while osteoblastic collagen synthesis (Fig. 6) and alkaline phosphatase were inhibited. Conversely we found that an increase in bicarbonate concentration, metabolic alkalosis, decreases calcium efflux from bone through an increase in osteoblastic bone formation and a decrease in osteoclastic bone resorption [22].

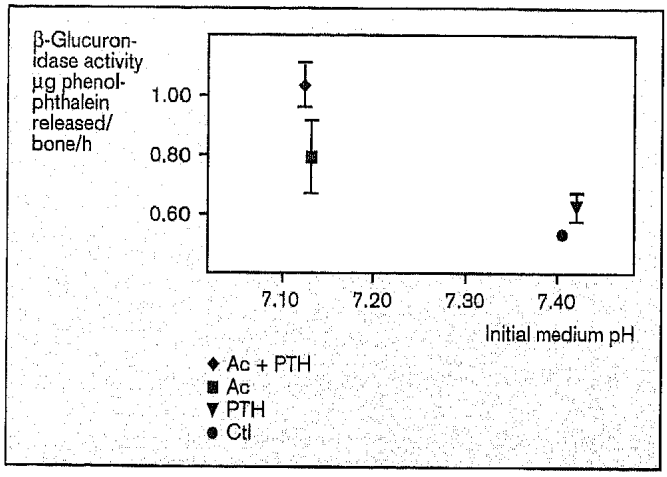
Further evidence that metabolic acidosis inhibits osteoblastic function was obtained utilizing primary osteoblasts in culture. Isolated osteoblasts cultured for three weeks synthesize collagen and form nodules of apatitic bone [103–106]. We found that metabolic acidosis leads

Figure 4. Net calcium flux during the final 51 hours of a 99 hour incubation for the six groups of calvariae studied



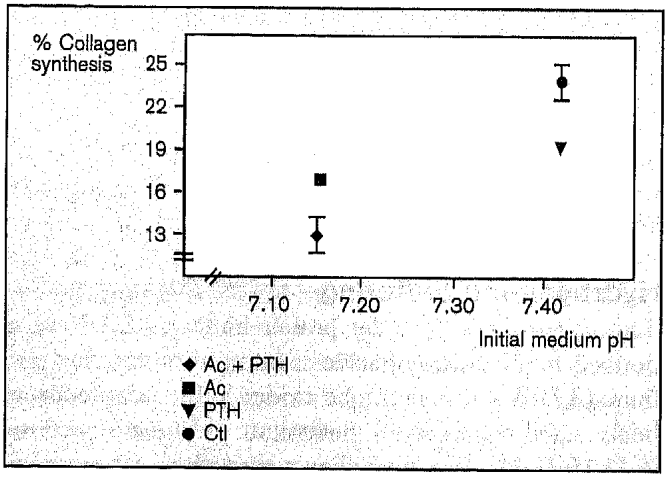
Live, calvariae cultured in living state; dead, calvariae subjected to three freeze-thaw cycles before culture; ctl, calvariae culture in unaltered medium; met, medium acidified by lowering the bicarbonate concentration; resp, medium acidified by increasing the partial pressure of carbon dioxide. Values are mean ± SEM. Reproduced with permission from [10].

Figure 5. Effect of acidosis and parathyroid hormone, alone and in combination, on osteoclastic β-glucuronidase activity



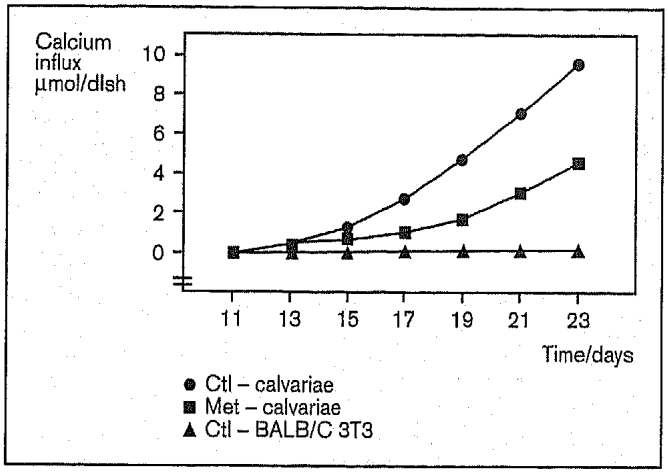
Ctl, calvariae incubated in control medium; met, medium acidified to a pH of approximately 7.10; PTH, medium with parathyroid hormone 10^{-10}; met+PTH, PTH added to acidic medium. Calvariae were incubated for 24 h and then transferred to similar fresh medium for an additional 24 h. At the end of the second 24 h incubation, aliquots of medium were removed for assay of β-glucuronidase activity. Values are mean ± SEM. Reproduced from [21] with permission.

Figure 6. Effect of acidosis and parathyroid hormone, alone and in combination, on osteoblastic collagen synthesis



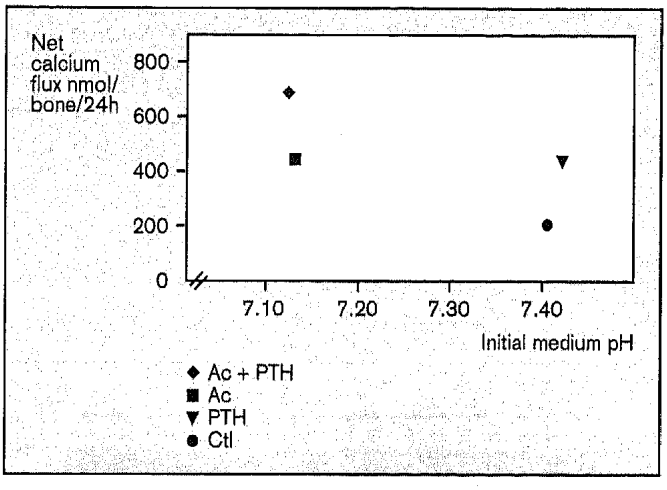
Calvariae were incubated in control medium (ctl), medium acidified to a pH of approximately 7.10 (met); medium with parathyroid hormone 10^{-10} M (PTH), or in parathyroid added to acidic medium (met+PTH) Calvariae were incubated for 24 h and then transferred to similar fresh medium for an additional 24 h. Incorporation of [^{3}H] proline into collagenase-digestible protein in calvariae was measured during the final 3 h of the second 24 h incubation. Values are mean ± SEM. Reproduced with permission from [21].

Figure 7. Cumulative calcium influx as a function of incubation time for cultured neonatal mouse calvarial cells



Cells were incubated in control medium until confluent (day 9) and then cultured for an additional 14 days in control medium (ctl–calvariae) or medium acidified by decreasing the medium bicarbonate concentration (met–calvariae). Balb/C 3T3 mouse fibroblasts were also incubated in control medium (ctl–BALB/C 3T3). Values are mean±SE. Changes in medium calcium concentration were calculated by subtracting the final from the initial calcium concentration and correcting for volume. Results are summed over the 14 day incubation period and represent calcium influx by the cell cultures. Reproduced with permission from [18].

Figure 8. Effect of acidosis and parathyroid hormone, alone and in combination, on net calcium efflux from cultured neonatal mouse calvariae



Calvariae were incubated in control medium (ctl), medium acidified to a pH of approximately 7.10 (met), medium with a final parathyroid hormone concentration of 10^{-10} M (PTH), or with parathyroid hormone added to acidic medium (met+PTH). Calvariae were incubated for 24 h and then transferred to similar fresh medium for an additional 24 h. At the end of the second 24 h incubation, aliquots of medium were removed for assay of net calcium flux. Values are mean±SEM. Reproduced with permission from [21].

not only to fewer nodules but also decreases calcium influx into the nodules [18] (Fig. 7). Thus, it appears that both augmentation of osteoclastic bone resorption and inhibition of osteoblastic bone formation have a prominent role in the hypercalciuria of chronic metabolic acidosis [13,18].

During renal failure there is often increased parathyroid hormone in addition to acidosis [107•,108]. To determine if acidosis and parathyroid hormone have additive effects on calcium efflux, calvariae were cultured in acidic medium with or without the hormone [21]. We found that acidosis and parathyroid hormone independently stimulated calcium efflux from bone, inhibited osteoblastic collagen synthesis and stimulated osteoclastic β-glucuronidase secretion. Their combination had a greater effect on each of these parameters than either alone (Fig. 8).

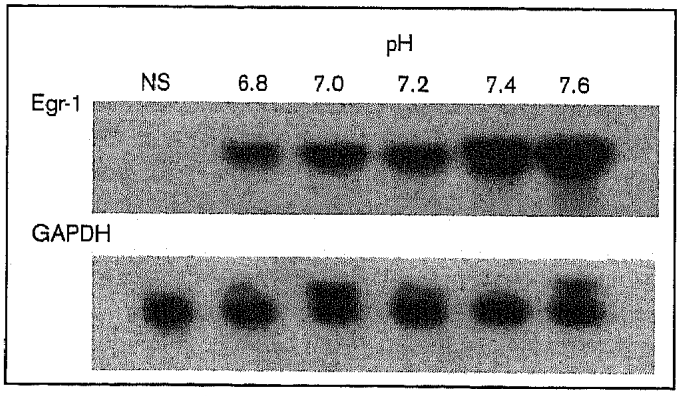
Acidosis induced alterations in gene expression

Based on the proton-induced increase in osteoclastic bone resorption and decrease in osteoblastic bone formation [13,19], we hypothesized that acidosis affects the pattern of gene expression in osteoblasts. As a model system we used primary neonatal mouse calvarial cells, which are principally osteoblasts and osteoblast precursors [101]. To assay acute effects of acidosis on gene expression, cells were cultured in a physiologically neutral pH medium until confluent and then stimulated with fresh medium at either neutral or acidic pH. RNA was harvested at various times after stimulation. Among a group of immediate early response genes, including *Egr-1*, *junB*, *c-jun*, *junD* and *c-fos*, only the magnitude of *Egr-1* stimulation was dependent on medium pH. At 40 min, *egr*-1 RNA levels were 10- to 30- fold higher than basal levels after stimulation with neutral medium. Acidic medium caused 30–50% less stimulation. A progressive decrease in medium pH to 6.8 led to a parallel decrease in *Egr-1* stimulation and an increase in pH to 7.6 led to an increase in *Egr-1* stimulation [101] (Fig. 9). Osteoblasts express type 1 collagen as the major component of the bone extracellular matrix, which subsequently becomes mineralized. Forty minutes after medium change, type I collagen RNA was stimulated approximately 3–5 fold. The stimulation was again decreased by acidosis and increased by alkalosis [101]. Inhibition of protein synthesis by cycloheximide caused a superinduction of *egr*-1 RNA while preserving the pH dependency of the process. In contrast, cycloheximide abolished the pH dependency of type 1 collagen RNA expression [101].

Cultured primary mouse calvarial cells differentiate and form sites of mineralization known as bone nodules [18,103–106]. During this process, osteoblasts express a number of matrix proteins distinct to bone, including bone sialoprotein, osteocalcin, osteonectin, osteopontin, and matrix gla protein [109]. We have shown that metabolic acidosis decreases bone nodule number, size

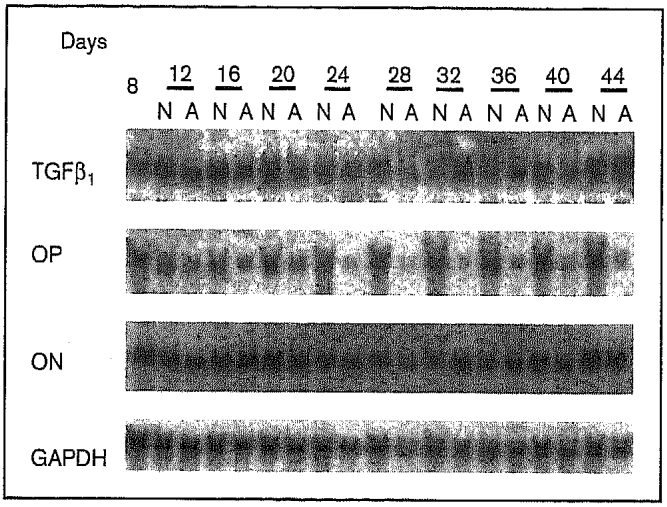
and calcium content [18]. We hypothesized that acidosis would alter the pattern of matrix gene expression in chronic cultures of bone cells resulting in a matrix that mineralizes less extensively than matrix from cultures incubated at neutral pH. After three to four weeks in neutral pH medium there was a dramatic increase in osteopontin RNA. In contrast there was no increase in osteopontin RNA in acidic cultures (Fig. 10). Osteopontin contains RGD (ARG-GLY-ASP) domains and serves as an anchoring protein for macrophages and osteoclasts. It may also be a chemoattractant for these cell types [110,111]. Downregulation of osteopontin expression may serve to limit recruitment of bone-resorbing cells during acidosis, perhaps a cause of low turnover renal osteodystrophy [3]. RNA for matrix Gla protein is also induced by neutral differentiation medium, reaching levels 20-30 fold greater than those before differentiation. Again, acidosis almost totally prevents the increase in matrix Gla protein RNA levels (Fig. 11). While matrix Gla protein expression is not limited to bone, it comprises about 10% of the carboxyglutamic acid found in bone [112]. The Gla residue coordinates with calcium and may serve to direct calcification [112]. The levels of RNA for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase have not been found to vary with pH, nor do the levels of two other RNA species expressed in osteoblasts, osteonectin and transforming growth factor β_1, indicating that there is not overall cellular toxicity. To determine if acidosis reversibly impairs cellular production of osteopontin and matrix Gla protein, cultures of primary calvarial bone cells were put in acidic differentiation medium at day 8, then switched to neutral medium at either day 15, 22, or 29 [28]. We found that a one-week exposure to acidosis had no lasting effect on osteopontin or matrix Gla protein expression, while a two-week exposure had a small inhibitory effect. There was partial recovery of RNA for osteopontin and matrix Gla protein after three weeks of

Figure 9. Response of *Egr-1* RNA to stimulation with medium of varying pH



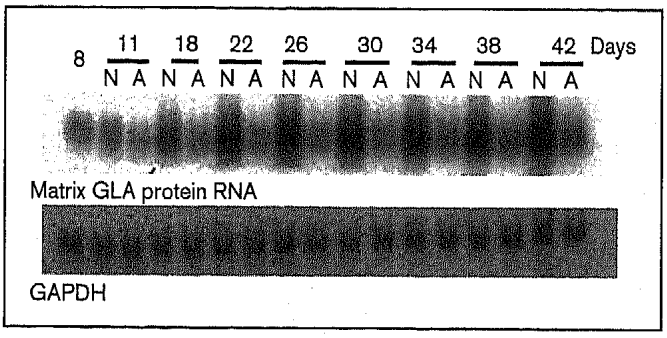
Cultured neonatal mouse calvarial cells were stimulated for 40 min with medium at the indicated pH and then harvested for RNA preparation. As a further control some cells were not stimulated (NS). Medium pH was altered by modification of the bicarbonate concentration at a constant carbon dioxide partial pressure of 40 mm Hg. Autoradiographs of a typical Northern filter as probed for *Egr-1* (a) then reprobed for glyceraldehyde-3-phosphate dehydrogenase (b). Twenty μg of total RNA were electrophoresed in each lane. Reproduced with permission from [101].

Figure 10. Northern analysis of transforming growth factor β_1, osteopontin and osteonectin expression



Cells were grown for 8 days in control medium (pH=75), prior to incubation in neutral (N, pH=75) or acidic (A, pH=71) differentiation medium, and were harvested for RNA at indicated times. Aliquots of RNA (20 μg) were electrophoresed, transferred to a single nylon membrane and then hybridized to transforming growth factor (TGF)β_1,osteopontin (OP), osteonectin (ON), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Images shown were acquired with PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA) β capture. Reproduced with permission from [28].

Figure 11. Northern analysis of matrix Gla protein RNA expression



Cells were grown for 8 days in control medium (pH=75) prior to incubation in neutral (N, pH=75) or acidic (A, pH=71) differentiation medium and were harvested for RNA at indicated times. Aliquots of RNA (20 μg) were electrophoresed, transferred to a single nylon membrane and then hybridized sequentially to matrix Gla protein and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Images shown were acquired with PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA) β capture. Reproduced with permission from [28].

acidosis. In the same samples, osteonectin and glyceraldehyde-3-phosphate dehydrogenase RNA expression were not affected.

Acidosis-induced changes in bone ion composition

We utilized a high resolution scanning ion microprobe with secondary ion mass spectroscopy to determine how hydrogen ion concentration alters the ion composition of bone mineral [6,11,15,23,24,97–99,113,114]. Our studies to date have shown that the calvarial surface is rich in sodium and potassium relative to calcium [6,11,15,23,24,29••,97–99,113]. The excess bone potassium is maintained through cell-mediated processes [98]. Loss of bone cell function produces an influx of calcium and marked release of bone potassium. There is a fall in the ratio of potassium to calcium, and to a lesser extent sodium to calcium, at the superficial surface of the mineral [98]. Metabolic acidosis causes release of mineral calcium and leads to a reduction in the surface ratio of sodium to calcium and potassium to calcium, indicating a greater relative release of mineral sodium and potassium than calcium [6]. However, the mineral and medium are in equilibrium [8] and there is movement of ions between the two [83] making it difficult to interpret the apparent ion fluxes, especially with respect to potassium and sodium. To help us better understand the effects of acidosis on potassium relative to calcium we labelled the mineral *in vivo* with the stable isotope ^{41}K and studied the response to acidosis *in vitro*. We found that mineral was indeed rich in potassium relative to calcium and that acidosis caused a fall in the potassium to calcium ratio indicating loss of this stable isotope from the bone mineral [24].

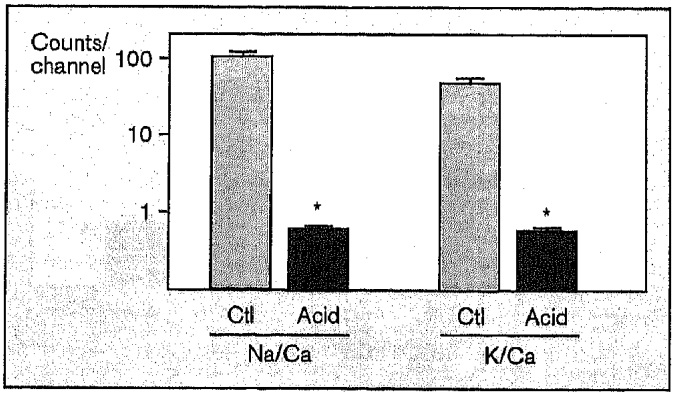
Since mineral in live bone is rich in potassium relative to calcium it was unclear if the osteoclasts selectively removed potassium or if they nonselectively remove the surface of the bone mineral. We isolated neonatal mouse bone cells and cultured them on bovine cortical bone slices in the presence of parathyroid hormone [23]. We then utilized the ion microprobe to compare the unresorbed bone with that at the base of the osteoclastic resorption pits. We found that in the presence of parathyroid hormone the osteoclasts nonselectively remove the potassium-rich surface of the bone mineral [23].

We also utilized the microprobe to study the acute physiochemical bone mineral dissolution caused by acidosis [15]. When we cultured calvariae with the osteoclastic inhibitor calcitonin there was a fall in the ratio of sodium to calcium coupled to an influx of calcium into bone, indicating little change in bone sodium. When calvariae were cultured in an acidic medium with calcitonin there was calcium release with no change in sodium to calcium, indicating that physicochemical bone mineral dissolution causes relatively equal calcium and sodium release [15]. Respiratory acidosis leads to some calcium release but there is no change in surface sodium to calcium or potassium to calcium, indicating apparent equimolar release of these three minerals [11].

All of our previous work with the ion microprobe involved investigation of bone cultured *in vitro*. To better understand the effects of acid on bone we established an in-vivo model. We utilized the microprobe to determine the mass spectra of important ion groups from femurs of mice acidified with oral ammonium chloride compared with mice drinking only distilled water [29••]. We examined an area in the midcortex (midway between the marrow space and the superficial cortex of the longitudinally split femur), midway down the bone shaft. We found that compared with mice given only oral distilled water the addition of NH_4Cl to the drinking water led to a marked change in the positive ion spectrum [29••]. In femurs from control mice the peak for potassium and sodium was far higher than that for calcium indicating that there is more potassium and sodium than calcium in the midcortex of the bone. However after oral ammonium chloride administration, there was a fall in the ratios of potassium and sodium relative to calcium (Fig. 12).

With respect to the negative ions we found that there was almost as much phosphate as carbon:carbon and carbon:nitrogen bonds in the midcortex of the control femurs. However, oral ammonium chloride led to a fall in the ratios of phosphate to carbon:carbon and phosphate

Figure 12. Ratio of sodium to calcium and potassium to calcium in the mid-cortex of neonatal mice femurs



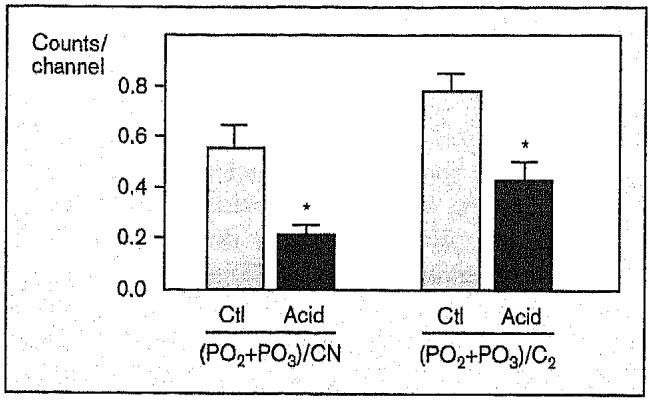
Ratios of sodium to calcium (Na/Ca) and potassium to calcium (K/Ca) in mice femurs are shown after drinking only distilled water (ctl) or water with 15% NH_4Cl (acid) for 7 days. Values are expressed as mean plus the upper 95% confidence limit. Compared with those drinking distilled water, there was a significant fall in the ratios of sodium to calcium and potassium to calcium after acid treatment. *, $P<0.05$. Reproduced with permission from [29••].

to carbon:nitrogen bond (Fig. 13). Additionally there was a marked decrease in the ratio of bicarbonate to carbon:carbon and bicarbonate to carbon:nitrogen bond with acidosis (Fig. 14).

Role of PCO_2 versus bicarbonate ion concentration

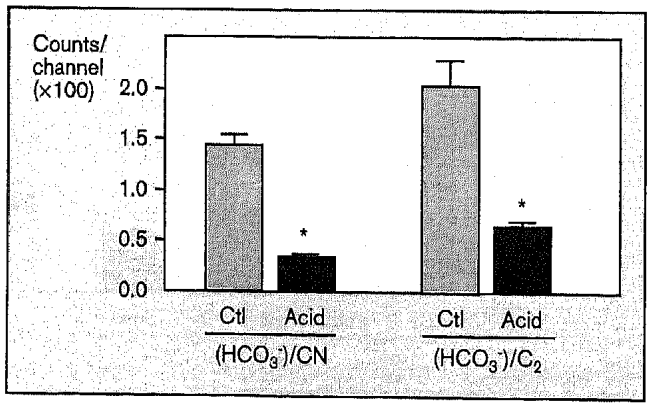
Most in-vivo and in-vitro studies have utilized hydrochloric acid or ammonium chloride to decrease bicarbonate as a model of metabolic acidosis. This non-anion gap acidosis mimics the clinical disorders of renal tubular acidosis and moderate to severe diarrhea [1]. *In vitro*, the type of acidosis appears to be critical in determining the magnitude of net calcium flux and proton buffering by bone. We found a clear distinction between the effects of metabolic (decreased bicarbonate) and respiratory (increased partial pressure of carbon dioxide) acidosis on cultured bone [4,8–12,14,16,18–20,41]. We tested the hypothesis that a decrement in pH alone was insufficient to induce maximal net calcium efflux from cultured bone. In acute studies we found there was greater net calcium efflux during culture in decreased bicarbonate medium than during culture in isohydric acidosis produced by an increase in the partial pressure of carbon dioxide [9]. The decreased net calcium efflux during respiratory acidosis compared with metabolic acidosis is due to decreased unidirectional calcium efflux from the mineral coupled to deposition of medium calcium on the bone surface during hypercapnia [12]. We found decreased bone carbonate in response to metabolic but not respiratory acidosis [16]. These results suggest that over this short time period acidosis affects the physicochemical driving forces for mineral formation and dissolution [5,8,14,15,17]. During metabolic acidosis the decreased bicarbonate favors dissolution while during respiratory acidosis the increased partial pressure of carbon dioxide and bicarbonate favors the deposition of carbonated apatite. Indeed there is no net proton influx into bone during respiratory acidosis [9]. Extending these studies to compensated metabolic and respiratory acidosis we found that at a constant pH, whether physiologically neutral or acidic, net calcium efflux from bone is dependent on bicarbonate concentration; lower medium bicarbonate concentrations cause greater calcium efflux from bone [14].

Figure 13. Ratio of total phosphate to the carbon nitrogen bond and total phosphate to carbon in the midcortex of the neonatal mouse femur



Ratios are shown after drinking only distilled water (ctl) or water with 15% NH_4Cl (acid) for 7 days. Values are expressed as mean plus the upper 95% confidence limit. Compared with only distilled water treatment, there was a significant fall in the ratios of total phosphate (PO_2+PO_3) carbon nitrogen bond (CN) and total phosphate to carbon (C_2) after acid treatment. *, $P<0.05$. Reproduced with permission from [29••].

Figure 14. Ratio of bicarbonate to the carbon nitrogen bond and bicarbonate to carbon in the mid-cortex of neonatal mouse femurs



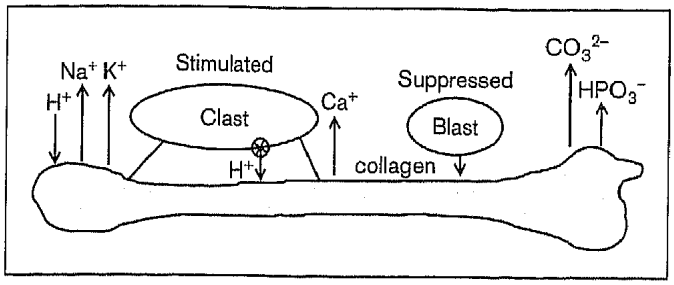
Ratios are shown after drinking only distilled water (ctl) or water with 15% NH_4Cl (acid) for 7 days. Values are expressed as mean plus the upper 95% confidence limit. Compared with only distilled water treatment, there was a significant fall in the ratios of bicarbonate (HCO_3^-) to carbon nitrogen bond (CN) and bicarbonate to carbon (C_2) after acid treatment. *, $P<0.05$. Reproduced with permission from [29••].

During more chronic incubations there is cell-mediated net calcium efflux from bone during models of metabolic but not respiratory acidosis [10,19]. A number of studies have shown that metabolic acidosis stimulates osteoclastic resorption [10,12,13,102,115–117]. We found that respiratory acidosis does not alter osteoclastic β-glucuronidase release, osteoblastic collagen synthesis or alkaline phosphatase activity as metabolic acidosis does [19]. Also, respiratory acidosis does not appreciably alter the surface ion composition of bone [6,11,15,23,24]. In contrast, however, both isohydric metabolic or respiratory acidosis caused a similar degree of inhibition of accumulation of osteopontin and matrix gla protein RNA [31••].

Relationship between calcium release and hydrogen ion buffering

During acute metabolic acidosis a reduction in pH causes both bone calcium release and proton buffering by bone. If all buffering was the result of mineral dissolution there should be a one to one ratio of protons buffered to calcium released in the case of calcium

Figure 15. Schematic of effects of metabolic acidosis on bone



Details are given in the text.

carbonate, a five to three ratio for apatite and one to one ratio for brushite [118,119]. However, with cultured calvariae the ratio was found to be 16–21 to one indicating that proton buffering could not simply be due to mineral dissolution [4]. That calcium release is only one component of proton buffering by bone is demonstrated by the microprobe studies, which show substantial sodium and potassium exchange for protons [6,11,15,23,24] and loss of bone phosphate and bicarbonate with acidosis [29••].

Conclusion

Metabolic acidosis appears to induce changes in bone mineral which are consistent with its purported role as a proton buffer (Fig. 15). The fall in mineral sodium, potassium, carbonate and phosphate will each buffer protons and lead to an increase in systemic pH toward the physiologic normal. These changes in mineral composition come about first through physicochemical mineral dissolution and later through alterations in bone cell function. The apparent protective function of bone to maintain systemic pH will come, in part, at the expense of its mineral stores. Future studies will be necessary to determine if the proton buffering properties of bone are described by a dynamic equilibrium: protonation of phosphate and carbonate and release of sodium and potassium during acidosis coupled to deprotonation and uptake of sodium and potassium during alkalosis. This attractive hypothetical mechanism has a clear survival advantage for mammals.

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