Chronic metabolic acidosis reversibly inhibits extracellular matrix gene expression in mouse osteoblasts

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Chronic metabolic acidosis reversibly inhibits extracellular matrix gene expression in mouse osteoblasts. Am. J. Physiol. 275 (Renal Physiol. 44): F840–F847, 1998.—Chronic metabolic acidosis induces net calcium efflux from bone mineral through an increase in osteoclastic resorption and a decrease in osteoblastic matrix deposition and mineralization. To determine the effects of chronic metabolic acidosis on the expression of genes necessary for mineralization, we grew primary bone cells, which are principally osteoblasts, to confluent in neutral pH (7.5) medium and then switched the cells either to a neutral pH or to an acidic pH (7.1) differentiation medium. Cells were harvested for RNA at 4- to 7-day intervals for up to 44 days. By 36 days, there was extensive bone nodule formation and mineralization in cells cultured in neutral medium; however, there was a substantial decrease in nodule formation and mineralization in cells cultured in acidic medium. There was a marked increase in matrix Gla protein RNA and an increase in osteopontin RNA in neutral cultures; however, acidic medium almost completely prevented any increase. In contrast, RNA levels for osteoectin and transforming growth factor-β1 were not altered by chronic acidosis. Additional cells were incubated in acid differentiation medium for 1, 2, or 3 wk and then transferred to neutral medium; in each case, there was recovery of matrix Gla protein RNA and osteopontin RNA expression. Thus metabolic acidosis appears to specifically inhibit RNA accumulation of certain genes whose products may be essential for formation of mature bone matrix.

Clinical studies have demonstrated that bone is significantly affected during chronic metabolic acidosis, resulting either from the acid generated by dietary protein intake (31, 41) or from the inability to excrete protons during renal failure (9).

We have modeled the effects of metabolic acidosis on bone using cultured neonatal mouse calvariae (6–8, 10–16, 30). There is net efflux of calcium from cultured calvariae incubated in physiologically acidic medium produced by a decrease in bicarbonate concentration at constant partial pressure of carbon dioxide, a model of metabolic acidosis (8, 17). During acute incubations (<24 h) in acidic medium, physicochemical mineral dissolution is the predominant mechanism for the calcium loss (17). However, over longer periods of time (>24 h), metabolic acidosis induces alterations in both osteoblastic and osteoclastic activity (8, 30). During this chronic metabolic acidosis, there is a decrease in calvarial collagen synthesis as well as diminished alkaline phosphatase activity, both of which indicate suppression of osteoblast function. In contrast, there is increased activity of osteoclastic β-glucuronidase during metabolic acidosis. Conversely, chronic metabolic alkalosis increases collagen synthesis and inhibits β-glucuronidase activity (10). These changes in bone cell function are consistent with the observed net calcium efflux from bone.

We have recently shown that after acute serum stimulation, the expression of specific genes in osteoblasts is dependent on medium pH (24). At a physiologically acidic pH, there was decreased expression of Egr-1 and type I collagen RNA compared with the expression observed at a neutral pH. In contrast, expression of c-fos, c-jun, junB, and junD RNA were not altered by incubation in acidic medium. These studies suggest that acidosis alters the expression of certain immediate early response genes.

We tested the hypothesis that chronic acidosis would alter the expression of genes important for osteoblast function. We utilized a model of isolated primary calvarial bone cells, which are predominantly osteoblasts and osteoblast precursors. These cells, when cultured in the presence of β-glycerophosphate and ascorbic acid, form regions of mineralization known as bone nodules (22). In addition, as the cells mature and form bone nodules, they sequentially exhibit a well-characterized pattern of gene and protein expression (43). We have previously shown that during metabolic acidosis, the number and calcium content of these nodules are significantly reduced (42).

In this study, we maintained isolated osteoblasts in differentiation medium either at neutral (7.5) or reduced (7.1) pH and examined the pattern of gene expression...
expression. We found that RNA levels for osteopontin and matrix Gla protein were dramatically inhibited by acidic medium, whereas there was no effect on expression of osteonectin or transforming growth factor-β1 (TGF-β1). The acid-induced inhibition of osteopontin and matrix Gla protein RNA is reversible by subsequent incubation in neutral medium pH, and this acid-induced inhibition occurs even after prolonged culture in neutral medium. These results suggest that expression of genes important for osteoblastic function is modulated by extracellular proton concentration.

METHODS

Cell culture. Bone cells were obtained from the calvariae (frontal and parietal bones of the skull) of 4- to 6-day-old CD-1 mice. Mice were killed by cervical dislocation, and the calvariae were dissected immediately and then placed in chilled HEPES. After accumulation of 20–50 calvariae, depending on the number of cells required, the bones were washed in saline-EDTA and then subjected to collagenase (Wako Pure Chemicals, Dallas, TX) digestion (26). The cells released by collagenase were plated on Primaria plates (Becton-Dickinson, Lincoln Park, NJ) in DMEM + 15% heat-inactivated horse serum at a density of 5 × 10⁴ cells per 100-mm dish, then cultured at 37°C in a CO₂ incubator at a partial pressure of carbon dioxide (PCO₂) of 40 mmHg. After 8 days, with medium changed every 3–4 days, the cells reached confluence. At this point, the cells were switched to differentiation medium (DMEM + 15% heat-inactivated horse serum + 10 mM β-glycerophosphate + 50 µg/ml ascorbic acid), either at neutral pH (7.5, “N” medium) or acidic pH (7.1, “A” medium). To closely replicate physiological conditions, only the HCO₃⁻/CO₂ buffer system was used to control pH. The initial medium pH in the neutral group was set at 7.5, rather than the physiological neutral pH of 7.4, as cells in culture acidify medium due to the ongoing release of metabolic acids (7). The acidic pH of 7.1 was produced by the addition of concentrated HCl to lower medium HCO₃⁻ concentration (HCO₃⁻) as a model of metabolic acidosis (7, 13).

Staining. At weekly intervals, cells on plates were washed three times in PBS, then fixed for 10 min in 7% neutral buffered formalin (100 mM NaPO₄, pH 7.2). Fixed cells were washed twice in PBS, then stained for 10 min in 7% neutral buffered formalin (100 mM NaPO₄, pH 7.2). Fixed cells were washed twice in PBS, then stained for 10 min with Alizarin Red S (1% wt/vol in water; Sigma, St. Louis, MO). To remove excess dye, the plates were washed twice with PBS then with water until the runoff appeared colorless.

Gene probes and labeling. Probes used for analysis of RNA included the following: TGF-β1, mouse, cDNA (generous gift of Harold Moses, Vanderbilt University, Nashville, TN) (4); osteonectin, bovine, cDNA (generous gift of Marian Young, National Institute of Dental Research, National Institutes of Health) (45); osteopontin, mouse, cDNA (generous gift of Gideon Rodan, Merck, Rahway, NJ) (37); matrix Gla protein, mouse, cDNA (generous gift of Dr. Gerard Karsenty, Anderson Cancer Center, Houston, TX) (29); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mouse, cDNA (Ambion, Austin, TX) (23). In each case, the inserts were removed from the vector by digestion with the appropriate restriction enzyme(s) and separated by electrophoresis on low-melting point agarose. Ethidium bromide-stained fragments were identified by ultraviolet (UV) transillumination, excised with a razor blade, and DNA purified from the gel with Wizard PCR Prep (Promega, Madison, WI). Radioactive probes were prepared by random primer extension using the DecaprimeII system (Ambion) and [α-³²P]dCTP (New England Nuclear, Boston, MA). Unincorporated nucleotides were removed by use of CentrSep spin columns (Princeton Separations, Adelphi, NJ).

RNA extraction and analysis. The cells were quickly scraped into a chaotrope solution (TRI-LS; Molecular Research, Cincinnati, OH), which dissociates RNA from protein complexes. RNA was purified from TRI-LS following manufacturer’s modification of the protocol of Chomczynski and Sacchi (19). After ethanol precipitation, the RNA was dissolved in water at a concentration of 10 µg/ml. Despite extensive mineralization in older neutral cultures, no consistent differences in bulk RNA recovery were noted. Aliquots (20 µg) were denatured in 50% formamide-6% formaldehyde by heating to 65°C for 15 min, then electrophoresed on 1% agarose in MOPS-formaldehyde buffer. Samples were routinely stained with ethidium bromide during electrophoresis to ensure the integrity of the RNA bands. After electrophoresis, the RNA was transferred to a charged nylon membrane (Zeta Probe; Bio-Rad, Richmond, CA) by capillary blotting with 10× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate). After blotting, the nucleic acid was fixed to the membrane by UV cross-linking (Stratalinker; Stratagene, La Jolla, CA). Filters were hybridized and washed according to manufacturers’ recommendations; prehybridization (at least 1 h) and hybridization (18–22 h) were conducted in 250 mM sodium phosphate, pH 7.2, 7% SDS, and 1 mM EDTA at 65°C. After hybridization, the spent solution was removed, and the filter(s) was washed twice in 40 mM sodium phosphate, pH 7.2, 5% SDS, and 1 mM EDTA at 65°C; then twice in 40 mM sodium phosphate, pH 7.2, 1% SDS, and 1 mM EDTA at 65°C. The washed filters were visualized and the signal was quantitated as above, and the process was repeated.

RESULTS

Mineralization. All cells were first cultured for 8 days to confluence in control medium (pH 7.5) prior to incubation in neutral (pH 7.5, N) or acidic (pH 7.1, A) differentiation medium. During incubation of the isolated bone cells in N medium, mineralization was initially detected at 29 days and progressively increased over the next 14 days to the conclusion of the study at 43 days (Fig. 1). In contrast, during incubation of the cells in A medium, mineralization was not detected at any time.

RNA expression. To determine the effects of continuous incubation in acidic, in comparison to neutral, medium on expression of genes important for bone mineralization, bone cells were harvested at intervals of 3–7 days after switching to differentiation medium at day 8. RNA was extracted, and levels of hybridization with specific probes were examined.

RNA levels for osteopontin increased gradually with time at neutral pH medium; however, there was no increase in osteopontin RNA accumulation in acidic pH medium (Fig. 2, Northern blot; and Fig. 3, densitometry of a different filter). In contrast, when the same filter used in Fig. 2 was probed to determine RNA levels for
osteonectin and TGF-β1, there was no difference, at any time, between the A and N samples.

RNA levels for matrix Gla protein increased substantially with time in the N medium; however, there was no increase in matrix Gla protein RNA accumulation in A medium (Fig. 4, Northern blot; and Fig. 5, densitometry of a different filter).

Recovery from acid incubation. Incubation in acidic medium could irreversibly alter the bone cells, rendering them incapable of expressing osteopontin or matrix Gla protein RNA, or the acid-induced decrease in expression could be reversible. To determine whether acid-induced inhibition was reversible, after the change to differentiation medium on day 8, cultures were incubated in A medium for 1, 2, or 3 wk. Cultures were then switched to N medium for the remainder of the experiment. Bone cells were harvested for RNA analysis weekly. Parallel cultures were maintained continuously in N or A medium, to serve as time controls.

RNA levels for matrix Gla protein and osteopontin, but not osteonectin, increased from day 8 to days 22, 29, 36, and 43 with continuous incubation in N medium (Fig. 6). Continuous incubation in A medium prevented the increase in matrix Gla protein and osteopontin RNA but did not affect osteonectin RNA levels at each time point (consistent with the results shown in Figs. 2–5).

When cells incubated in A medium for 1 wk were switched to N medium on day 15 (R1), there was a substantial reversal of the acid-induced inhibition of matrix Gla protein and osteopontin RNA accumulation (Fig. 6, Northern blot; Fig. 7A, quantitation of matrix Gla protein; Fig. 7B, quantitation of osteopontin). When cells incubated in A medium for 2 wk were switched to N medium on day 22 (R2), there was again a reversal of the acid-induced inhibition of matrix Gla protein and osteopontin RNA. When cells incubated in A medium for 3 wk were switched to N medium on day 29 (R3),...
there was a partial reversal of the acid-induced inhibition of matrix Gla protein and osteopontin RNA. As the duration of incubation in A medium increased (R1 to R2 to R3), there was a decrease in the magnitude of the subsequent accumulation of both matrix Gla protein and osteopontin RNA after switching to N medium.

To more closely determine the rate of recovery of matrix Gla protein and osteopontin RNA levels, after the change to differentiation medium on day 8, cultures were incubated in A medium for 2 wk. Cultures were then switched to N medium for the remainder of the experiment, and bone cells were harvested for RNA analysis on days 23, 24, 27, 29, 32, and 36. Parallel cultures were maintained continuously at N or A medium, to serve as time controls (data not shown). Matrix Gla protein RNA levels did not exhibit significant recovery until 10 days after the change to neutral medium while osteopontin RNA levels recovered within 5 days (Fig. 8).

Acid inhibition after neutral medium pH incubation. After initial induction of matrix Gla protein and osteopontin RNA expression by neutral pH differentiation medium, the expression may continue irreversibly, or it may be inhibitable by subsequent incubation in acid medium. To determine whether A medium could inhibit RNA expression even after induction in N differentiation medium, following the change to differentiation medium on day 8, cultures were incubated in N medium for 1, 2, or 3 wk. Cultures were then switched to A medium for the remainder of the experiment. Bone cells were harvested for RNA analysis weekly. Parallel cultures were maintained continuously at N or A medium, to serve as time controls.

RNA for matrix Gla protein and osteopontin, but not osteonectin, again increased from day 8 to days 22, 29, 36, and 43 with continuous incubation in N medium (Fig. 9). Continuous incubation in A medium again prevented the increase in matrix Gla protein and osteopontin RNA but did not affect osteonectin RNA levels at each time point illustrated.

When cells initially cultured in N medium for 1 wk were switched to A medium on day 15 (I1), on day 22 (I2), or on day 29 (I3), there was complete inhibition of matrix Gla protein and osteopontin RNA accumulation but not osteonectin RNA (Fig. 9).

To more closely determine the rate of inhibition of matrix Gla protein and osteopontin, after the change to differentiation medium on day 8, cultures were incubated in neutral medium for 2 wk to permit expression of matrix Gla protein and osteopontin RNA. Cultures were then switched to acidic medium for the remainder of the experiment, and bone cells were harvested for RNA analysis on days 23, 24, 25, 27, 29, and 32. Parallel
cultures were maintained continuously at N or A medium, to serve as time controls (data not shown). Both matrix Gla protein and osteopontin RNA levels decreased rapidly, to less than 50% of maximal values within 3 days after medium switch (Fig. 10). Osteopontin RNA levels appeared to decrease more rapidly than matrix Gla protein RNA levels.

**DISCUSSION**

Clinically, chronic metabolic acidosis has significant effects on bone (9, 31, 41). In organ culture, metabolic acidosis induces the release of bone calcium, mediated acutely through physicochemical dissolution (17) and chronically through decreased cell-mediated matrix deposition and increased cell-mediated resorption (8, 30). Acidosis decreases osteoblastic collagen synthesis and alkaline phosphatase release and increases osteoclastic β-glucuronidase release (8, 30). When bone cells, primarily osteoblasts, are placed in long-term culture in neutral pH medium, there is substantial matrix formation with subsequent mineralization (22); similar cultures maintained in acidic medium exhibit substantially less mineralization (42). We now show that although incubation of bone cells in physiologically neutral medium upregulates RNA levels for two proteins found in mature bone matrix, matrix Gla protein and osteopontin, incubation in acidic medium almost totally abolishes this increase. Concurrent measurements of RNA levels for two other matrix proteins, TGF-β1 and osteonectin, were unaffected by acid incubation. Thus a decrease in medium bicarbonate concentration producing a decrease in medium pH, a model of physiological metabolic acidosis (8, 17), appears to have

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**Fig. 7.** Quantitation of recovery after acid treatment. At day 8, cultures were switched to differentiation medium, either at neutral pH (7.5) or acidic pH (7.1). One group (N, •) was continuously maintained at neutral pH, whereas a second group (A, ●) was continuously maintained at the acidic pH. Cells in group R1 (▲) were cultured in acidic medium for 1 wk (day 8 to day 15) and then switched to neutral medium for the remainder of the experiment. Cells in group R2 (▲) were cultured in acidic medium for 2 wk (day 8 to day 22) and then switched to neutral medium for the remainder of the experiment. Cells in group R3 (▲) were cultured in acidic medium for 3 wk (day 8 to day 29) and then switched to neutral medium for the remainder of the experiment. Aliquots of RNA (20 µg) were electrophoresed (METHODS), transferred to a single nylon membrane, and then hybridized sequentially to the indicated probes. A: all values are expressed as the ratio of MGP RNA to GAPDH RNA on that day divided by the ratio on day 8 (predifferentiation). B: all values are expressed as the ratio of OP RNA to GAPDH RNA on that day divided by the ratio on day 8 (predifferentiation).

**Fig. 8.** Time course of recovery for OP RNA and MGP RNA after acid treatment. At day 8, cultures were switched to differentiation medium at acidic pH (7.1). Cells were cultured in acidic medium for 2 wk (day 8 to day 22) and then switched to neutral pH (7.5) medium for the remainder of the experiment. Aliquots of RNA (20 µg) were electrophoresed (METHODS), transferred to a single nylon membrane, and then hybridized sequentially to MGP, OP, and GAPDH. Values are expressed as the ratio of the indicated RNA to GAPDH. Left axis (●): MGP RNA level after medium switch as a function of time. Right axis (▲): OP RNA level after medium switch as a function of time.

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**Fig. 9.** Inhibition after onset of differentiation. At day 8, cultures were switched to differentiation medium, either at neutral pH (7.5) or acidic pH (7.1). One group (N) was continuously maintained at neutral pH, whereas a second group (A) was continuously maintained at the acidic pH. Cells in group I1 were cultured in neutral medium for 1 wk (day 8 to day 15) and then switched to acidic medium for the remainder of the experiment. Cells in group I2 were cultured in neutral medium for 2 wk (day 8 to day 22) and then switched to acidic medium for the remainder of the experiment. Cells in group I3 were cultured in neutral medium for 3 wk (day 8 to day 29) and then switched to acidic medium for the remainder of the experiment. Aliquots of RNA (20 µg) were electrophoresed (METHODS), transferred to a single nylon membrane, and then hybridized sequentially to MGP, OP, ON, and GAPDH.
of RNA (20 µg) were electrophoresed (METHODS), transferred to a single nylon membrane, and then hybridized to MGP, OP, and GAPDH. Values are expressed as the ratio of the indicated RNA to GAPDH. Left axis (■): MGP RNA level after medium switch as a function of time. Right axis (●): OP RNA level after medium switch as a function of time.

Fig. 10. Time course of inhibition. At day 8, cultures were switched to differentiation medium at neutral pH. Cells were cultured in neutral medium for 2 wk (day 8 to day 22) and then switched to acidic pH (7.1) medium for the remainder of the experiment. Aliquots of the same RNA preparations were analyzed on 2 separate filters. Aliquots of RNA (20 µg) were electrophoresed (METHODS), transferred to a single nylon membrane, and then hybridized to MGP, OP, and GAPDH. Values are expressed as the ratio of the indicated RNA to GAPDH. Left axis (■): MGP RNA level after medium switch as a function of time. Right axis (●): OP RNA level after medium switch as a function of time.

a specific regulatory role to inhibit RNA accumulation of certain genes.

Stimulation of matrix Gla protein RNA and osteopontin RNA is inhibited by metabolic acidosis, whereas TGF-β1 and osteonectin are not, indicating that the inhibition is not due to a generalized toxic effect of protons on cells. The inhibitory effect of metabolic acidosis is fully reversible, further evidence that the increase in proton concentration has a regulatory role and is not simply toxic to the bone cells.

The bone cells utilized in this study are principally mature osteoblasts and osteoprogenitor cells which can be expected to mature in culture (43). Even after 4 wk in culture, matrix Gla protein RNA and osteopontin RNA were each rapidly downregulated on exposure to acid medium, indicating that even these relatively differentiated cells retain their responsiveness to pH. Further studies will be required to determine whether early, purely osteoprogenitor cells also respond to an increase in medium proton concentration.

The regulatory effect of metabolic acidosis does not appear to be mediated simply by alterations in extracellular proton concentration. Acidosis can be produced either by decreasing the medium \([HCO_3^-]\), at a constant \(PCO_2\), which is a model of metabolic acidosis, or by increasing the \(PCO_2\), at a relatively constant \([HCO_3^-]\), which is a model of respiratory acidosis (9). Isohydric metabolic acidosis and respiratory acidosis have profoundly different effects on osteoblastic function (6–8, 14, 16, 42). There is a marked decrease in osteoblastic collagen synthesis and alkaline phosphatase activity during metabolic acidosis, whereas these parameters of osteoblastic activity do not change during isohydric respiratory acidosis (8). The regulatory role of metabolic acidosis also does not appear to be mediated solely by alterations in intracellular proton concentration. Respiratory acidosis results in a greater increase in intracellular proton concentration than does isohydric metabolic acidosis, yet has no measurable effect on osteoblastic function (38).

A primary function of the osteoblast is to synthesize an extracellular matrix that is subsequently mineralized, either spontaneously or through a cell-mediated process (43). The premineralized bone matrix, termed osteoid, is ~90% type 1 collagen, but also contains other proteins including osteopontin, osteonectin, osteocalcin, matrix Gla protein, bone sialoprotein, decorin, and biglycan (46). The function of each of these proteins is incompletely understood. Bone sialoprotein can initiate apatite crystal formation (28), and other matrix proteins may also act to initiate or accelerate matrix mineralization. The γ-carboxylated glutamine (Gla) residues found in osteocalcin and matrix Gla protein are known to coordinate with ionized calcium, suggesting a role in matrix mineralization (25). However, null mutants for each of these genes in mice have no apparent impairment of bone formation (20, 34). Osteocalcin knockout mice have a denser and stronger bone than normal littermates (20), whereas matrix Gla protein knockouts die at an early age from ectopic calcification of the aorta and great vessels (34). These results suggest that the bone Gla proteins act to limit or slow mineralization, rather than promote it.

Like the Gla proteins, the phosphorylated poly-Asp sequence in osteopontin is an inhibitor of apatite crystal growth (5, 28). Osteopontin is found at cement lines in living bone, where new bone and old bone join (35). Osteopontin contains GRGDS sequences, which can act as promoters of attachment and spreading of fibroblasts, osteoblasts, and osteoclasts; the RGD sequences may also trigger intracellular signaling events through interaction with integrins (39).

Several studies have shown that matrix Gla protein is produced exclusively by chondrocytes and smooth muscle cells (21, 27, 33); however, Barone and co-workers have reported its expression in primary rat osteoblast cultures (2, 3). Our study is consistent with that of Barone and co-workers in that we find substantial expression of matrix Gla protein during long-term cultures of primary mouse osteoblasts. Why others have not found expression of matrix Gla protein in bone cells remains to be determined.

Both matrix Gla protein RNA and osteopontin RNA are inhibited during incubation in acid medium, suggesting the possibility that their respective genes share regulatory motifs. Whereas the 5′-flanking sequence of mouse osteopontin is available (GenBank accession no. X51834) (36), corresponding sequences of mouse matrix Gla protein are not published. Using the 5′-flanking sequence of human matrix Gla protein (GenBank accession no. M55270) (18) and searching each for established transcription factor binding motifs, >30 potential binding sites were found in each sequence (TESS; URL is http://agave.humgen.upenn.edu/tess/index.html) (40). At this time, it is not possible to conclude which sites or combination of sites may confer the pH dependence. A sequence alignment (MEME, see Ref. 1; URL is http://www.sdsc.edu/MEME/) between these frag-
mements indicates the presence of several repeated domains with lengths up to 43 nucleotides. The potential role of these sequences in the regulation of matrix Gla protein RNA and osteopontin RNA expression during chronic metabolic acidosis remains to be elucidated.

Previously, we have shown that induction of the immediate early response gene Egr-1 and type 1 collagen were both inhibited by acidic medium and stimulated by alkaline medium (24). In contrast, the expression of other immediate early response genes including c-fos, c-jun, junB, and junD was not appreciably altered by differences in pH. Whether the acute effects of metabolic acidosis on gene expression are related to the more chronic effects demonstrated here were not addressed in this study.

We have previously shown that incubation in acidic medium produced by lowering the [HCO₃⁻] decreases bone nodule formation (42). However, in the previous study formation of bone nodules was evident by 14 days after cells were placed in differentiation medium, whereas in the current study nodules were not evident until day 21. This difference in the time to first detection of nodules may be due to a difference in culture technique. In our former study the medium was changed every other day, whereas in the current study medium was changed twice weekly. The more frequent addition of fresh nutrients may have resulted in an earlier appearance of bone nodules. In our previous study, bone nodules were detected by Von Kossa staining, whereas in the current study detection was by Alizarin Red S staining. Although both stains detect calcium deposits, it is unclear whether there is a difference in sensitivity of these two stains and, if so, how a difference would alter the apparent time to nodule formation. Despite these small differences in technique, both studies confirm that lowering the medium pH, through a decrease in [HCO₃⁻], will decrease the number of bone nodules.

Metabolic acidosis stimulates release of bone calcium through a decrease in osteoblastic bone formation and an increase in osteoclastic bone resorption (8, 30). In bone organ culture, osteoblastic collagen synthesis is decreased during metabolic acidosis (8, 30) and increased during metabolic alkalosis (10). Acute metabolic acidosis inhibits the induction of the immediate early response gene Egr-1 and type 1 collagen gene expression in osteoblasts (24). We now show that chronic metabolic acidosis reversibly inhibits accumulation of RNA encoding matrix Gla protein and osteopontin, but not osteonectin and TGF-β1, suggesting metabolic acidosis influences osteoblastic bone matrix production and subsequent mineralization.

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