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Acid pH Increases Carbonic Anhydrase II and Calcitonin Receptor mRNA Expression in Mature Osteoclasts

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Abstract. Numerous resorptive stimuli have been shown to enhance osteoclast differentiation, increasing osteoclast numbers and accelerating bone resorption. Currently, there is much less understanding of regulation of mature osteoclast activity. Indeed, there is presently only minimal evidence of changes in gene expression as a mechanism for altering bone resorption. We investigate here, in the mature osteoclast, regulation of 2 genes—carbonic anhydrase II (CAII) and calcitonin receptor (CTR) in response to acidosis, which is known to increase bone resorption. We studied the effect of acid pH on CAII and CTR mRNA expression in mature osteoclasts raised in coculture of ST-2 and primary marrow cells. On day 6 of culture, stromal cells were removed with collagenase, the remaining osteoclasts were incubated overnight, and then exposed to varying pH. RT-PCR was performed on total RNA using primers for CAII, CTR, or glyceraldehyde dehydrogenase phosphate (GAP). Expression of CTR mRNA was increased 2.14 ± 0.41 and 2.56 ± 0.45 ($P < 0.05$)-fold by a 4-hour exposure to pH 6.75 and 6.5, respectively. CAII mRNA was similarly increased 2.18 ± 0.42 and 2.63 ± 0.48 ($P < 0.05$)-fold by pH 6.75 and 6.5, respectively. Increased expression of CAII and CTR mRNA was seen by 2 hours and maximally by 4 hours. Increased expression of CTR and CAII mRNA was not explained by increases in osteoclast numbers: pH 7.4–100 ± 3.7, 6.75–133 ± 8.3, 6.5–124 ± 7.8. These results demonstrate upregulation of two osteoclast genes in response to acidosis, illustrating the ability of the mature osteoclast to respond to resorptive signals with increased functional gene expression.

Normal bone remodeling requires both bone resorption by the osteoclast and bone formation by the osteoblast. The predominant mechanism of osteoporosis is accelerated bone resorption. Modulation of bone resorption can occur via two distinct mechanisms: alterations in osteoclast differentiation resulting in a change in osteoclast numbers or modification of mature osteoclast activity. Increased bone resorption characteristic of postmenopausal osteoporosis results from augmented osteoclast formation [1]. Various cytokines that stimulate osteoclast differentiation *in vitro* and *in vivo* have been identified [1]. During differentiation, osteoclast specific gene expression increases [2, 3] providing the machinery necessary for bone resorption [4].

However, it is unclear whether, after recruitment of osteoclasts from marrow to a site of bone resorption, the activity of mature osteoclasts can be stimulated. Specifically, evidence for regulation of osteoclast gene expression in the mature cell is equivocal. Oursler et al. [5] described, in avian osteoclasts, decreased lysosomal gene expression after treatment with 17-β-estradiol, implying the ability to decrease function. *In vivo*, rat CAII and vacuolar proton pump expression were increased in both the immobilized and contralateral limb in mRNA from whole bone [6]. The increased expression may be due to the increase in osteoclast numbers seen in both limbs rather than induced expression. Recently, Nordstrom et al. [7] have shown enhanced activity of the vacuolar H^+-ATPase pump in the setting of acidosis but effects on gene expression were not explored. Evidence for stimulated osteoclast gene expression comes from Asotra et al. [8] who showed, by RT-PCR, increased CAII mRNA expression in individual rabbit osteoclasts exposed to acid pH.

Increased bone resorption due to acidosis has been demonstrated *in vitro* and *in vivo*. Previous investigators have demonstrated increased bone resorption in whole organ cultures [9, 10] as well as increased pit number in response to acidosis [11–14]. At the level of the osteoclast, acidosis has been shown to increase CAII mRNA expression [8] and vacuolar pump H^+-ATPase activity [7]. In addition, a generalized activation of the osteoclast has been described with increased podosome formation in response to lowered pH [15].

To better understand the response of the mature osteoclast to a resorptive stimulus such as acidosis, we studied expression of two osteoclast genes, CAII and CTR, at varying pH in osteoclast-like cells raised in murine marrow culture. CAII expression is required for normal osteoclast function to catalyze the production of protons from CO_2 and H_2O. The protons are secreted into the resorption pit forming the acidic milieu necessary for effective bone resorption. CTR, on the other hand, is an osteoclast-specific gene that allows modulation of osteoclast function. Calcitonin binding to its receptor results in contraction of the mature osteoclast and decreased bone resorption [3]. Demonstration of the upregulation of these two genes essential to osteoclast function in response to acidosis provides further evidence of the ability of the mature osteoclast to respond to changes in the local environment.

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Materials and Methods

Materials

C57BL/6NCR male mice were obtained from Frederick Cancer Center (Frederick, MD). Dr. Ed Greenfield (Case Western Reserve, Cleveland, OH) kindly provided the immortalized stromal ST-2 cell line. 1,25$(OH)_2D_3$ was purchased from Biomol (Plymouth Meeting, PA). Fetal calf serum (FCS) and media were obtained from Atlanta Biological (Atlanta, GA). Molecular biology reagents were obtained from Promega (Madison, WI). Other chemicals were obtained from Sigma (St. Louis, MO).

Cell Culture

ST-2 cells were plated overnight in 24-well plates at a density of 60,000/well in alpha minimal essential medium (α-MEM) with 10% FCS. Six to 28-week-old mice were sacrificed by cervical dislocation after CO_2 anesthesia. Femorae and tibiae were aseptically removed and dissected free of adhering tissue. Bone ends were cut off and marrow cavity was flushed with α-MEM using a 25-gauge needle and marrow cells were washed twice with α-MEM. Marrow cells were plated over ST-2 cells at a density of 2×10^6/well. All co-cultures were treated with 10^{-7} M dexamethasone and 10^{-8} M 1,25$(OH)_2D_3$. All cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C. After 6 days, cultures were treated with 1.5 mg/ml of collagenase for 20 minutes to remove stromal cells. The remaining cells were cultured overnight in 10% FCS and treated the next morning with varying pH. The pH of the culture media was lowered by the addition of concentrated hydrochloric acid (HCl). Replicate cultures were fixed and stained for tartrate-resistant alkaline phosphatase (TRAP). The number of TRAP+ multinucleated cells containing three or more nuclei were counted.

Reverse Transcription PCR

RNA was extracted using Trizol reagent from Life Technologies (Grand Island, NY). The RNA was precipitated, washed with 75% ethanol, and resuspended in 20 μl DEPC-treated water. The isolated RNA (0.5 μg) was added to a 10 μl reverse transcriptase reaction containing 2.5 mM dNTPs, 1× buffer, 2 μM reverse primer, recombinant RNAse H-moloney murine leukemia virus reverse transcriptase, and RNAsin, incubated for 30 minutes at 37°C. The RT reactions were performed in separate tubes using specific primers [CAII RT primer: 5′-CGCCAGTTGTCC-3′ (734–723), CTR RT primer: 5′-AGCTCCTACTACATAAGC-3′ (3612–3629), GAP RT primer 5′-CAAAGTTGTCATGGATGACC-3′ (545–526)]. PCR was performed on the 10 μl of reverse transcriptase product. PCR reaction mixture contains 200 μM dNTPs, 0.5 μM of PCR primers, PCR buffer, 1.6 μM $MgCl_2$. The reaction was heated to 95°C for 2 minutes and then Taq polymerase was added. The forward primer was end-labeled with ^{32}P-γ-ATP using T_4 kinase to allow visualization and quantitation. PCR products (15 μl) were run on a 15% acrylamide gel, silver stained, and exposed to a phosphorimager to measure density. GAP was used as control. Data are expressed as CTR/GAP or CAII/GAP. All data are normalized to a value of 1 for control cells to allow comparison between experiments. The CTR reaction was carried out for 30 cycles, annealing at 60°C; CAII for 23 cycles, annealing at 60°C; GAP for 21 cycles, annealing at 56°C. Cycle experiments were carried out to ascertain optimal conditions; CTR PCR product plateau at 35 cycles, CAII at 25 cycles, and GAP at 25 cycles. PCR conditions were selected for log-phase expansion. Primers were CAII reverse primer: 5′-ATCCAGGTCACACATTCCAGC-3′ (626–606), CAII forward primer: 5′-CTCTCAGGACAATGCAGTGC-3′ (216–235), CTR reverse primer: 5′-GGAAATGAATCAGAGAGTGC-3′ (1746–1765), CTR forward primer: 5′-TTGCAACTACTTCTGGATGC-3′ (1311–1330), GAP reverse primer: 5′-CAAAGTTGTCATGGATGACC-3′ (545–526), and GAP forward primer: 5′-TGTCATCAACGGGAAGC-3′ (239–257).

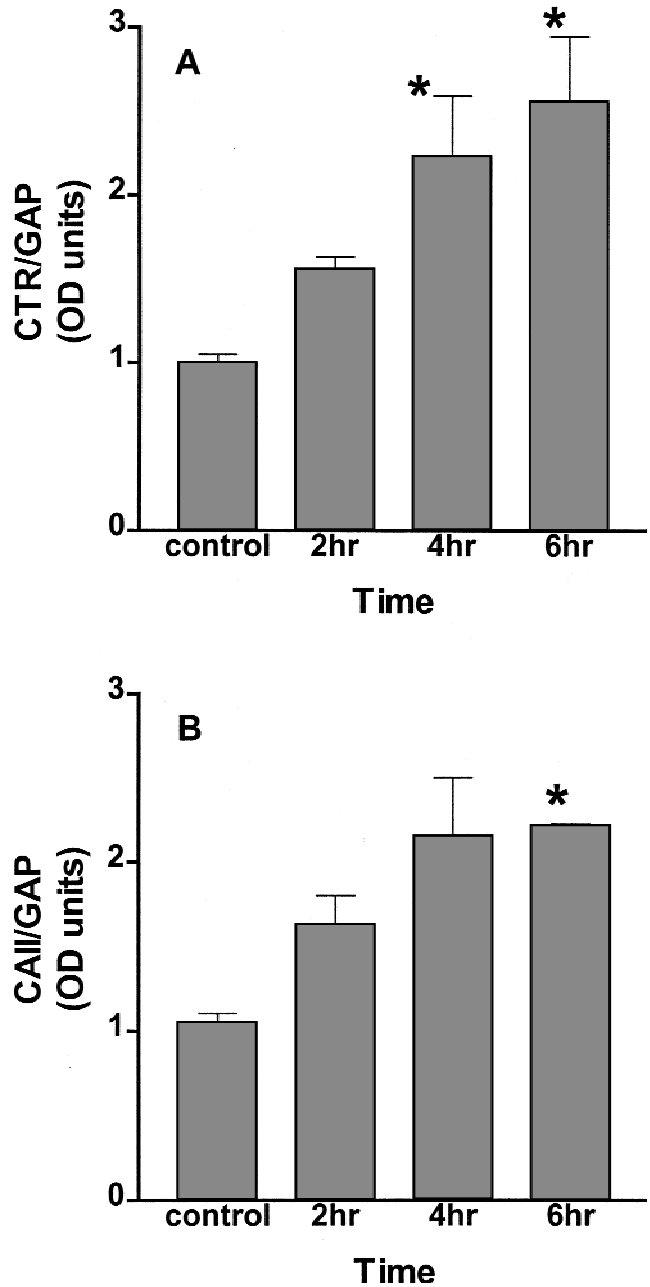

Fig. 1. Time course of increased CTR and CAII mRNA expression. Mature osteoclasts were exposed to pH 7.4 (control) or 6.5 for the specified time. Total RNA was extracted, and RT-PCR was performed for CTR, CAII, and GAP in separate reactions using a ^{32}P-labeled forward primer to allow for visualization. PCR products were run on an acrylamide gel and exposed to a phosphorimager for quantitation. Densitometric readings are expressed as CTR/GAP or CAII/GAP with normalization to control. **(A)** CTR mRNA was significantly increased compared with control at 4 and 6 hours, 2.24 ± 0.36 and 2.56 ± 0.39-fold, respectively. **(B)** CAII mRNA was significantly increased 2.22 ± 0.008-fold at 6 hours compared with control. (*$P < 0.05$ compared with control).

Calcitonin Labeling and Autoradiography

Salmon calcitonin (sCT) was labeled using chloramine-T. Briefly, 10 μg of sCT was mixed with 100 μl 0.2 M $NaPO_4$, 1 mCi ^{125}I-Na, and 10 μl chloramine T. Chloramine T was readded at 60 and 120 seconds. The reaction was stopped at 3 minutes and transferred to a C-18 column. For autoradiography, mature osteoclasts were treated with differing pH for 6 hours. Cells were then treated with 0.1% bovine serum albumen (BSA) for 15 minutes, then 0.2 nM ^{125}I-sCT was added. For nonspecific binding (swamp), 300 nM cold sCT was added. Cultures were incubated for 1 hour at room temperature (RT), washed in α-MEM ×3 and fixed with 0.1 M Na cacodylate, 2% formaldehyde, and 2% gluteraldehyde. Slides were dipped in LM-1 emulsion and developed after 7 days at 4°C.

Statistics

Statistics were analyzed using the program Graph Pad Prism (Graph Pad Softward, San Diego, CA). Differences between means in each group were evaluated by analysis of variance (ANOVA). Bartlett's test was performed for equal variances. A post-test for trend was then performed. Turkey's multiple comparison test was performed to compare individual groups.

Results

Mature osteoclasts were raised in coculture of primary marrow and ST-2 cells for 6 days. Stromal cells were removed with collagenase and the remaining osteoclasts were left overnight in 10% FCS. Osteoclasts were then exposed to pH 6.5 or 7.4 (control) for the specified time. RNA was extracted and RT-PCR was performed for CTR, CAII, and GAP. A 1.56 ± 0.05, 2.24 ± 0.36, and 2.56 ± 0.39-fold increase in CTR mRNA expression was seen after 2, 4, or 6 hour exposure, respectively, to pH 6.5 (Fig. 1A). Analysis of variance (ANOVA) confirmed significant differences between groups ($P = 0.003$) and a trend of increased CTR with decreasing pH ($P = 0.003$). Multiple comparisons showed only a significant difference between control and CTR at 4 and 6 hours. The pattern of CAII mRNA expression was similar to that of CTR. Expression was increased 1.64 ± 0.17, 2.16 ± 0.35, and 2.22 ± 0.008 fold at 2, 4, and 6 hours, respectively (Fig. 1B). ANOVA confirmed statistically significant differences between groups ($P = 0.04$) and a trend of increased CAII with decreasing pH ($P = 0.01$). Multiple comparisons showed only a significant difference between control and CAII at 6 hours. The time course was repeated twice with two replicates per time in each experiment. The 4-hour time point was then chosen for further experiments.

A dose response performed at 4 hours showed that expression of CTR mRNA was increased 2.14 ± 0.41 and 2.56 ± 0.45 ($P < 0.05$)-fold by exposure to pH 6.75 and 6.5, respectively (Fig. 2A). ANOVA confirmed statistically significant difference between groups ($P = 0.003$) and a statistically significant trend ($P = 0.001$) of increasing CTR mRNA with decreasing pH. CAII mRNA was similarly increased 2.18 ± 0.42 and 2.63 ± 0.48 ($P < 0.05$)-fold by pH 6.75 and 6.5, respectively (Fig. 2B). ANOVA also confirmed statistically significant difference between groups ($P = 0.007$) and a statistically significant trend ($P = 0.002$). Multiple comparisons demonstrated a statistical difference between control and pH 6.5 for both CTR and CAII. There was no statistical difference between pH 6.75 and 6.5. The results shown are an average of eight separate experiments with two replicates for each condition per experiment.

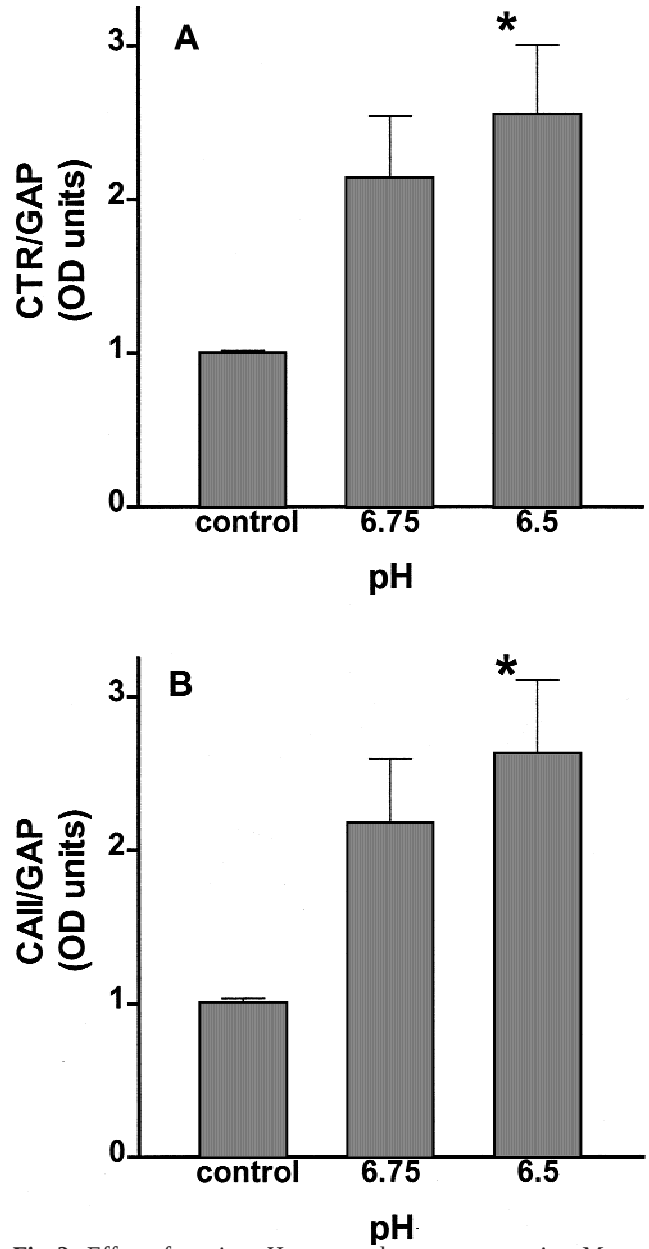

Fig. 2. Effect of varying pH on osteoclast gene expression. Mature osteoclasts were exposed to control conditions, pH 6.75 for 4 hours. As in Figure 1, RT-PCR was performed and PCR products were measured. **(A)** Results show increased CTR mRNA expression in response to decreasing pH, at pH 6.75, and 6.5 CTR/GAP was increased 2.14 ± 0.41 and 2.56 ± 0.45-fold, respectively. **(B)** CAII mRNA expression was increased 2.18 ± 0.42 and 2.63 ± 0.48-fold at pH 6.75 and 6.5, respectively (*$P < 0.05$ compared with control).

^{125}I-Calcitonin binding was demonstrated in osteoclasts at both control and low pH. Photomicrographs demonstrate a higher number of silver grains in osteoclasts exposed to pH 6.5 versus pH 7.4 (Fig. 3). We were unable to quantitate the difference in CTR number by a binding assay because of small numbers of osteoclasts in the culture system.

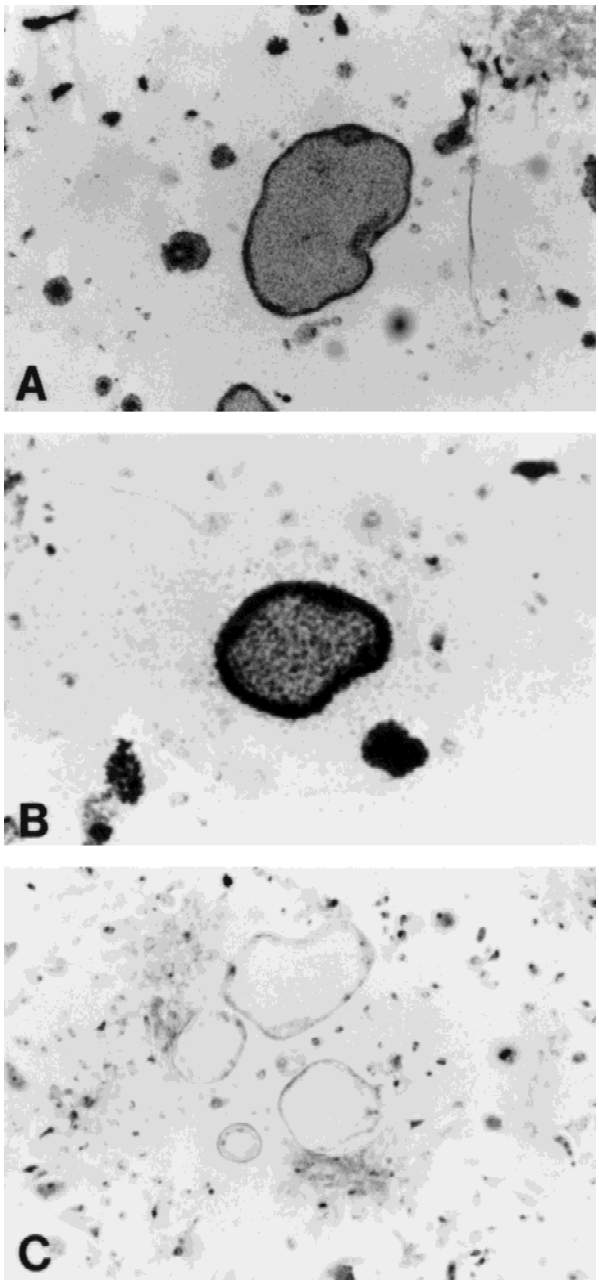

Fig. 3. Calcitonin binding at acid pH. Osteoclasts were raised in coculture of primary marrow and ST-2 cells. After 7 days, stromal cells were removed and the remaining cells were exposed to pH 6.5 for 6 hours. Cells were then labeled with ^{125}I-calcitonin as described in Methods. **(A)** Photomicrograph of CTR binding to control cells showing binding to large multinucleated cells as well as mononuclear cells. **(B)** Photomicrograph of CTR binding in cells exposed to pH 6.5 showing dense binding. **(C)** Photomicrograph of CTR binding in cells treated with excess unlabeled calcitonin (swamp) showing no binding.

Osteoclasts were counted in parallel cultures to ensure cell viability at the lower pH. After a 4-hour exposure to various pH, osteoclast numbers were as follows: pH 7.4–100 ± 3.7, 6.75–133 ± 8.3, 6.5–124 ± 7.8 (Table). Though the number of osteoclasts was significantly increased at the lower pH, the increase in osteoclasts was minimal compared with the 2.5-fold increase in mRNA expression.

Discussion

In mature osteoclasts these results demonstrate a 2.5-fold increase in CTR and CAII mRNA in response to an acidic milieu. This response could be seen as early as 2 hours but continued to increase at 4 and 6 hours. In addition, a dose response to decreasing pH was suggested for both gene products. Because of the difficulty in culturing mature, isolated osteoclasts, gene regulation in these unique cells has been poorly understood. Here we provide further evidence of the responsiveness of the mature osteoclast to its local environment by altering gene expression.

CTR expression is one of the features that distinguish osteoclasts from other multinucleated macrophage-like cells [16]. Though the role of the CTR to decrease osteoclast activity is well documented, regulation of CTR expression is only beginning to be understood. We demonstrated increased CTR mRNA expression under acidic conditions. The effect of acid pH on CTR expression has not been previously studied. Increased CTR mRNA expression has been described in response to glucocorticoid treatment [17], whereas decreased expression has been demonstrated with calcitonin treatment [3, 16, 17, 18]. The rise in CTR mRNA expression in the setting of increased bone resorption seems paradoxical since calcitonin binding to its receptor inhibits bone resorption. In the setting of accelerated resorption seen in response to acid pH, an increase in CTR expression may augment the capacity for rapid inhibition of bone resorption.

Metabolic acidosis in whole animals and organ cultures results in a negative calcium balance and increased bone resorption [19]. Teti et al. [15] have described increased podosome formation at low pH, indicating a generalized activation of the osteoclast in response to acidosis. Several investigators [11–13] have shown increased pit number and area resorbed by isolated rat osteoclasts in response to low pH. Our data showing increased CAII mRNA expression imply that enhanced expression of osteoclastic enzymes involved in bone resorption may be the mechanism for the increased resorption.

Increased osteoclast expression of CAII mRNA under acidic conditions has previously been shown by RT-PCR in individual osteoclasts [8]. We have expanded on the findings of Asotra et al. [8] demonstrating a dose response of increased CAII mRNA expression with decreasing pH in mature osteoclasts. This may represent a generalized response to acidosis since similar findings of increased CAII mRNA are seen in renal cortical cells exposed to acidosis [20, 21]. In the kidney it is thought that increased CAII expression is an adaptive response to chronic metabolic acidosis which facilitates acid secretion [20]. At the level of the osteoclast there appears to be a universal activation of machinery necessary for acidification since, in addition to increased CAII mRNA expression, the vacuolar H-ATPase pump activity is increased, enhancing the capacity for acidification of the resorption pit [7].

We demonstrated an increase in osteoclast-like numbers in response to acid pH. Others have reported no change or a minimal decrease in osteoclast numbers in the setting of low pH for 24–72 hours [11, 12, 14]. However, increased numbers of osteoclasts were reported in organ culture after 7-day exposure to low pH [10]. Since the 4-hour time period

Table. Effect of pH on gene expression and osteoclast number

	pH 7.4	pH 6.75	pH 6.5
CTR/GAP (n = 14)	1 ± 0.08 (n = 19)	2.14 ± 0.41 (n = 14)	2.56 ± 0.45[a] (n = 18)
CAII/GAP	1 ± 0.03 (n = 16)	2.18 ± 0.42 (n = 14)	2.63 ± 0.48[a] (n = 16)
Osteoclast no.	100 ± 3.7 (n = 32)	133 ± 8.3[a] (n = 23)	124 ± 7.8[a] (n = 28)

After mature osteoclasts were exposed to variable pH for 4 hours, RT-PCR was performed for CTR, CAII and GAP. Both CTR and CAII expression increased progressively, as shown in Figures 1 and 2 and this table. In replicate cultures cells were fixed and stained for TRAP. TRAP+ multinucleated cells containing three or more nuclei were counted as osteoclasts.
[a] $P < 0.05$.

in which we measure increased osteoclast numbers would be too short to demonstrate osteoclast recruitment, the increased number of TRAP+MNC likely represents fusion of mononuclear TRAP+ cells to multinucleated TRAP+ cells. Interestingly, Goldhaber and Rabadjija [10] suggested that fusion of mononuclear precursors may be induced at low pH. In isolated rabbit osteoclast cultures maintained at pH 6.5, the number of TRAP+MNCs increased progressively over 48 hours whereas TRAP+ mononuclear cells initially increased at 24 hours but then decreased at 48 hours [13]. The increase in multinucleated cells in the last 24 hours of culture implies that fusion of mononuclear cells into multinucleated cells was occurring. These findings by our laboratory and others demonstrating increased osteoclast numbers at low pH may be due to increased fusion of mononuclear TRAP+ cells into mature, multinuclear TRAP+ cells. Fusion into multinucleated osteoclasts may facilitate the accelerated resorption seen in response to acidosis since osteoclasts with a greater number of nuclei exhibit greater bone resorption [22–24].

These results demonstrate regulation of two genes in the mature osteoclast, illustrating the ability of the osteoclast to respond to resorptive signals with increased functional gene expression. We have shown increased expression of two very distinct genes with opposing effects. Increased CAII expression augments the capacity for bone resorption, whereas increased CTR expression enhances the ability to inhibit osteoclast activity. We hypothesize that upregulation of CTR occurs as the cell becomes activated to allow for rapid downregulation of the cell once the resorptive stimuli are removed. These results supply evidence for modulation of mature osteoclast activity and imply that as the osteoclast is "turned on" by resorptive stimuli, overall gene expression is increased.

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