Cortisol Inhibits Acid-Induced Bone Resorption \textit{In Vitro}

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Abstract. Metabolic acidosis increases urine calcium excretion without an increase in intestinal calcium absorption, resulting in a net loss of bone mineral. \textit{In vitro}, metabolic acidosis has been shown to initiate physicochemical mineral dissolution and then enhance cell-mediated bone resorption. Acidic medium stimulates osteoblastic prostaglandin \(E_2\) production, which mediates the subsequent stimulation of osteoclastic bone resorption. Glucocorticoids are also known to decrease bone mineral density, and metabolic acidosis has been shown to increase glucocorticoid production. This study tested the hypothesis that glucocorticoids would exacerbate acid-induced net calcium efflux from bone. Neonatal mouse calvariae were cultured in acid (Acid; \(pH\) \(7.06 \pm 0.01; [HCO_3^-] = 10.6 \pm 0.3\) mM) or neutral (Ntl; \(pH\) \(7.43 \pm 0.01; [HCO_3^-] = 26.2 \pm 0.5\) mM) medium, with or without \(1\) \(\mu\)M cortisol (Cort), and net calcium efflux and medium prostaglandin \(E_2\) (PGE\(_2\)) levels and osteoclastic \(\beta\)-glucuronidase activity were determined. Compared with Ntl, Cort alone decreased calcium efflux, medium PGE\(_2\), and osteoclast activity; Acid led to an increase in all three parameters. The addition of Cort to Acid led to a reduction of calcium efflux, medium PGE\(_2\) levels and \(\beta\)-glucuronidase activity compared with Acid alone. There was a significant direct correlation between medium PGE\(_2\) concentration and net calcium efflux \((r = 0.944; n = 23; P < 0.0001)\), between osteoclastic \(\beta\)-glucuronidase activity and net calcium efflux \((r = 0.663; n = 40; P < 0.001)\), and between medium PGE\(_2\) concentration and \(\beta\)-glucuronidase activity \((r = 0.976; n = 4; P < 0.01)\). Thus, \textit{in vitro} cortisol inhibits acid-induced, cell-mediated osteoblastic bone resorption through a decrease in osteoblastic PGE\(_2\) production. These results suggest that the osteopenia observed in response to metabolic acidosis \textit{in vivo} is not due to an increase in endogenous cortisol production.

Metabolic acidosis, which is observed during renal insufficiency or failure or with defects in renal tubular acid excretion, increases renal calcium excretion without altering intestinal calcium absorption (1–3). As bone is the largest repository of calcium in the body, bone is thought to be the source of this additional urine calcium (4). Indeed metabolic acidosis is associated with a decrease in bone mineral content (5,6). \textit{In vitro} studies have described the direct response of bone to an acid challenge. A physiologic reduction in medium pH has been shown to initially induce physicochemical mineral dissolution (7,8) over the first 3 h of culture. With prolonged incubation in acidic medium, there is decreased osteoblastic bone formation and increased osteoclastic resorption (9,10), all of which contribute to a net efflux of calcium from bone (6,11). This cell-mediated resorption appears due to increased osteoblastic prostaglandin \(E_2\) (PGE\(_2\)) synthesis, leading to a suppression of osteoblastic bone formation and subsequent stimulation of osteoclastic bone resorption (12–14).

Glucocorticoids lead to a dramatic decrease in bone mineral density, either when endogenously in excess or when administered exogenously (15). The mechanism by which glucocorticoids decrease bone density is multifactorial. The osteopenia appears due to a complex combination of direct effects on bone formation (16–19) and resorption (16,17,20) and indirect effects on calcium homeostasis, including decreased intestinal calcium absorption (20).

There is evidence from both animal and human studies to suggest that metabolic acidosis stimulates an increase in cortisol production (21–23). The mechanism by which acidosis stimulates cortisol is not clear; in acute studies, infusion of acid into isolated canine adrenal glands did not stimulate cortisol secretion (24), suggesting there is not direct regulation of glucocorticoid synthesis in the adrenal gland. Chronic metabolic acidosis can increase cortisol production, and both acidosis and cortisol induce osteopenia; it is therefore important to determine whether glucocorticoids directly exacerbate acid-induced net calcium efflux from bone. That glucocorticoids could enhance acid-induced bone resorption has precedent in the study of muscle proteolysis (21,25,26). In muscle, acidosis is known to stimulate protein and essential amino acid breakdown through the ubiquitin-proteasome proteolytic pathway, a mechanism that requires glucocorticoids (21,27). This permissive effect of glucocorticoids appears to be due to regulation of mRNA levels of key enzymes in the degradative pathway (28).

In this study, however, we found that cortisol inhibited acid-induced net calcium efflux, bone cell PGE\(_2\) production, and osteoclastic activity. These results suggest that cortisol does not contribute to acid-induced bone resorption.

Materials and Methods

\textit{Organ Culture of Bone}

Neonatal (4- to 6-d-old) CD-1 mice (Charles River, Wilmington, MA) were killed, and their calvariae were removed by dissection.
(7,10,12,29). Exactly 2.8 ml of Dulbecco’s modified Eagle medium (DMEM) containing 15% heat-inactivated horse serum, heparin sodium (10 U/ml), and potassium penicillin (100 U/ml) were preincubated at a fixed partial pressure of carbon dioxide (Pco2, 40 mmHg) at 37°C for 3 h in 35-mm dishes. After preincubation, 1 ml of medium was removed to determine initial medium pH and Pco2 and total calcium concentration and two calvariae were placed in each dish on a stainless steel wire grid.

Calvariae were incubated for an initial 24 h and then moved to fresh similar preincubated medium for an additional 24 h. At the beginning and end of each incubation period, medium was removed and analyzed for pH, Pco2, and calcium. After the 24 to 48 h incubation period, medium was also immediately analyzed for medium PGE2 or β-glucuronidase concentrations.

**Experimental Groups**

Calvariae were divided into four groups. Calvariae were incubated in medium either at neutral (Ntl) pH (approximately 7.4) or acidic (Acid) pH (approximately 7.1) produced by a primary decrease in the [HCO3−], to model metabolic acidosis (Met), with or without 1 µM cortisol (Cort). To closely replicate physiologic conditions, only the HCO3−/CO2 buffer system was used (30). To reduce medium pH, 2.4 N HCl was added to the medium to obtain the desired reduction of [HCO3−] and thus pH.

**Prostaglandin E2 Enzyme Immunoassay**

Culture medium was analyzed for PGE2 production by the calvariae immediately after the end of the second 24-h incubation, using an enzyme immunoassay kit obtained from Cayman Chemical (Ann Arbor, MI). Quantitation was done using a Dynatech MR700 microplate reader and Immunosoft computer program.

**β-Glucuronidase Activity**

Culture medium was collected at the end of the second 24-h incubation. Medium β-glucuronidase activity was determined colorimetrically using phenolphthalein glucuronidate (Sigma, St. Louis, MO) as a substrate (31).

**Conventional Measurements**

Medium pH and Pco2 were determined with a blood-gas analyzer (Radiometer model ABL 5, Copenhagen, Denmark) and calcium by ion-selective electrode (Nova Biomedical, Waltham, MA). The concentration of bicarbonate ([HCO3−]) was calculated from pH and Pco2 as described previously (29). Net calcium (Ca) flux was calculated as Vm × ([Ca]f − [Ca]i), where Vm is the medium volume (1.8 ml) and [Ca]f and [Ca]i are the final and initial medium Ca concentrations, respectively. A positive flux value indicates movement of Ca from the bone into the medium, and a negative value indicates movement from the medium into the bone.

**Statistical Analyses**

All tests of significance were calculated using ANOVA, with Bonferroni correction for multiple comparisons, and regression analysis using conventional computer programs (BMDP New System, Statistical Solutions, Cork, Ireland). Values are expressed as mean ± SEM, and P ≤ 0.05 was considered significant.

**Results**

**Medium pH, Pco2, and [HCO3−]**

When compared with Ntl, there was no difference in the initial medium pH and [HCO3−] in the Cort group during either the 0 to 24 h time period or the 24 to 48 h time period (Table 1). When compared with Ntl and Cort during each of the two time periods, there was a significant decrease in medium pH due to a decrease in medium [HCO3−] in both the Acid group and the Acid + Cort group. There was no difference in initial medium pH or [HCO3−] between the two groups incubated in Acid medium. There was no difference in the Pco2 in any group during either time period.

**Net Calcium Flux**

During the first and the second 24-h period and over the cumulative 48-hr time period, there was significantly less calcium efflux from calvariae incubated with 1 µM cortisol (Cort) when compared with Ntl (Figure 1). When compared

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**Table 1. Initial medium ion concentrations**

<table>
<thead>
<tr>
<th></th>
<th>Ntl</th>
<th>Cort</th>
<th>Acid</th>
<th>Acid + Cort</th>
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<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>19</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
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<tr>
<td>0 to 24 h</td>
<td>7.43 ± 0.01</td>
<td>7.43 ± 0.01</td>
<td>7.06 ± 0.01</td>
<td>7.05 ± 0.01</td>
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<tr>
<td>24 to 48 h</td>
<td>7.43 ± 0.01</td>
<td>7.43 ± 0.01</td>
<td>7.06 ± 0.01</td>
<td>7.06 ± 0.01</td>
</tr>
<tr>
<td>Pco2 (mm Hg)</td>
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<tr>
<td>0 to 24 h</td>
<td>39.4 ± 0.7</td>
<td>38.1 ± 1.7</td>
<td>39.2 ± 0.5</td>
<td>39.8 ± 0.6</td>
</tr>
<tr>
<td>24 to 48 h</td>
<td>39.8 ± 0.6</td>
<td>39.4 ± 0.4</td>
<td>39.6 ± 0.7</td>
<td>39.6 ± 0.6</td>
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<tr>
<td>[HCO3−] (meq/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 24 h</td>
<td>26.2 ± 0.5</td>
<td>26.0 ± 0.5</td>
<td>10.6 ± 0.3</td>
<td>10.6 ± 0.4</td>
</tr>
<tr>
<td>24 to 48 h</td>
<td>26.2 ± 0.5</td>
<td>26.5 ± 0.6</td>
<td>10.8 ± 0.4</td>
<td>10.7 ± 0.4</td>
</tr>
</tbody>
</table>

*Data are the mean ± SEM. Neonatal mouse calvariae were cultured in neutral (Ntl) or acid (Acid) medium in the absence or presence of 1 µM cortisol (Cort) as indicated for 24 h (0 to 24 h) and reincubated in similar fresh medium for a second 24 h (24 to 48 h). Pco2, partial pressure of carbon dioxide; [HCO3−], medium bicarbonate concentration.

*this P < 0.05 versus Ntl.

**this P < 0.05 versus Cort.**
with both Ntl and Cort, incubation in Acid medium with or without Cort led to a significant increase in net calcium efflux during each individual 24-h period and during the cumulative 48-hr period. However, when compared with Acid alone, incubation in Acid + Cort led to a significant decrease in net calcium efflux during each of the individual 24-h periods and during the cumulative 48-hr period.

Medium PGE₂ Concentration

When compared with Ntl, there was a decrease in medium PGE₂ concentration from calvariae incubated with Cort (Figure 2). Compared with Ntl, incubation in Acid with or without Cort led to an increase in medium PGE₂ concentration. However, incubation in Acid + Cort led to a fall in medium PGE₂ concentration compared with Acid alone. There was a significant direct correlation between medium PGE₂ concentration and net calcium flux (Figure 3).

Medium β-Glucuronidase Activity

When compared with Ntl, there was decrease in medium osteoclastic β-glucuronidase activity from calvariae incubated with Cort (Figure 4). Compared with Ntl, incubation in Acid led to an increase in medium β-glucuronidase activity. However, incubation in Acid + Cort led to a fall in medium β-glucuronidase activity compared with Acid alone. There was a significant direct correlation between medium β-glucuronidase activity and net calcium flux (Figure 5).

Correlation of Medium PGE₂ Concentration and Medium β-Glucuronidase Activity

There was a significant direct correlation between the medium PGE₂ concentration of each group and the medium β-glucuronidase activity of each group (Figure 6). β-glucuronidase activity is indicative of osteoclast activation; therefore, this suggests that the stimulation of osteoblastic PGE₂ production by acidosis directly leads to increased osteoclast activation and subsequent bone resorption.

Discussion

During renal insufficiency and renal failure and with specific abnormalities of renal tubular function, there is less hydrogen ion excretion than endogenous acid production, which results in a decrease in serum pH and bicarbonate, a disorder termed metabolic acidosis (30). Metabolic acidosis, both in vivo and in vitro, induces net calcium efflux from bone (6,11,32). Chronic metabolic acidosis, produced by feeding rats NH₄Cl (21) or infusing dogs with NH₄Cl (22), has also been shown to cause a significant increase in corticosteroid excretion. In a small human study, NH₄Cl-induced acidosis was also associated with an increase in cortisol excretion (23); however, another similar study in humans did not show any increase in cortisol

Figure 1. Effect of cortisol on acid-induced net calcium flux. Neonatal mouse calvariae were cultured in neutral (Ntl) or acid (Acid) medium in the absence or presence of 1 µM cortisol (Cort) as indicated for 24 h (0 to 24 h) and reincubated in similar fresh medium for a second 24-h period (24 to 48 h). Data are the mean ± SEM for 19 to 20 pairs of bones in each group; *P < 0.05 versus Ntl; †P < 0.05 versus Cort; ‡P < 0.05 versus Acid alone.

Figure 2. Effect of cortisol on acid-induced prostaglandin E₂ (PGE₂) concentration. Neonatal mouse calvariae were cultured in Ntl or Acid medium in the absence or presence of 1 µM Cort as indicated for 24 h (0 to 24 h) and reincubated in similar fresh medium for a second 24-h period (24 to 48 h). Data shown are for the second incubation. Calcium fluxes are for the incubations subsequently used for PGE₂ measurements. Data are the mean ± SEM for 5 to 6 pairs of calvariae in each group; *P < 0.05 versus Ntl; †P < 0.05 versus Cort; ‡P < 0.05 versus Acid alone.

Figure 3. Correlation between PGE₂ concentration and net calcium flux. Neonatal mouse calvariae were cultured in neutral (squares) or acid (triangles) medium in the absence (open symbols) or presence (closed symbols) of 1 µM cortisol for 24 h and reincubated in similar fresh medium for a second 24 h. Data shown are for the second incubation, and each symbol represents a pair of calvariae.
secretion, although plasma aldosterone levels significantly increased (33). Chronic metabolic acidosis could stimulate endogenous glucocorticoid production; we therefore tested the hypothesis that a glucocorticoid, cortisol, would directly exacerbate acid-induced net calcium efflux from bone. However, in contrast, we found that cortisol inhibited acid stimulation of net calcium efflux, bone cell PGE2 production, and osteoclastic activity. These results suggest that the osteopenia observed in vivo in response to acidosis is not augmented by an increase in cortisol production.

When bone is cultured in acidic medium, there is an increase in net calcium efflux initially due to physicochemical dissolution (8,34) followed by a cell-mediated increase in bone resorption and decrease in bone formation (7,10). Acid medium stimulates osteoblastic PGE2 production, which mediates the subsequent stimulation of osteoclastic bone resorption (12–14,35). Cortisol has previously been shown to inhibit prostaglandin production (36) as well as hormonal stimulation of the rate-limiting enzyme that converts arachidonic acid to PGE2 (prostaglandin G/H synthase, PGHS-2) in neonatal mouse calvariae (37). Cortisol also inhibited serum stimulation of PGHS-2 mRNA levels in MC3T3-E1 mouse osteoblastic cells (38). In the results presented here, we demonstrate that cortisol inhibits acid-induced bone resorption, apparently through its ability to inhibit PGE2 production in the osteoblast. There is a direct correlation between net calcium efflux and PGE2 production both in the presence and absence of cortisol as well as a direct correlation between medium PGE2 concentration and glucuronidase activity, a measure of osteoclastic activity. This supports the idea that PGE2 production stimulated by acidosis mediates the subsequent acid-induced osteoclastic bone resorption.

Glucocorticoids stimulate net bone resorption in vivo; however, both inhibition (36,39,40) as well as stimulation (41,42) of bone resorption have been reported using bone organ culture systems. Glucocorticoids have been shown to inhibit bone cell proliferation (43) and have a biphasic effect on type I collagen synthesis, a principal function of the osteoblast (16,44–46). In addition to being dose- and treatment time-dependent, the effects of glucocorticoids also seem to depend on the stage of osteoblast differentiation. Glucocorticoids decrease bone formation via suppression of osteoblast maturation and promotion of apoptosis (19). Glucocorticoids also inhibit production of osteoprotegerin, a soluble neutralizing receptor produced by
osteoblasts, which limits osteoclastogenesis (47). Glucocorticoids stimulate the expression and action of bone morphogenetic proteins, which could account for their ability to promote osteoblast differentiation in some model systems. However, they also suppress the effect of CBFA1, a nuclear transcription factor critical for bone formation (16,48). It is not certain how the net response to glucocorticoids in vivo manifests itself with respect to these opposing responses.

Glucocorticoid-induced osteopenia in vivo has been well characterized (19,49), although the exact mechanism of induction of the resultant loss of bone mineral is not entirely understood. In general, there appears to be an uncoupling of bone remodeling to favor bone resorption over bone formation (20). Presumably this is due to an overall inhibition of osteoblastic proliferation and biosynthetic activity and stimulation of osteoclastic activity. In addition to direct effects on skeletal function, glucocorticoids also decrease intestinal calcium absorption and at higher doses increase urinary calcium excretion. Thus, the net osteopenia observed in vivo after glucocorticoid treatment is probably due to a complex combination of direct effects on bone formation and resorption as well as indirect effects to inhibit intestinal calcium absorption and increase renal calcium excretion (17,20,50,51).

In this study, we examined a direct effect of cortisol on acid-induced bone mineral resorption. We did not study any potential interaction of glucocorticoids and acidosis on overall body calcium homeostasis. Thus we cannot rule out that an acid-induced increase in cortisol in vivo could contribute to the net loss of bone mineral through more indirect mechanisms, such as alteration of intestinal calcium absorption or renal calcium excretion, that were not studied here. We have demonstrated that in vitro the addition of cortisol to acid medium does not increase, but rather inhibits, acid-induced calcium efflux from bone, suggesting that any acidosis-induced increase in cortisol will not directly increase acid-induced bone resorption. However, even though cultured neonatal mouse calvariae respond to protons and calcium-regulating hormones, synthesize DNA and protein, and have functioning osteoblasts and osteoclasts (52), as human bone does in vivo, it is not clear whether these results obtained in vitro are applicable to human bone perfused by blood. Without clinical studies confirming this in vitro work, it is premature to consider the use of cortisol, or any inhibitor of prostaglandin synthesis, to inhibit acid-induced bone resorption in humans.

Acknowledgment
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References


