Cortisol Inhibits Acid-Induced Bone Resorption *In Vitro*

NANCY S. KRIEGER, KEVIN K. FRICK, and DAVID A. BUSHINSKY

Department of Medicine, Nephrology Unit, University of Rochester School of Medicine, Rochester, New York.

Abstract. Metabolic acidosis increases urine calcium excretion without an increase in intestinal calcium absorption, resulting in a net loss of bone mineral. *In vitro*, metabolic acidosis has been shown to initially induce physicochemical mineral dissolution and then enhance cell-mediated bone resorption. Acidic medium stimulates osteoblastic prostaglandin E₂ 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 ± 0.01; [HCO₃⁻] = 10.6 ± 0.3 mM) or neutral (Ntl; pH 7.43 ± 0.01; [HCO₃⁻] = 26.2 ± 0.5 mM) medium, with or without 1 μM cortisol (Cort), and net calcium efflux and medium prostaglandin E₂ (PGE₂) levels and osteoclastic β-glucuronidase activity were determined. Compared with Ntl, Cort alone decreased calcium efflux, medium PGE₂, 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 PGE₂ levels and β-glucuronidase activity compared with Acid alone. There was a significant direct correlation between medium PGE₂ concentration and net calcium efflux (r = 0.944; n = 23; P < 0.0001), between osteoclastic β-glucuronidase activity and net calcium efflux (r = 0.663; n = 40; P < 0.001), and between medium PGE₂ concentration and β-glucuronidase activity (r = 0.976; n = 4; P < 0.01). Thus, *in vitro* cortisol inhibits acid-induced, cell-mediated osteoblastic bone resorption through a decrease in osteoblastic PGE₂ production. These results suggest that the osteopenia observed in response to metabolic acidosis *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 urinary calcium (4). Indeed metabolic acidosis is associated with a decrease in bone mineral content (5,6). *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 osteoclastic prostaglandin E₂ (PGE₂) 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 (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 PGE₂ production, and osteoclastic activity. These results suggest that cortisol does not contribute to acid-induced bone resorption.

Materials and Methods

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...
Medium PGE2 or incubation period, medium was also immediately analyzed for cortisol (Cort). To closely replicate physiologic conditions, only the Prostaglandin E2 Enzyme Immunoassay

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 $[\text{HCO}_3^-]$, to model metabolic acidosis (Met), with or without 1 μM cortisol (Cort). To closely replicate physiologic conditions, only the $\text{HCO}_3^-/\text{CO}_2$ buffer system was used (30). To reduce medium pH, 2.4 N HCl was added to the medium to obtain the desired reduction of $[\text{HCO}_3^-]$ 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).

Table 1. Initial medium ion concentrations

<table>
<thead>
<tr>
<th></th>
<th>Ntl</th>
<th>Cort</th>
<th>Acid</th>
<th>Acid + Cort</th>
</tr>
</thead>
<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>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 24 h</td>
<td>7.43 ± 0.01</td>
<td>7.43 ± 0.01</td>
<td>7.06 ± 0.01&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.05 ± 0.01&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>24 to 48 h</td>
<td>7.43 ± 0.01</td>
<td>7.43 ± 0.01</td>
<td>7.06 ± 0.01&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>7.06 ± 0.01&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>$\text{Pco}_2$ (mm Hg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<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>
</tr>
<tr>
<td>$[\text{HCO}_3^-]$ (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&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>10.6 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</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&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>10.7 ± 0.4&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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). $\text{Pco}_2$, partial pressure of carbon dioxide; $[\text{HCO}_3^-]$, medium bicarbonate concentration.

<sup>b</sup> $P < 0.05$ versus Ntl.

<sup>c</sup> $P < 0.05$ 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 /H11001 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 PGE 2 Concentration**

When compared with Ntl, there was a decrease in medium PGE 2 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).

**Correlation of Medium PGE 2 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 NH4 Cl (21) or infusing dogs with NH4 Cl (22), has also been shown to cause a significant increase in corticosteroid excretion. In a small human study, NH4 Cl-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 concentration.

**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 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 total 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 calvaria 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

This work was supported in part by National Institutes of Health Grants AR 46289 and DK 56788 funds from the Renal Research Institute.

References