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Role of Carbonic Anhydrase in Bone Resorption: Effect of Acetazolamide on Basal and Parathyroid Hormone-Induced Bone Metabolism

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Summary. The effects of the carbonic anhydrase inhibitor acetazolamide on basal and parathyroid hormone (PTH)-induced bone metabolism were studied to evaluate the manner in which acetazolamide inhibits bone resorption. Half-calvaria from 5 to 6-day-old mice were cultured using the following treatments: control; acetazolamide (10, 33, or 100 μM); PTH (16.7 nM bovine PTH 1-34); acetazolamide + PTH. The effects of acetazolamide on PTH-induced cAMP accumulation and protein synthesis were determined. Media from bones cultured for 48 hours were analyzed for calcium to assess bone resorption, glucose to assess calvarial glucose utilization, and lactic acid to assess calvarial lactic acid release. Media were also assayed for β-glucuronidase activity as an indicator of lysosomal enzyme release and for lactate dehydrogenase activity as an indicator of cytosolic enzyme release and cytotoxicity. Acetazolamide at 100 μM completely inhibited PTH-induced bone resorption. This inhibition did not appear to be due to cell death, as acetazolamide did not increase lactate dehydrogenase release. Acetazolamide had no effect on PTH-enhanced cAMP levels, indicating that receptor binding and adenylate cyclase activation were unaffected. Acetazolamide alone did not alter calvarial protein synthesis, but did significantly inhibit protein synthesis in the presence of PTH. PTH significantly enhanced calvarial glucose utilization, lactic acid release, and β-glucuronidase release. Acetazolamide inhibited all of these PTH-induced parameters in a manner that roughly paralleled its inhibition of bone resorption; acetazolamide alone had no effect on the basal values. Our results indicate that acetazolamide inhibition of bone resorption *in vitro* may involve general alterations in hormonally stimulated bone cell metabolism secondary to carbonic anhydrase inhibition.

Key words: Acetazolamide — Parathyroid hormone — Bone resorption — Bone metabolism — Calvarium.

The carbonic anhydrase inhibitor acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) has been shown by our laboratory [1–4] and by other laboratories [5–6] to inhibit bone resorption *in vivo* and *in vitro*. These findings, in combination with studies demonstrating the presence of carbonic anhydrase in osteoclasts [7, 8], suggest that carbonic anhydrase plays a fundamental role in the process of bone resorption. This concept is supported by recent clinical results which have identified a carbonic anhydrase II deficiency as the defect responsible for one form of osteopetrosis [9].

Osteoclastic bone resorption is a twofold mechanism in which bone mineral is solubilized and organic matrix is degraded. The manner in which carbonic anhydrase has been postulated to facilitate osteoclastic bone resorption is through the generation of hydrogen ions from carbon dioxide by the CO_2 hydration reaction. The acid so formed can then be actively transported across the membrane of the ruffled border into the site of resorption, where it can assist in both demineralization and matrix degradation, as described by Vaes [10]. Inhibition of carbonic anhydrase by agents such as acetazolamide might thus inhibit bone resorption by preventing the acidification of Howship's lacunae. This hypothesis is supported by a recent study in which osteoclasts exposed to acetazolamide were

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unable to maintain an acidic extracellular resorbing fluid [11]. Whether this effect of acetazolamide is due specifically to an inhibition of hydrogen ion secretion, or whether it occurs secondarily to other, more general, metabolic alterations induced by carbonic anhydrase inhibition is unclear. The possibility that acetazolamide action on bone is independent of carbonic anhydrase also cannot be discounted.

The present study was undertaken to evaluate the manner in which acetazolamide inhibits PTH-induced bone resorption by examining the effects of acetazolamide on several metabolic parameters of bone. Experiments were designed to determine whether acetazolamide, at concentrations that inhibit bone resorption (1) has a toxic effect on bone cells; (2) alters PTH-induced cAMP accumulation, an effect which would suggest alterations of hormone-receptor interaction and/or adenylate cyclase activation; (3) affects either basal or PTH-induced protein synthesis; (4) affects either basal or PTH-induced glucose metabolism; and (5) inhibits basal or PTH-induced lysosomal enzyme release.

Materials and Methods

Materials

PTH (synthetic bovine 1-34) was obtained from Bachem Fine Chemicals (Torrance, CA). Culture media and Antibiotic-Antimycotic solution were obtained from Gibco Laboratories (Grand Island, NY). ^{3}H-cAMP (20 Ci/mmol) and ^{3}H amino acid mixture (255 mCi/mg) were obtained from ICN Radiochemicals (Irvine, CA). IBMX (3-isobutyl-l-methylxanthine), lactic acid, lactate dehydrogenase, 4-methylumbelliferyl-β-D-glucuronide, 4-methylumbelliferone, and assay kits 226-UV and 510 were from Sigma Chemical Co. (St. Louis, MO). Acetazolamide was from Lederle Laboratories (Pearl River, NY). All other chemicals used were reagent grade or better. Water used was deionized and glass distilled.

Bone Culture System

The experimental system used was an *in vitro* bone culture system in which half-calvaria from 5 to 6-day-old mice were maintained, free-floating, in 1.5 ml Dulbecco's Modified Eagle Medium (high glucose) plus 1% (v/v) Antibiotic-Antimycotic solution without protein supplementation. Bones were cultured at 37° C in a water-saturated atmosphere of 5% CO_2 in air.

cAMP Determination

The effects of acetazolamide on basal and PTH-enhanced cAMP levels in cultured half-calvaria were determined using the following groups: (1) control; (2) acetazolamide (100 μM); (3) PTH (16.7 nM); (4) acetazolamide + PTH. Bones were precultured for 24 hours in media containing neither PTH nor acetazolamide. Each bone was then transferred to 1.5 ml medium containing 0.5 mM IBMX (3-isobutyl-l-methylxanthine). After 15 minutes, 0.5 ml of appropriate medium was added to each culture dish to yield the final PTH and acetazolamide concentrations described above. Cultures were maintained for an additional 15 minutes, at which time bones were removed from culture media, blotted gently to remove excess medium, and placed in 0.7 ml 90% l-propanol [12]. cAMP was extracted for 24 hours as described by Ng et al. [13]. A 0.5 ml aliquot of each sample was evaporated and redissolved in cAMP assay buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.5). cAMP was assayed by the method of Gilman [14] as modified by Tovey et al. [15].

Evaluation of Acetazolamide Cytotoxicity

Acetazolamide cytotoxicity was evaluated by determining the release of the cytosolic enzyme lactate dehydrogenase into media from bones cultured for 48 hours. The following treatments were used: (1) control; (2) acetazolamide (10, 33, or 100 μM); (3) PTH (16.7 nM); (4) acetazolamide + PTH. Lactate dehydrogenase activity was assayed as described in Sigma technical bulletin No. 226-UV. Assays were carried out at room temperature, and enzyme activities were corrected to 30°C according to Sigma technical bulletin No. 226-UV.

Effect of Acetazolamide on Calvarial Protein Synthesis

The effects of acetazolamide on calvarial protein synthesis were determined by measuring the incorporation of ^{3}H amino acids into half-calvaria cultured in the presence or absence of PTH. In this experiment, only 100 μM acetazolamide was used. Bones treated for 47 hours were transferred to 0.5 ml appropriate media containing 5 μCi ^{3}H amino acid mixture. After incubation for an additional 60 minutes, bones were homogenized by hand in ice-cold 10% trichloroacetic acid (TCA) using a ground glass homogenizer. Homogenates were washed three times with ice-cold 10% TCA, and final pellets were solubilized with 0.1 N NaOH for counting and protein determination [16]. After three TCA washes, remaining TCA soluble radioactivity was not above background levels.

Effect of Acetazolamide on Calvarial Glucose Metabolism

The effects of acetazolamide on basal and PTH-induced glucose utilization and lactic acid release were studied in cultures maintained for 48 hours using the treatments described above for the evaluation of acetazolamide cytotoxicity. Media glucose concentrations were determined using the colorimetric method described in Sigma technical bulletin No. 510. Glucose utilization was determined by subtracting final glucose concentrations of media cultured in the presence of bone from the glucose concentration of medium cultured without bone. Media lactic acid concentrations were determined by the method of Gutmann and Wahlefeld [17].

Table 1. Effect of PTH and acetazolamide (AZ) on 48 hour calcium release from neonatal mouse half-calvaria *in vitro*

Treatment	Medium calcium (mg/dl)	% Inhibition of PTH response
Control	7.2 ± 0.02[a]	
AZ (10 μM)	7.2 ± 0.06[a]	
PTH (16.7 nM)	9.3 ± 0.18[b]	
AZ + PTH	9.3 ± 0.08[b]	0
Control	7.2 ± 0.08[a]	
AZ (33 μM)	7.1 ± 0.04[a]	
PTH (16.7 nM)	9.9 ± 0.13[b]	
AZ + PTH	8.2 ± 0.10[c]	63[d]
Control	7.1 ± 0.07[a]	
AZ (100 μM)	6.6 ± 0.05[c]	
PTH (16.7 nM)	10.3 ± 0.12[b]	
AZ + PTH	6.9 ± 0.06[a,c]	100[d]

All values shown represent the mean ± SEM for 5 bones per group in 3 separate experiments

[a,b,c] Groups with like superscripts are not significantly different from each other ($P > 0.05$); groups with different superscripts are significantly different from each other ($P < 0.01$)

[d] PTH response significantly inhibited ($P < 0.01$ compared to group treated with PTH alone)

Effect of Acetazolamide on Calvarial Lysosomal Enzyme Release

The effect of acetazolamide on basal and PTH-induced release of the lysosomal enzyme β-glucuronidase from half-calvaria cultured for 48 hours was studied using the treatments described above for the evaluation of acetazolamide cytotoxicity. Media β-glucuronidase activities were assayed using 1.0 mM 4-methylumbelliferyl-β-D-glucuronide as substrate according to the method of Robins et al. [18]. Assay buffer was 0.08 M sodium acetate, 0.05% bovine serum albumin, pH 3.75. Product formation was quantitated by comparison with 4-methylumbelliferone standards.

Effect of Acetazolamide on Calvarial Calcium Balance

The effect of acetazolamide on basal and PTH-induced calcium balance was determined in 48-hour cultures using the treatments described for the evaluation of acetazolamide cytotoxicity. Changes in calcium balance were evaluated by measuring medium calcium concentrations using a Technicon II AutoAnalyzer (Technicon Instruments, Tarrytown, NY) fitted with a calcium manifold.

Statistical Analyses

All statistical analyses were made using analysis of variance followed by Duncan's multiple range test. All measured parameters except ^3H amino acid incorporation were normalized per half-calvarium. In no experiment was there any significant difference in half-calvarial weights between groups.

Table 2. Effect of PTH and acetazolamide (AZ) on 15-minute accumulation of cAMP in neonatal mouse half-calvaria *in vitro*

Treatment	Calvarial cAMP (pmol/half-calvarium)	% Inhibition of PTH response
Control	8.4 ± 1.0[a]	
AZ (100 μM)	8.6 ± 1.0[a]	
PTH (16.7 nM)	111.9 ± 6.2[b]	
AZ + PTH	121.3 ± 11.0[b]	0

All values shown represent the mean ± SEM for 5 bones per group

[a,b] Groups with like superscripts are not significantly different from each other ($P > 0.05$); groups with different superscripts are significantly different from each other ($P < 0.01$)

Results

Calcium Balance

PTH significantly enhanced bone resorption over control levels at 48 hours, elevating media calcium concentrations from 7.1 to 9.8 mg/dl [average of three experiments ($P < 0.01$)]. Acetazolamide significantly inhibited PTH-induced calcium release, with complete inhibition observed at 100 μM acetazolamide (Table 1). These results confirm previous findings from our laboratory and other laboratories that PTH-induced bone resorption is inhibited by acetazolamide. In the absence of PTH, acetazolamide treatment at the highest concentration (100 μM) resulted in culture media calcium levels that were low relative to control media (Table 1). This result is consistent with acetazolamide's inhibition of spontaneous bone resorption occurring in the absence of exogenous PTH.

cAMP Accumulation

The effects of acetazolamide on basal and PTH-induced cAMP accumulation in cultured half-calvaria are shown in Table 2. Acetazolamide (100 μM) had no effect on calvarial cAMP levels either in the absence or in the presence of PTH, indicating that acetazolamide did not interfere with PTH receptor binding or adenylate cyclase activation even at a concentration that completely inhibited bone resorption.

Acetazolamide Cytotoxicity

The release of the cytosolic enzyme lactate dehydrogenase from cultured bones was used as an indicator of cell death. The effects of acetazolamide

Table 3. Effect of PTH and acetazolamide (AZ) on 48-hour lactate dehydrogenase release into the media from neonatal mouse half-calvaria *in vitro*

Treatment	Lactate dehydrogenase release (nmol NADH · half-calvarium^{-1} · min^{-1})
Control	45.4 ± 2.5
AZ (10 μM)	41.6 ± 2.8
PTH (16.7 nM)	46.9 ± 4.3
AZ + PTH	46.5 ± 2.9
Control	38.4 ± 2.3
AZ (33 μM)	41.2 ± 3.3
PTH (16.7 nM)	44.4 ± 2.5
AZ + PTH	44.6 ± 2.4
Control	56.1 ± 3.7
AZ (100 μM)	60.1 ± 4.0
PTH (16.7 nM)	62.2 ± 3.6
AZ + PTH	64.0 ± 3.0

All values shown represent the mean ± SEM for 5 bones per group in 3 separate experiments
Within each experiment, all statistical analyses were not significant ($P > 0.05$)

and PTH, alone and in combination, on calvarial lactate dehydrogenase release are shown in Table 3. Acetazolamide had no significant effect on lactate dehydrogenase release at 48 hours, either in the presence or absence of PTH. Treatment of bones with 10 mM sodium azide for 48 hours resulted in a 2.4-fold increase in lactate dehydrogenase release (data not shown). This result indicates that the inhibition of bone resorption observed with acetazolamide was not due simply to acetazolamide-induced bone cell death.

Protein Synthesis

The effects of 100 μM acetazolamide on the incorporation of ^{3}H amino acids into calvarial TCA insoluble material are shown in Table 4. Acetazolamide alone had no significant effect on incorporation. PTH neither stimulated nor inhibited overall protein synthesis during the time frame studied. However, acetazolamide treatment significantly decreased protein synthesis by approximately 20% in the presence of PTH. One possible explanation for this unexpected finding is detailed in the following discussion.

Glucose Metabolism

The effects of acetazolamide on 48-hour basal and PTH-induced glucose utilization and lactic acid release are shown in Table 5. PTH significantly enhanced calvarial glucose utilization to 189% of control values [average of three experiments ($P < 0.01$)]. Acetazolamide at 33 μM significantly inhibited this PTH-induced enhancement; inhibition was essentially complete at 100 μM acetazolamide. These results closely parallel the inhibition of bone resorption observed with acetazolamide. Acetazolamide alone had no effect on basal calvarial glucose utilization. PTH significantly enhanced calvarial lactic acid release to 197% of control values [average of three experiments ($P < 0.01$)]. Again, this PTH-induced enhancement was inhibited partially by 33 μM and completely by 100 μM acetazolamide. Acetazolamide alone had no effect on basal calvarial lactic acid release.

Table 4. Effect of PTH and acetazolamide (AZ) on protein synthesis by half-calveria *in vitro*

Treatment	Trichloroacetic acid insoluble DPM/mg protein ($\times 10^{-4}$)
Control	10.1 ± 0.3[a]
AZ (100 μM)	10.9 ± 0.3[a]
PTH (16.7 nM)	10.0 ± 0.9[a]
AZ + PTH	8.1 ± 0.5[b]

All values shown represent the mean ± SEM for 5 bones per group
[a,b] Groups with like superscripts are not significantly different from each other ($P > 0.05$); groups with different superscripts are significantly different from each other ($P < 0.05$)

Lysosomal Enzyme Release

The effects of acetazolamide on 48-hour basal and PTH-induced release of the lysosomal enzyme β-glucuronidase are shown in Table 6. PTH significantly enhanced calvarial β-glucuronidase release to 161% of control values [average of three experiments ($P < 0.01$)]. This PTH-induced enhancement was inhibited by acetazolamide, but significant inhibition was observed only with 100 μM acetazolamide. Inhibition was partial rather than complete. Acetazolamide alone had no effect on basal calvarial β-glucuronidase release.

Discussion

Carbonic anhydrase inhibitors such as acetazolamide inhibit hormonally induced bone resorption *in vivo* and *in vitro* [1–6]. Whether this inhibition is due to specific inhibition of carbonic anhydrase-mediated acid secretion, nonspecific alterations of osteoclast metabolism secondary to carbonic anhydrase inhibition, or carbonic anhydrase indepen-

Table 5. Effect of PTH and acetazolamide (AZ) on 48-hour glucose consumption and lactic acid release into the media from neonatal mouse half-calvaria *in vitro*

Treatment	Glucose consumption (μg/half-calvarium)	% Inhibition of PTH response	Lactic acid release (μg/half-calvarium)	% Inhibition of PTH response
Control	930 ± 60[a]		727 ± 30[a]	
AZ (10 μM)	855 ± 75[a]		587 ± 43[a]	
PTH (16.7 nM)	1500 ± 150[b]		1232 ± 88[b]	
AZ + PTH	1500 ± 60[b]	0	1182 ± 87[b]	10
Control	600 ± 45[a]		473 ± 33[a]	
AZ (33 μM)	675 ± 90[a]		558 ± 59[a]	
PTH (16.7 nM)	1440 ± 45[b]		1195 ± 49[b]	
AZ + PTH	1125 ± 60[c]	38[d]	908 ± 70[c]	40[d]
Control	945 ± 105[a]		778 ± 14[a]	
AZ (100 μM)	1020 ± 105[a]		765 ± 56[a]	
PTH (16.7 nM)	1590 ± 90[b]		1319 ± 61[b]	
AZ + PTH	1020 ± 75[a]	88[d]	781 ± 39[a]	99[d]

All values shown represent the mean ± SEM for 5 bones per group in 3 separate experiments

[a,b,c] Groups with like superscripts are not significantly different from each other ($P > 0.05$); groups with different superscripts are significantly different from each other ($P < 0.01$)

[d] PTH response significantly inhibited ($P < 0.01$ compared to group treated with PTH alone)

dent alterations is uncertain. The likelihood that carbonic anhydrase inhibitors do, in fact, act on bone through carbonic anhydrase inhibition is underscored by several studies. In a study utilizing several carbonic anhydrase inhibitors, the potency of inhibition of bone resorption has been shown to parallel the potency of carbonic anhydrase inhibition [6]. The acetazolamide analogue CL 13,850 (N-t-butylacetazolamide), which does not inhibit carbonic anhydrase, is likewise ineffective as an inhibitor of bone resorption, suggesting that acetazolamide inhibition is not due to nonspecific drug action [1, 2]. The effectiveness of acetazolamide inhibition increases as ambient CO_2 levels decrease [5], a finding consistent with carbonic anhydrase involvement [19].

In the present study, two possible nonspecific actions of acetazolamide on bone were addressed: (1) the possibility that acetazolamide inhibits bone resorption toxically; and (2) the possibility that acetazolamide interferes with PTH-receptor interaction. We found that 100 μM acetazolamide, a concentration that completely inhibited PTH-induced bone resorption, had no effect on bone cell viability, as judged by the release of the cytosolic enzyme lactate dehydrogenase. This result indicates that acetazolamide inhibition of bone resorption cannot be explained by a nonspecific, cytotoxic mechanism. Acetazolamide at 100 μM likewise had no effect on PTH-enhanced calvarial cAMP accumulation, an early PTH response that appears to be osteoblastic and therefore carbonic anhydrase independent. This result is consistent with a recent finding of Pierce et al. [20], and indicates that acetazolamide does not interfere with PTH-receptor interaction and adenylate cyclase activation. While these findings do not eliminate the possibility that acetazolamide inhibits bone resorption in a carbonic anhydrase independent fashion, they do provide additional evidence that acetazolamide inhibition of hormonally stimulated bone resorption occurs as a consequence of acetazolamide inhibition of carbonic anhydrase.

One possible carbonic anhydrase-dependent mode of acetazolamide action on bone is a specific inhibition of carbonic anhydrase-mediated acid secretion resulting in inhibition of bone demineralization and matrix degradation, which appear to be acid-dependent processes [10]. A recent study by Fallon [11] demonstrating that acetazolamide both inhibits bone resorption and prevents the acidification of osteoclast extracellular resorbing fluid supports this hypothesis. However, a cause/effect relationship linking acetazolamide action specifically to an inhibition of extracellular acidification is difficult to demonstrate, and it is possible that this action of acetazolamide is an indirect effect secondary to general alterations of bone cell metabolism. Inhibition of bone resorption might thus be due to metabolic alterations sufficient to disrupt the biochemistry of osteoclastic bone resorption. Our rationale for studying the effects of acetazolamide on bone metabolism was that if acetazolamide acts by specifically inhibiting the secretion of acid across the ruffled border membrane, then other, more general, aspects of bone metabolism should remain unaffected. The parameters selected for study (glucose utilization, lactic acid release, and lysosomal enzyme release) are all parameters that are enhanced by PTH, allowing for the study of acetazolamide

Table 6. Effect of PTH and acetazolamide (AZ) on 48-hour β-glucuronidase release into the media from neonatal mouse half-calvaria *in vitro*

Treatment	β-glucuronidase release (pmol product · half-calvarium^{-1} · min^{-1})	% Inhibition of of PTH response
Control	184 ± 7[a]	
AZ (10 μM)	181 ± 8[a]	
PTH (16.7 nM)	286 ± 16[b]	
AZ + PTH	283 ± 20[b]	3
Control	167 ± 10[a]	
AZ (33 μM)	185 ± 12[a]	
PTH (16.7 nM)	276 ± 14[b]	
AZ + PTH	251 ± 12[b]	23
Control	263 ± 10[a]	
AZ (100 μM)	287 ± 10[a]	
PTH (16.7 nM)	430 ± 16[b]	
AZ + PTH	314 ± 18[c]	69[d]

Values shown represent the mean ± SEM for 5 bones per group in 3 separate experiments

[a,b,c] Groups with like superscripts are not significantly different from each other ($P > 0.05$); groups with different superscripts are significantly different from each other ($P < 0.01$)

[d] PTH response significantly inhibited ($P < 0.01$ compared to group treated with PTH alone)

effects on both the basal and PTH-stimulated states.

In the present study, acetazolamide alone caused no changes in basal glucose utilization, lactic acid release, or lysosomal enzyme release. Acetazolamide did, however, inhibit the PTH-induced enhancements of all three parameters in a manner that roughly paralleled its inhibition of calcium release. Acetazolamide alone had no effect on calvarial protein synthesis, measured as ^{3}H amino acid incorporation into trichloroacetic acid-insoluble material. Unexpectedly, acetazolamide significantly inhibited protein synthesis in the presence of PTH. This result might be due to a differential effect of PTH on protein synthesis, wherein an inhibition of protein synthesis in one cell type is counterbalanced by an enhancement of protein synthesis in another. Acetazolamide might thus inhibit PTH-enhanced osteoclastic protein synthesis, much as it inhibits other PTH-enhanced metabolic parameters, unmasking PTH inhibition of protein synthesis elsewhere.

The results of our studies suggest that acetazolamide inhibition of bone resorption involves general alterations of hormonally induced bone cell metabolism rather than, or in addition to, inhibition of hydrogen ion secretion. These alterations are likely, but not necessarily, a result of osteoclastic carbonic anhydrase inhibition. Since acetazolamide appears to alter hormonally stimulated bone cell metabolism without affecting basal bone cell metabolism, our results confirm the potential usefulness of acetazolamide (or similar carbonic anhydrase inhibitors) in the therapeutic treatment of bone loss.

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