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# Optimal Bone Resorption by Isolated Rat Osteoclasts Requires Chloride/Bicarbonate Exchange

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SUMMARY

We have examined the effect of DIDS (4,4'-diisothiocyanatostilbene sulfonic acid) a potent, specific and irreversible inhibitor of chloride/bicarbonate exchange on bone resorption by disaggregated rat osteoclasts, using an in vitro bone slice assay. DIDS inhibited bone resorption in concentration dependent fashion, without affecting osteoclast viability or survival on bone slices. The role of anion exchange in the resorptive process is discussed.

**Key words:** Bone resorption - Anion exchange - Osteoclasts.

## INTRODUCTION

Osteoclasts (OCs) resorb bone by binding onto bone surfaces and then transporting protons into the hemivacuole between the cell and the mineralized bone surface (reviewed in [1]). Protons are generated by carbonic anhydrase (CA) from carbon dioxide and water and current evidence (from immunohistochemical studies) suggests that OCs contain CA isozyme II [2]. The acidic milieu, created by proton secretion into the hemivacuole (pHs as low as 4.7 have been recorded in vivo [3]), solubilizes bone mineral - primarily calcium phosphate. This provides optimal conditions for the activity of OC enzymes, mainly acid hydrolases and proteases, which degrade the organic bone matrix [1]. Bicarbonate ions are also produced by the CA catalysed reaction and their removal from OCs would seem to be essential in order to maintain the intracellular pH at physiological levels during bone resorption. Recently, Teti et al. [4] have demonstrated the presence of a chloride/bicarbonate (anion) exchange in avian OCs, using pH-sensitive fluorescent probes to measure cytoplasmic pH changes while varying the ionic composition of the medium and using inhibitors of the anion exchange.

We have used the bone resorption assay [5] to examine the effect of DIDS (4,4'-diisothiocyanatostilbene sulfonic acid), a potent, specific irreversible inhibitor of the erythrocyte anion exchange [6], on bone resorption by disaggregated rat OCs. The results show that DIDS can inhibit bone resorption in concentration dependent fashion and indicate that control of intra-cellular pH by anion exchange is important for optimal osteoclastic bone resorption.

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## MATERIALS AND METHODS

The bone resorption assay has been described in detail previously [5]. Briefly, OCs disaggregated from neonatal rat long bones were settled onto bovine cortical bone slices and incubated for 6 hr in the presence or absence of DIDS (Sigma Chemical Co., St. Louis, Mo.). After incubation, OCs were removed by immersion of the bone slices in 10% NaOCl for 10 min, washed, dehydrated in ethanol and sputter coated with gold for scanning electron microscopy. The entire surface of each bone slice was examined "blind" and the number of resorption pits and their plan surface area (the area of pits in the plane of the bone slice surface when viewed perpendicularly from above) was recorded.

The depth of OC resorption pits was measured with the Petran version of the Tandem Scanning Reflected Light Microscope [7]. Pit depth was computed as the distance through which the objective, focussed on the surface of the slice, had to be moved to become focussed on the base of the excavation under observation.

Morphological examination of OCs incubated on plastic for 6 hr with 100 $\mu$M DIDS revealed no evidence of drug-mediated cytotoxicity or cell death. Osteoclast survival on bone slices was assessed after incubation with 100 $\mu$M DIDS. Bone

slices were stained with toluidine blue (0.1 % w/v in phosphate buffered saline) and multinucleate OCs were counted by transmistted light microscopy.

Differences between groups were analysed by paired Student's t-test.

## RESULTS

As shown in Fig. 1, DIDS inhibited OC bone resorption in a concentration dependent manner, as assessed both by the plan area resorbed per bone slice and the number of resorption pits per bone slice.

OC survival on bone slices was not affected by incubation with 100 $\mu$M DIDS. As shown in Table 1, similar numbers of OCs were observed on bone slices incubated with DIDS (100 $\mu$M) or medium control, while resorption was inhibited (~36 %) in the presence of DIDS.

It has been shown previously that there is a good correlation between the plan surface area and volume of bone resorbed [5]. We have measured pit depths in order to rule out the unlikely possibility that although the surface area of pits was reduced by DIDS that deeper pits were made, meaning that inhibition of total bone resorption by DIDS was actually less than estimated by measurement of plan surface area.

The average area per resorption pit was reduced ~30 % from 71 $\pm$ 5 $\mu M^2$ in control cultures to 50 $\pm$ 2 $\mu M^2$ with 100 $\mu$M DIDS (mean $\pm$ SEM for 15 bone slices; $p = 0.05$). The average pit depth of 20 pits chosen at random was 6.1 $\pm$ 0.5 $\mu$M (control) and 5.6 $\pm$ 0.7 with 100 $\mu$M DIDS (mean $\pm$ SEM; $0.15 > p > 0.1$). Thus, the average volume of bone resorbed per pit (calculated by 2/3 mean pit area x mean pit depth) was reduced from 289 $\mu M^3$ (control) to 187 $\mu M^3$ with 100 $\mu$M DIDS, representing ~35 % inhibition of total bone resorbed. These results indicate that the resorptive capacity of OCs was inhibited at the level of individual excavations as measured by the plan surface area resorbed, pit depth or total volume of bone resorbed, in the presence of DIDS.

## DISCUSSION

While it has been known for many years that OCs are the cells that resorb bone, it is only recently that the mechanisms involved have been elucidated [1]. After binding onto the bone surface, OCs secrete CA derived protons into the hemivacuole in order to solubilize the mineral component of bone. Exocytosis of lysosomal acid-dependent hydrolases and proteases results in degradation of the organic bone matrix and maintains exposure of calcified material. Thus, OCs are capable of resorbing all components of bone, as previously shown by morphological evidence [8]. The accumulation of bicarbonate ions in OCs also produced by the CA catalysed reaction which generates protons, could lead to detrimental elevation of intracellular pH in OCs during bone resorption. Recent evidence from Teti et al. [4] has indicated that avian OCs possess a DIDS inhibitable anion transport system, which exchanges intracellular bicarbonate ions for extracellular chloride ions.

We have examined the effect of DIDS, a potent, specific irreversible inhibitor of chloride/bicarbonate exchange [6], on bone resorption by isolated mammalian (rat) OCs in vitro. The results show that DIDS can partially inhibit bone resorption in a dose dependent fashion (Fig. 1). Maximal inhibition of ~40 % (plan surface area) was seen with 100 $\mu$M DIDS, at which concentration OC viability and survival on bone slices was not affected. DIDS also inhibited bone resorption as assessed by the number of pits made per bone slice, the mean area per pit and the depth of resorption pits. These results indicate that the resorptive process itself is impaired by DIDS, resulting in fewer and smaller pits.

Proton transport by the Na+/H+ antiporter and anion transport by the chloride/bicarbonate exchanger are known to be coupled processes in other cell systems [9,10], and in isolated rabbit proximal tubule cells it has been shown that Na+/H+ antiport activity is inhibited when the intracellular pH increases [11]. There is evidence in the literature for the Na+/H+ antiporter and anion exchanger regulating OC acidity [12] and we have found that inhibitors of the Na+/H+ antiporter are potent inhibitors of OC bone resorption (manuscript in preparation), indicating a major role for this proton transport system in osteoclastic bone resorption. Thus, by analogy with the kidney tubule system, it is possible that an increase of intracellular pH in resorbing OCs, due to inhibition of anion exchange by

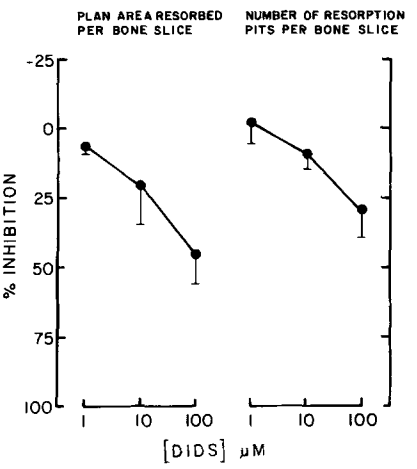


Figure 1. Each point is the mean $\pm$ SEM from 3 experiments, 5 bone slices per experiment. The base line plan surface area resorbed was 911 $\pm$ 267 $\mu M^2$ and number of pits resorbed per bone slice was 14.5 $\pm$ 4.9 (mean $\pm$ SEM of the 3 experiments).

Table 1.

| | PLAN AREA RESORBED PER BONE SLICE (n=5) ($\mu M^2$; MEAN $\pm$ SEM) | NUMBER OF OCs PER BONE SLICE (MEAN $\pm$ SEM) |
|---|---|---|
| CONTROL | 1454 $\pm$ 271 | 6.2 $\pm$ 1.6 |
| DIDS (100 $\mu$M) | 926 $\pm$ 253 ($p < 0.01$) | 5.8 $\pm$ 2.0 ($p > 0.7$) |

DIDS, results in diminished proton secretion and impaired bone resorption.

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## REFERENCES

1. Marks, S.C., Popoff, S.N.: Bone cell biology: the regulation of development, structure and function in the skeleton. Am. J Anat. 183: 1-44, 1988.
2. Gay, C.V., Schraer, H., Anderson, R.E., Cao, H.: Currents studies on the location and function of carbonic anhydrase in osteoclasts. Ann. N.Y. Acad. Sci. 429: 473-478, 1984.
3. Silver, I.A., Murrills, R.J. and Etherington, D.J.: Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. Exp. Cell Res. 175: 266-276, 1988.
4. Teti, A., Blair, H.C., Teitlebaum, S.L., Kahn, A.J., Koziol, C., Konsek, J., Sambonin-Zallone, A., Schelsinger, P.H.: Cytoplasmic pH regulation and chloride/bicarbonate exchange in avian osteoclasts. J. Clin. Invest. 83: 227-233, 1989.
5. Chambers, T.J., McSheehy, P.M.J., Thomson, B.M., Fuller, K.: The effect of calcium-regulating hormones and prostaglandins on bone resorption by osteoclasts disaggregated from neonatal rabbit bones. Endocrinology 116: 234-239, 1985.
6. Cabantchik, Z.I., Knauf, P.A., Rothstein, A.: The anion transport of the red blood cell. Biochim. Biophys. Acta 515: 239-302, 1978.
7. Tobias, J., Chambers, T.J.: Glucocorticoids impair bone resorptive activity and viability of osteoclasts disaggregated from neonatal rat long bones. Submitted, 1989.
8. Chambers, T.J., Revell, P.A., Fuller, K. and Athanasou, N.A.: Resorption of bone by isolated rabbit osteoclasts. J. Cell Sci., 66: 383-399, 1984.
9. Moolenaar, W.H.: Regulation of cytoplasmic pH by Na+/H+ exchange. Trends Biochem. Sci., 11: 141-143, 1986.
10. Chaillet, J.R., Amsler, K., Boron, W.F.: Optical measurements of intracellular pH in single LLC-$PK_1$ cells: demonstration of Cl-$HCO_3$ exchange. Proc. Natl. Acad. Sci., 83: 522-526, 1986.
11. Kurtz, I.: Basolateral membrane Na+/H+ antiport, Na+/base cotransport and Na+-independent $Cl^-$/base exchange in the rabbit $S_3$ proximal tumbule. J. Clin. Invest., 83: 616-622, 1989.
12. Anderson, R.E., Woodbury, D.M., Jee, W.S.S.: Humoral and ionic regulation of osteoclast acidity. Calc. Tiss. Int., 39: 252-258, 1986.

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