

Inhibitory mechanisms of H⁺-ATPase inhibitor bafilomycin A₁ and carbonic anhydrase II inhibitor acetazolamide on experimental bone resorption

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Abstract The effects of the vacuolar-type H⁺-ATPase inhibitor bafilomycin A₁ (baf. A₁) and the carbonic anhydrase II inhibitor acetazolamide (AZ) on bone resorption and procathepsin L secretion of rat osteoclasts were investigated using the bone slice assay method, pit formation test. Baf. A₁ completely suppressed osteoclastic bone resorption stimulated by parathyroid hormone (PTH), but did not affect procathepsin L secretion, while AZ suppressed both bone resorption and procathepsin L secretion. These findings suggest that bone resorption by procathepsin L secretion and its processing are regulated by proton production and proton secretion.

Key words: Procathepsin L; Bone resorption; Bafilomycin A₁; Acetazolamide

1. Introduction

Bone resorption depends on the secretion of protons and lysosomal proteinases from osteoclasts into the extracellular microenvironment (Howship's lacuna) under their ruffled border [1,2]. In osteoclasts, protons are generated by carbonic anhydrase II (CAII) [3,4] and actively transported by the proton pump driven by vacuolar-type H⁺-ATPase at the ruffled border into the lacuna [5], which participate in bone resorption. The lysosomal proteinases, possibly cysteine proteinases, had been thought to play an important role in osseous collagenolysis [2,6–11] and protons may assist the protease secretion. The secretion of protons and lysosomal proteinases may play an essential role in the bone resorption mechanism. During osteoclastic bone resorption, it is possible that removal of calcium precedes the degradation of the bone matrix collagen [2,12].

We previously reported that the bone pit formation stimulated by PTH was significantly suppressed by specific inhibitors of cathepsin L and L-type proteinases, but not by those of cathepsins B and D [13]. It was also demonstrated using bone slice assays that an increase in pit formation occurs in parallel with the secretion of procathepsin L induced by PTH and that both increases were completely inhibited by the addition of calcitonin [14]. These findings suggest that procathepsin L is secreted from osteoclasts indirectly stimulated by PTH, and plays an important role in PTH-induced bone resorption. Procathepsin L is synthesized in the endoplasmic reticulum (ER), transported into the Golgi system and then secreted into the lacuna via the ruffled border membrane

[1,2,15]. The secreted procathepsin L appears to be converted to its active mature form, cathepsin L, in the lacuna [16]. However, the regulatory mechanisms of procathepsin L secretion from osteoclasts are still unclear.

The secretion of both protons and procathepsin L is indispensable for osteoclastic bone resorption. In the present study, in order to clarify whether procathepsin L secretion is influenced by proton pumping and/or proton production in osteoclasts, the secretion of procathepsin L into the medium of the bone slice culture was assayed. Pit formation was arrested by administration of the vacuolar-type H⁺-ATPase inhibitor bafilomycin A₁ (baf. A₁) and also the CAII inhibitor acetazolamide (AZ).

2. Materials and methods

2.1. Materials

Baf. A₁ and AZ were purchased from Wako Pure Chemical (Osaka, Japan). Rabbit anti-rat cathepsin L was donated by Dr. E. Kominami (Department of Biochemistry, Juntendo University, Tokyo, School of Medicine, Japan). Human PTH (1–34) was obtained from Peninsula Laboratories, Inc. (Belmont, CA). Sprague-Dawley (SD) rats (1–2 days old) came from Charles River, Japan.

2.2. Assay of bone resorption (Pit formation test)

Bone resorption was assayed according to the method of McSheehy and Chambers [17] with modifications as described in detail previously [13,14,16]. Unfractionated bone cells were prepared from tibiae, femora, and humeri of 1–2-day-old SD rats. The bones were dissected free of soft tissues in iced tissue culture medium (pH 7.2) [alpha-minimum essential medium (α-MEM) (Dainippon Pharmaceutical Co., Japan) supplemented with 100 IU per ml benzylpenicillin] containing 10 mM HEPES. For preparation of bone cells the long bones were minced with scissors in the same medium, and then the suspension was triturated. 250 µl of the cell suspension (4 × 10⁶ cells per ml) was allowed to settle onto a bovine cortical bone slice (400-µm thick circle, 6 mm in diameter) in each well of a 96-well plate. After 1 h of incubation at 37°C in a CO₂ incubator, unattached cells were washed away with α-MEM, and bone slices with attached cells were transferred to fresh medium containing various concentrations of inhibitors and 10^{−7} M PTH and then incubated for 24–72 h. Osteoclast-induced pits on the slices were stained with acid hematoxylin for 5 min. The total area of pits was measured under an optical microscope.

2.3. Observation of osteoclasts on slices

Observation of osteoclasts on slices was made following tartrate-positive acid phosphatase (TRAP) staining according to Akatsu et al. [18]. The cells were washed with phosphate-buffered saline (PBS, pH 7.4), and then fixed with 10% formalin in PBS for 10 min. After treatment with ethanol-acetone (50:50, v/v) for 1 min, the slice surface with cells was dried and stained for TRAP. The cells were observed under an optical microscope.

2.4. Electrophoresis and Western blotting analysis

SDS-polyacrylamide gel electrophoresis was carried out according

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to the method of Laemmli [19]. For Western blotting analysis the medium did not contain fetal bovine serum. In the experiment utilizing baf. A₁, the media were collected at 72 h after addition of PTH and baf. A₁ and concentrated 100 times. Samples were resolved by SDS-PAGE in a 15–25% gradient gel and electrophoretically transferred to a nitrocellulose blotting membrane (Millipore). Immunoblotting for detection of 39-kDa procathepsin L was conducted with rabbit anti-rat cathepsin L as the primary antibody, and the bound antibodies were detected with goat anti-rabbit IgG-conjugated alkaline phosphatase (E·Y Laboratories, Inc.). The alkaline phosphatase reaction was performed with a Picoblue Immunoscreening Kit (Stratagene, CA). The SDS-PAGE low-range standards (Bio-Rad, Richmond) used as molecular weight markers were BSA (80 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa), and lysozyme (18.5 kDa). In the experiment with AZ, the media were collected at 72 h after addition of PTH and AZ and concentrated 30 times. Samples were electrophoresed in a SDS-12.5% polyacrylamide gel and transferred electrophoretically to a nitrocellulose blotting membrane. The membrane was probed with the primary antibody and then with a biotinylated donkey anti-rabbit Ig whole antibody (Amersham). Following incubation with streptavidin-horseradish peroxidase (Amersham), the blots were developed by the enhanced chemiluminescence method (Amersham) and then exposed to Polaroid type T667 film (Polaroid) that had been loaded into an ECL Mini-camera (Amersham). The biotinylated SDS-PAGE broad-range standards (Bio-Rad) used as molecular weight markers were myosin (200 kDa), β -galactosidase (116.25 kDa), phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa).

2.5. Statistical analysis

Statistical comparison between groups was performed by the Mann-Whitney U-test.

3. Results and discussion

The present study was performed in order to clarify the inter-related regulation between proton and procathepsin L secretion from osteoclasts. Baf. A₁ inhibited osteoclastic bone resorption by blocking vacuolar-type H⁺-ATPase in the ruffled border membrane [20]. As shown in Fig. 1, PTH-

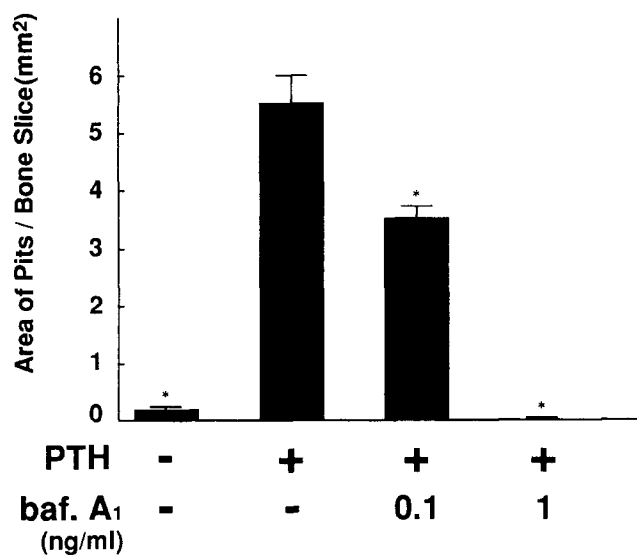


Fig. 1. Inhibitory effect of baf. A₁ on pit formation induced by PTH. Cells were stimulated with PTH at a concentration of 10^{-7} M. Each value of bone resorption indicates the mean \pm S.E.M. * $p < 0.05$, vs. value for PTH alone. Before staining of the resorption pits, the number of osteoclasts per bone slice was counted. The number for PTH, PTH plus 0.1, or 1 ng/ml of baf. A₁ treatment was 111 ± 16 , 121 ± 5 , or 90 ± 12 , respectively.

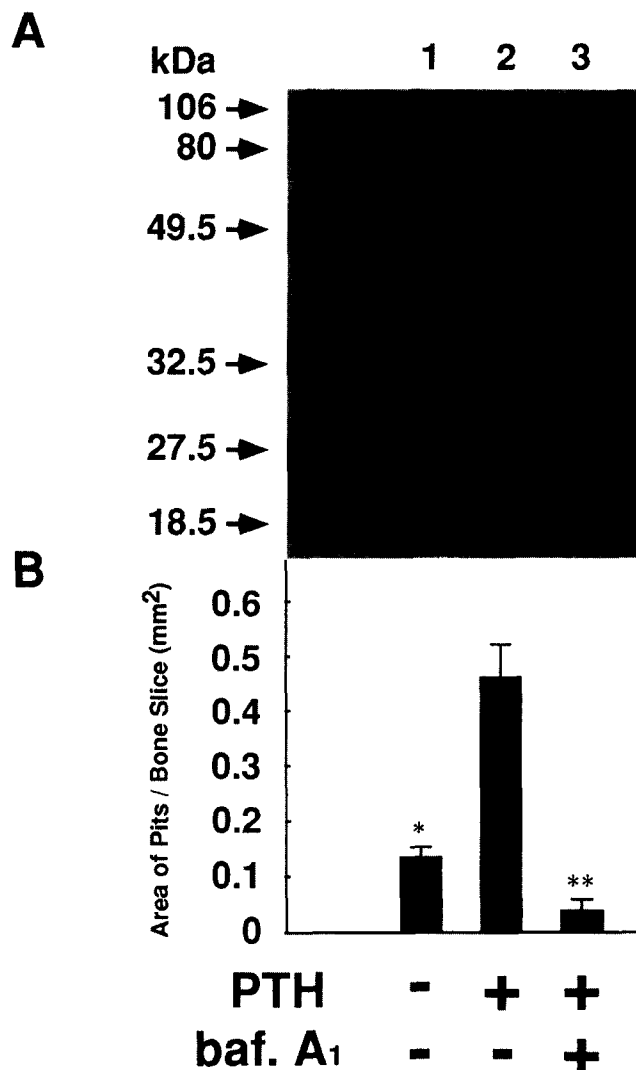


Fig. 2. (A) Effect of baf. A₁ on procathepsin L secretion. Lanes: 1, untreated group; 2, PTH group; 3, PTH and baf. A₁ group. Concentrations of PTH and baf. A₁ were 10^{-7} M and 1 ng/ml, respectively. The media were collected at 72 h after addition of PTH and baf. A₁, concentrated 100 times, and subjected to SDS-PAGE. (B) Pit formations under the same conditions as those in (A). Bone resorption was assayed according to the method described in Section 2. Each value indicates the mean \pm S.E.M. * $p < 0.05$, ** $p < 0.01$; vs. value for PTH alone. The number of osteoclasts per bone slice was 6 ± 3 and 12 ± 6 in the treatment with PTH and PTH plus baf. A₁, respectively.

enhanced bone resorption was suppressed by baf. A₁ in a dose-dependent manner at concentrations of 0.1 and 1 ng/ml. The number of osteoclasts per bone slice was not significantly affected by baf. A₁ at concentrations less than 1 ng/ml (Fig. 1), suggesting no toxicity in this range. When the proton pump was specifically inhibited by baf. A₁ treatment, we measured the amount of procathepsin L secreted into the culture medium using Western blotting analysis. Although sufficient baf. A₁ (1 ng/ml) was added, the amount of 39-kDa procathepsin L secreted was not inhibited as shown in Fig. 2A, whereas complete inhibition of bone resorption was observed for the same dose (Fig. 2B). These findings suggest that procathepsin L secretion is independent of proton transport and baf. A₁ addition inhibits the conversion of procathepsin L to the mature form in the lacuna. Then, to in-

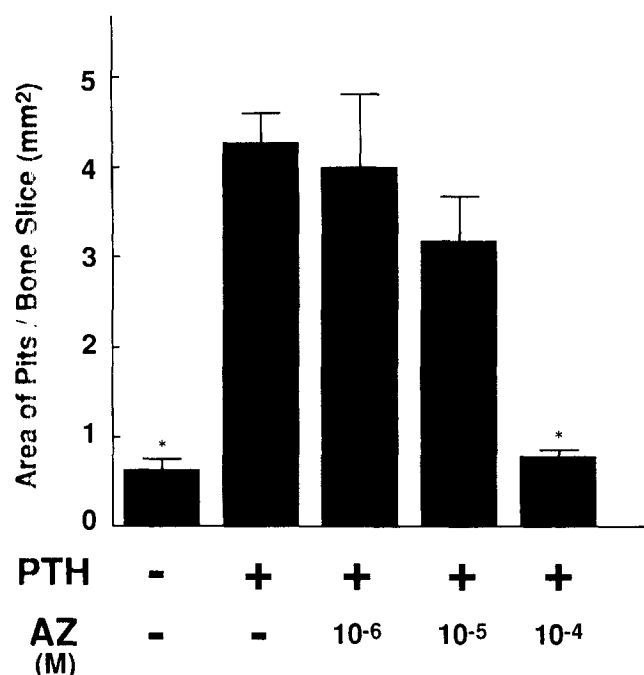


Fig. 3. Inhibitory effect of AZ on pit formation induced by PTH. Cells were stimulated by PTH at a concentration of 10^{-7} M. Each value of bone resorption indicates the mean \pm S.E.M. * $p < 0.01$, vs. value for PTH alone. The number of osteoclasts per bone slice was 7 ± 4 , 8 ± 4 , 5 ± 2 or 13 ± 2 in the treatment with PTH, PTH plus 10^{-6} , 10^{-5} or 10^{-4} M AZ, respectively.

investigate whether proton production positioned upstream of the proton pump is associated with procathepsin L secretion, we assayed procathepsin L secretion under inhibition of proton production by AZ. AZ treatment markedly reduced PTH-enhanced procathepsin L secretion into the culture medium as shown in Fig. 4A. Therefore, procathepsin L secretion is associated with proton production in the osteoclasts, but not with proton transport. The inhibitory effects on bone resorption by AZ have previously been reported [21–24], and the present study has developed this effect in more detail on the correlation with procathepsin L secretion. AZ treatment resulted in a significant decrease in PTH-induced bone resorption at a concentration of 10^{-4} M, and did not influence the number of osteoclasts on the slices as shown in Figs. 3 and 4B.

The mechanism by which AZ prevents procathepsin L secretion is unclear. Hall et al. [23] reported that the release of lysosomal enzyme β -glucuronidase and protein synthesis stimulated by PTH were inhibited by AZ in mouse half-calvaria, suggesting the possibility of alteration of hormonally stimulated bone cell metabolism. Accordingly, the decrease in procathepsin L secretion may be influenced by the response to various intracellular signals by the inhibition of proton production by AZ.

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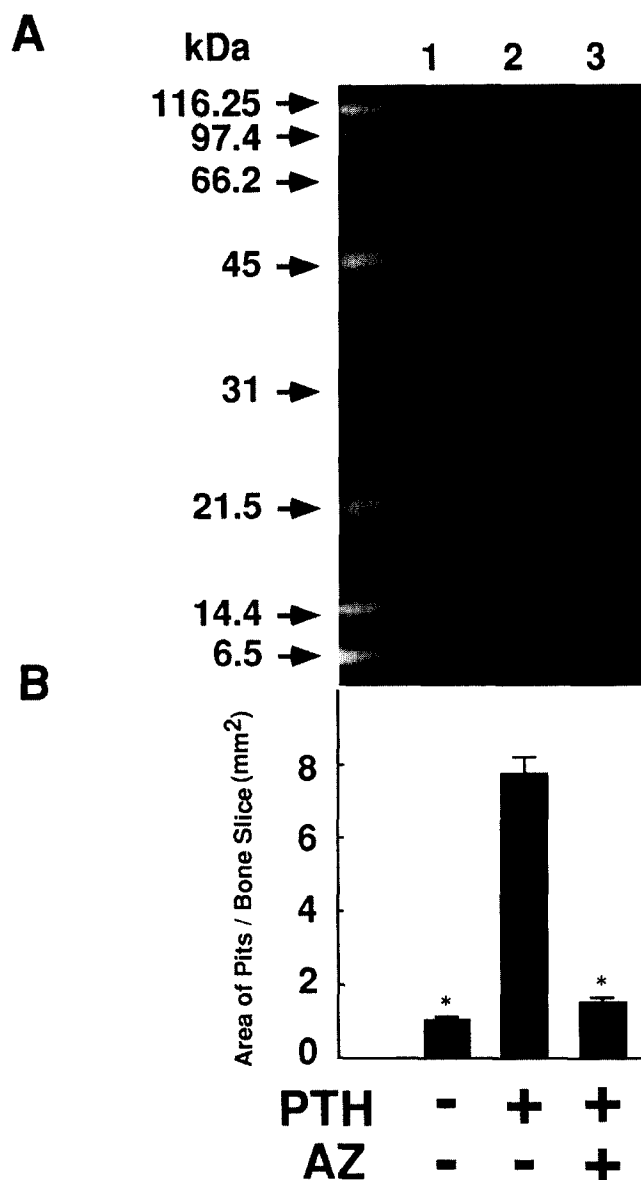


Fig. 4. (A) Suppression of procathepsin L secretion by AZ. Lanes: 1, untreated group; 2, PTH group; 3, PTH and AZ group. Concentrations of PTH and AZ were 10^{-7} and 10^{-4} M, respectively. The media were collected at 72 h, concentrated 30 times, and subjected to SDS-PAGE. (B) Pit formations under the same conditions as those in (A). Bone resorption was assayed according to the method described in Section 2. Each value indicates the mean \pm S.E.M. * $p < 0.01$, vs. value for PTH alone. The number of osteoclasts per bone slice was 5 ± 1 and 4 ± 1 in the treatment with PTH and PTH plus AZ, respectively.

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