Stimulatory Effect of β -Alanyl-L-histidinato Zinc on Alkaline Phosphatase Activity in Bone Tissues from Elderly Rats: Comparison with Zinc Sulfate Action

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The capability of β -alanyl-L-histidinato zinc (AHZ) to increase alkaline phosphatase activity in the femoral diaphysis from elderly rats was investigated. The femoral-diaphyseal tissues were removed from weanling (3-week-old) and elderly (10-month-old) female rats. Bone tissues were cultured in Dulbecco's modified Eagle medium (high glucose, 4.5%) supplemented with antibiotics and bovine serum albumin. Among various other bone-stimulating factors (AHZ; 10^{-5} M, zinc sulfate; 10^{-4} M, sodium fluoride; 10^{-3} M, insulin; 10^{-8} M, and β -estradiol; 10^{-9} M), AHZ had a potent effect on increasing alkaline phosphatase activity in the diaphyseal tissues from both rat groups. In the bone tissues from elderly rats, the effect was concentration dependent (10^{-7} – 10^{-5} M). At 10^{-5} M the effect of AHZ was seen for a longer time during 72-h culture, although the zinc sulfate (10^{-5} M) effect was no longer. The effect of AHZ to increase bone alkaline phosphatase activity was completely abolished by the presence of cycloheximide (10^{-6} M). AHZ thus appears able to directly stimulate alkaline phosphatase activity dependent on protein synthesis in the bone tissues from elderly rats.

Keywords β -alanyl-L-histidinato zinc; alkaline phosphatase; osteoporosis; aged rat femur

It is known that zinc is essential for growth in humans and many animals.¹⁾ Bone growth retardation is a common finding in various conditions associated with zinc deficiency.^{2,3)} Zinc has a stimulatory effect on bone formation and mineralization *in vivo* and *in vitro*.^{4,5)} Recently, it was reported that a new zinc compound, β -alanyl-L-histidinato zinc (AHZ), in which zinc is chelated to β -alanyl-L-histidine, can stimulate bone growth in weanling rats,⁶⁾ and thereafter it was demonstrated that AHZ has a direct anabolic effect on osteoblastic cells (MC3T3-E1) *in vitro*.⁷⁾ This compound has a more potent effect than zinc sulfate on bone formation,^{6,7)} suggesting that it may have a role in the therapeutics for osteoporosis. Whether AHZ can demonstrate an anabolic effect in bone tissues from elderly rats, however, remains to be elucidated, because it has been reported that bone metabolism deteriorates with increasing age.^{8,9)}

The present study was undertaken to clarify the effect of AHZ on alkaline phosphatase activity, which is involved in bone calcification, in tissue culture using the femoral diaphysis from elderly rats. AHZ showed a direct stimulatory effect on the alkaline phosphatase activity in this rat group dependent on protein synthesis, suggesting that it may be beneficial in the treatment of osteoporosis with increasing age.

MATERIALS AND METHODS

Chemicals Dulbecco's modified Eagle medium and penicillin-streptomycin solution (5000 units/ml penicillin; 5000 μ g/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, N.Y., U.S.A.). Bovine serum albumin (fraction V), cycloheximide, insulin (bovine pancreas, 26.2 IU/mg), and β -estradiol were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). AHZ, which was supplied by Zeria Pharmaceutical Co. (Tokyo, Japan), was dissolved in hydrochloric acid and neutralized

with sodium hydroxide. Zinc sulfate and other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan). All water used was glass-distilled.

Animals Weanling female (3-week-old) and elderly female (10-month-old) Wistar rats were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium, 1.1% phosphorus and 0.012% zinc, and distilled water. The rats were killed by decapitation.

Bone Culture The femurs from both age groups were removed aseptically after bleeding and soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and epiphysis (containing metaphyseal tissue) were separated and weighed. The femoral-diaphyseal tissues were cut into small pieces and fragments were cultured in a 35-mm dish in 2.0 ml medium consisting of Dulbecco's modified Eagle's medium (high glucose, 4.5 g/dl) supplemented with 0.25% bovine serum albumin plus antibiotics (100 units penicillin and 100 μ g streptomycin/ml of medium).⁵⁾ Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO₂ and 95% air.

Analytical Procedures Alkaline phosphatase activity in the bone tissues was determined by the method of Walter and Schutt.¹⁰⁾ The bone tissues were immersed in 3.0 ml of ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized in a Potter-Elvehjem homogenizer with a Teflon pestle, and disrupted for 60 s with an ultrasonic device. The supernatant fraction, centrifuged at 600 g for 5 min, was used for measurement of the enzyme activity. The efficiency of the extraction of enzyme component from bone tissues was greater than 90% and the enzyme analysis was reproducible. The enzyme assay was carried out under optimal conditions. Enzyme activities were expressed as micromoles of *p*-nitrophenol liberated per minute per milligram of protein. Protein was determined by the method of Lowry *et al.*¹¹⁾

Statistical Analysis The significance of the difference between values was estimated by Student's *t*-test. *p*-Values of less than 0.05 were considered to indicate statistically significance differences.

RESULTS

The effect of AHZ on alkaline phosphatase activity in the femoral-diaphyseal tissues from weanling and elderly rats was compared to that of various bone-stimulating factors (Table I). The bone tissues were cultured for 24 h in medium containing various reagents, which have a maximum effect on bone formation *in vitro*. Alkaline phosphatase activity in the femoral-diaphyseal tissues from weanling rats was significantly increased by the presence of AHZ (10^{-5} M), zinc sulfate (10^{-4} M), insulin

(10^{-8} M) or β -estradiol (10^{-9} M), while sodium fluoride (10^{-3} M) had no effect. Moreover, in the bone tissues from elderly rats, the enzyme activity was significantly elevated by the presence of AHZ, zinc sulfate or insulin, while sodium fluoride and β -estradiol did not have an appreciable effect. The effect of AHZ or zinc sulfate to increase the enzyme was about 3- or 2-fold the control value, respectively. Thus, AHZ revealed a potent effect on bone alkaline phosphatase from elderly rats, although the enzyme activity in elderly rat bone tissues dropped markedly lower than that from weanling rat bone tissues.

The effect of increasing concentrations of AHZ or zinc sulfate on bone alkaline phosphatase activity is shown in

TABLE I. Effects of AHZ and Various Factors on Alkaline Phosphatase Activity in the Femoral-diaphyseal Tissues from Weanling and Elderly Rats

Treatment	Alkaline phosphatase ($\mu\text{mol}/\text{min}/\text{mg}$ protein)	
	Weanling	Elderly
Control	1.339 ± 0.083	0.335 ± 0.033
AHZ (10^{-5} M)	2.452 ± 0.266^b	1.099 ± 0.089^b
Zinc sulfate (10^{-4} M)	2.170 ± 0.111^b	0.697 ± 0.087^b
Sodium fluoride (10^{-3} M)	1.591 ± 0.288	0.279 ± 0.020
Insulin (10^{-8} M)	1.855 ± 0.086^b	0.467 ± 0.018^b
β -Estradiol (10^{-9} M)	1.657 ± 0.048^a	0.379 ± 0.020

Each value is the mean $\pm$ S.E.M. of five bones from different rats. The femoral-diaphyseal fragments obtained from weanling and elderly rats were cultured for 24 h in the presence of various reagents. *a)* $p < 0.05$ and *b)* $p < 0.01$, as compared with the control value.

Fig. 1. Effect of Increasing Concentrations of AHZ or Zinc Sulfate on Alkaline Phosphatase Activity in the Femoral-diaphyseal Tissues from Elderly Rats

The bone tissues were cultured for 24 h in medium containing either AHZ (10^{-7} to 10^{-5} M) or zinc sulfate (10^{-7} to 10^{-5} M). Each value is the mean $\pm$ S.E.M. of 5 bone tissues from different rats. *a)* $p < 0.01$, as compared with the control value. *b)* $p < 0.01$, as compared with the value from zinc sulfate at each concentration. $\circ$, zinc sulfate; $\bullet$, AHZ.

Fig. 2. Time Course of the Effect of AHZ or Zinc Sulfate to Increase Alkaline Phosphatase Activity in the Femoral-diaphyseal Tissues from Elderly Rats

The bone tissues were cultured for up to 72 h in medium containing either AHZ (10^{-5} M) or zinc sulfate (10^{-5} M). Each value is the mean $\pm$ S.E.M. of 5 bone tissues from different rats. *a)* $p < 0.01$, as compared with the control value. $\square$, control; $\square$ (hatched), zinc sulfate; $\blacksquare$, AHZ.

Fig. 3. Effect of Cycloheximide on the AHZ-Increased Alkaline Phosphatase Activity in the Femoral-diaphyseal Tissues from Elderly Rats

The bone tissues were cultured for 24 h in medium containing either vehicle, AHZ (10^{-5} M), cycloheximide (10^{-6} M) or AHZ plus cycloheximide. Each value is the mean $\pm$ S.E.M. of 5 bone tissues from different rats. *a)* $p < 0.01$, as compared with the control value. $\square$, control; $\blacksquare$, AHZ.

Fig. 1. The femoral-diaphyseal tissues from elderly rats were cultured for 24 h in medium containing either AHZ or zinc sulfate (10^{-7} to 10^{-5} M). AHZ had a potent effect, more than that of zinc sulfate, on bone alkaline phosphatase activity. An appreciable effect of AHZ was seen at 10^{-7} M.

The time course of the effect of AHZ or zinc sulfate on bone alkaline phosphatase activity is shown in Fig. 2. The femoral-diaphyseal tissues from elderly rats were cultured for up to 72 h in medium containing either AHZ (10^{-5} M) or zinc sulfate (10^{-5} M). With longer periods of culture, the effect of either compound to increase bone alkaline phosphatase activity was weakened. At 72 h of culture, the bone enzyme activity was significantly increased by the presence of AHZ, although the effect of zinc sulfate was no longer seen.

The effect of AHZ in increasing bone alkaline phosphatase activity was completely abolished by the presence of cycloheximide, an inhibitor of protein synthesis, when the femoral-diaphyseal tissues from elderly rats were cultured for 24 h in medium containing either AHZ (10^{-5} M), cycloheximide (10^{-6} M) or AHZ plus cycloheximide (Fig. 3).

DISCUSSION

AHZ has a stimulatory effect on bone formation in the bone tissues of weanling rats.⁶ Its effect of bone metabolism in aged rats, however, has been unknown. It is important to know whether AHZ can play a therapeutic role in the development of osteoporosis with increasing age. In the present study, it was found that AHZ increases alkaline phosphatase activity, which relates to bone calcification, in the femoral-diaphyseal tissues from elderly rats, *in vitro*. The effect of AHZ to increase the enzyme activity in the bone tissues of these rats was completely abolished by the presence of cycloheximide, an inhibitor of protein synthesis, *in vitro*. The enzyme is known to be a zinc-protein.¹⁴ In a separate experiment, however, the addition of AHZ or zinc sulfate (10^{-8} – 10^{-4} M) to the enzyme reaction mixture did not cause a significant increase in the enzyme activity in the homogenate of these tissues (data not shown). Thus the effect of AHZ increasing bone alkaline phosphatase activity in tissue culture appears to be based on bone protein synthesis, and, although this deteriorates in the femoral tissues of aged rats,⁹ it is possible that AHZ stimulates this bone protein synthesis which deteriorates with increasing age.

AHZ had a more potent effect than that of zinc sulfate, on alkaline phosphatase activity in the femoral-diaphyseal tissues from elderly rats *in vitro*. In this new zinc

compound zinc is chelated to β -alanyl-L-histidine. The zinc in AHZ may accumulate in bone cells without difficulty, because the metal easily binds to the hydroxyapatite of bone tissue.¹⁵ Also, AHZ in bone cells (osteoblasts) may be retained for a longer period and have a prolonged effect, since the effect to increase bone alkaline phosphatase activity was seen after 72-h of culture (Fig. 2). Meanwhile, it is reported that zinc can activate aminoacyl-tRNA synthetase, a rate-limiting enzyme in the translational process of protein synthesis, in bone cells.¹⁶ Likewise, AHZ in bone cells may activate the enzyme in bone cells, although this remains to be elucidated.

Among various bone-stimulating factors used, AHZ caused the most effective increase of alkaline phosphatase activity in the femoral-diaphyseal tissues from elderly rats. Bone alkaline phosphatase activity was markedly decreased with increasing age in comparison with that of weanling rats. Bone tissues from elderly rats weakened in their response to insulin¹⁷ and estrogen,¹⁸ which can stimulate bone formation and osteoblastic cell proliferation. Aging presumably deteriorates the hormonal effect on bone metabolism. In addition, aging induces a decrease in bone cellular zinc content.⁹ The presence of AHZ may have a preventive and therapeutic role in the deterioration of bone metabolism with increasing age.

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