Biochemical Pharmacology, Vol. 35, No. 5, pp. 773–777, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

ACTION OF ZINC ON BONE METABOLISM IN RATS

INCREASES IN ALKALINE PHOSPHATASE ACTIVITY AND DNA CONTENT

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(*Received* 5 *March* 1985; *accepted* 7 *August* 1985)

Abstract—The effects of zinc on the enzymes of femoral tissue were investigated in weanling rats that had been given zinc sulfate (1.0 mg Zn^{2+}/100 g body wt) p.o. for 3 days. Administration of zinc caused a marked elevation of alkaline phosphatase and acid phosphatase activities, whereas it did not cause significant changes in succinate dehydrogenase, 5'-nucleotidase, ATPase, pyrophosphatase and β-*N*-acetylglucosaminidase activities. The effect of zinc was greater on alkaline phosphatase of the femoral diaphysis. Zinc content of the femoral diaphysis was raised significantly by administration of zinc. The addition of zinc in concentrations of $10^{-2} - 10^{2}$ μM did not produce a significant increase in alkaline phosphatase activity in the femoral diaphysis, indicating that zinc could not activate the enzyme. Administration of cycloheximide or actinomycin D completely inhibited the increase in alkaline phosphatase activity produced by administration of zinc. DNA content of the femoral diaphysis, but not epiphysis, was increased markedly by administration of zinc. The increases in both alkaline phosphatase activity and DNA content of the femoral diaphysis were not caused by administration of copper, manganese, cobalt, nickel and chromium(III). The present investigation suggests that zinc may induce the increase in alkaline phosphatase related to DNA synthesis and, as a result, stimulate bone growth.

It is well known that zinc is essential for the growth of man and many animals [1]. Bone has one of the highest zinc concentrations of all tissues [2]. Bone growth retardation is a common finding in various conditions associated with zinc deficiency [3, 4]. Thus, a physiological role of zinc in the growth and calcification of bone tissue has been suggested in zinc-deficient rats. The action of zinc on the cellular metabolism of bone, however, is not clarified fully.

Earlier publications from this laboratory have indicated that bone is a target organ of zinc and that a comparatively low dose of zinc has a stimulatory effect on the bone growth and calcification of weanling rats [5, 6]. The present study was undertaken, therefore, to investigate the action of zinc on bone metabolism after administration of zinc sulfate to weanling rats. We found that zinc stimulates the induction of alkaline phosphatase, which is a physiologically important enzyme in bone calcification, related to DNA synthesis in rat bone cells.

MATERIALS AND METHODS

Weanling male Wistar rats weighing 60–65 g were obtained from the Nippon Bio Supp. Center Co., Tokyo. The animals were fed commercial laboratory chow (solid) containing 1.1% calcium, 1.1% phosphorus and 0.012% zinc, and distilled water freely. Zinc sulfate was dissolved in distilled water to a concentration of 1.0 mg as Zn^{2+}/ml. This solution (1.0 ml/100 g body wt) was orally administered to rats for 3 days. The animals were killed 24 hr after the last administration of zinc. Likewise, solutions of copper sulfate, manganese chloride, cobalt chloride, nickel chloride or chromium (III) chloride (15.3 μmoles as metal ion/100 g) were administered. Cycloheximide or actinomycin D was dissolved in distilled water to concentrations of 300 μg/ml and 40 μg/ml. These solutions (0.5 ml/100 g) were injected i.p. in rats, 1 hr before administration of zinc (1.0 mg/100 g), three times at 24-hr intervals. The rats were killed 24 hr after the last administration of zinc.

The femur was removed, soaked in ice-cold 0.25 M sucrose solution, and cleaned of soft tissue and marrow; the diaphysis and epiphysis (containing metaphyseal tissue) were separated and weighed. The femoral tissue was 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 sec with an ultrasonic device. The supernatant fraction centrifuged at 600 *g* for 5 min was used for measurement of enzyme activities. All of the enzyme assays described below were carried out under optimal conditions. Acid and alkaline phosphatase activities were determined by the method of Walter and Schutt [7]. The enzyme activity was expressed as μmoles of *p*-nitrophenol liberated per min per mg protein. Succinate dehydrogenase [8], 5'-nucleotidase [9], β-*N*-acetylglucosaminidase [10], ATPase and pyrophosphatase [11] activities were measured by the method described elsewhere. Protein was determined by the method of Lowry *et al.* [12].

Zinc content in the diaphysis and epiphysis tissues of femur was determined by atomic absorption spectrophotometry after digestion with nitric acid.

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Table 1. Effect of zinc administration on the activities of various enzymes in the homogenate of whole femure of weanling rats

Enzyme	Activity	
	Control	Zinc
Succinate dehydrogenase*	4.80 ± 0.16	4.90 ± 0.23
5′-Nucleotidase†	41.1 ± 4.36	44.7 ± 4.27
Acid phosphatase†	344 ± 40.5	596 ± 21.9‡
Alkaline phosphatase†	1524 ± 50.0	1757 ± 66§
ATPase†	196 ± 10.2	196 ± 6.9
Pyrophosphatase†	62.2 ± 1.45	61.4 ± 2.31
β-*N*-acetylglucosaminidase†	19.0 ± 1.43	22.2 ± 0.43

Zinc (1.0 mg/100 g) was administered orally for 3 days, and the rats were killed 24 hr after the last administration of zinc. Each value is the mean ± S.E.M. of five animals.

* Expressed as 10^{-2} × absorbance at 490 nm per min per mg protein.

† Expressed as nmoles per min per mg protein.

‡ $P < 0.01$, compared with the control.

§ $P < 0.05$, compared with the control.

Zinc content was expressed as μg of zinc per g wet bone tissue. Also, zinc content in the supernatant fraction (600 *g* for 5 min) of the femoral tissue homogenate was measured likewise. Zinc concentration was expressed as μg of zinc per mg protein.

To measure DNA content in the femoral tissue, the diaphyseal and epiphyseal fragments of the femoral tissue were shaken with 4.0 ml of ice-cold 0.1 N NaOH solution for 24 hr [13]. After alkali extraction, the samples were centrifuged at 10,000 *g* for 5 min, and the supernatant fraction was collected. DNA content in the supernatant fraction was determined by the method of Ceriotti [14] and expressed as the amount of DNA (mg) per g wet weight of bone tissue.

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 significant differences.

RESULTS

Effect of zinc administration on activities of enzymes in femur. The effect of zinc administration on various enzymes in the homogenate of femoral tissue (containing the diaphyseal and epiphyseal fragments) was examined after oral administration of zinc sulfate (1.0 mg Zn^{2+}/100 g) for 3 days in rats as shown in Table 1. Of a number of enzymes tested, acid and alkaline phosphatase activities were increased significantly. Succinate dehydrogenase, 5′-nucleotidase, ATPase, pyrophosphatase and β-*N*-acetylglucosaminidase activities were not altered significantly.

The increase in alkaline phosphatase activity of the femoral tissue after zinc administration was observed only in the diaphysis, and not in the epiphysis (Fig. 1A). The increase in acid phosphatase activity was seen in both the diaphysis and the epiphysis (Fig. 1B). The effect of zinc administration on these two enzymes, however, was greater on alkaline phosphatase in the diaphysis.

Zinc accumulation in femur after zinc administration. The increase in zinc accumulation in the femoral tissue after administration of zinc sulfate (1.0 mg Zn^{2+}/100 g) for 3 days in rats is shown in Table 2. The accumulation of zinc in the femoral tissue was greater in the diaphysis than in the epiphysis, although zinc content in both tissues increased significantly. Zinc concentrations in the supernatant fraction of the diaphyseal and epiphyseal tissue homogenates were increased markedly by zinc administration. The zinc that accumulated in the diaphyseal and epiphyseal tissues was distributed about 70 and 35% respectively. Thus, the administered zinc accumulated largely in the diaphyseal tissue. Meanwhile, zinc concentration in the serum increased significantly after administration of zinc for 3 days, being raised from 205.1 ± 9.1 to 255.0 ± 12.2 (μg/100 ml) for five animals.

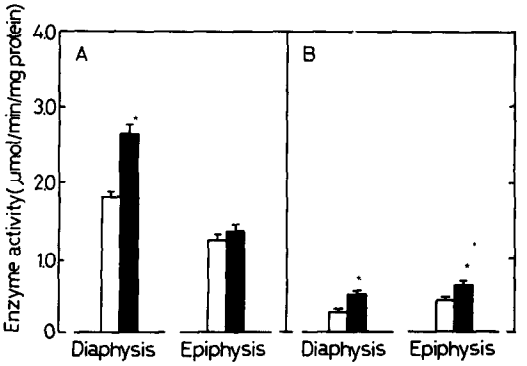

Fig. 1. Effect of zinc on the activities of alkaline phosphatase and acid phosphatase in the diaphysis and the epiphysis of the femur of weanling rats. The rats were administered zinc sulfate (1.0 mg Zn^{2+}/100 g) orally for 3 days and were killed 24 hr after the last administration of zinc. Each bar is the mean of five animals. Vertical lines represent the S.E.M. Key: (*) $P < 0.01$, compared with the control; (□) control; (■) zinc; (A) alkaline phosphatase; and (B) acid phosphatase.

Table 2. Zinc content in the diaphysis and epiphysis of the femur of weanling rats after administration of zinc sulfate

Treatment	Zinc in bone tissue (μg/g wet bone tissue)	Zinc in the supernatant of bone homogenate (μg/mg protein)
Diaphysis		
Control	187.6 ± 10.9	0.63 ± 0.02
Zinc	254.2 ± 14.3*	4.16 ± 0.65*
Epiphysis		
Control	67.6 ± 3.9	0.38 ± 0.02
Zinc	85.0 ± 4.0*	3.14 ± 0.58*

Zinc (1.0 mg/100 g) was administered orally for 3 days, and the rats were killed 24 hr after the last administration of zinc. Each value is the mean ± S.E.M. of five animals.
* $P < 0.01$, compared with the control.

The addition of Zn^{2+}, in the range of 10^{-2}–10^{2} μM, to the enzyme reaction mixture did not cause a significant increase in alkaline phosphatase activity of the diaphysis and the epiphysis, as shown in Fig. 2. The enzyme activities were inhibited at 10 and 100 μM Zn^{2+}.

Effects of inhibitors on the increase in alkaline phosphatase activity by zinc administration. The effects of cycloheximide and actinomycin D on alkaline phosphatase activity in the diaphyseal tissue are shown in Fig. 3. Administration of cycloheximide (150 μg/100 g) or actinomycin D (20 μg/100 g) to the control rats did not produce a significant alteration of alkaline phosphatase activity. Administration of these inhibitors completely prevented the increase in alkaline phosphatase activity after administration of zinc (1.0 mg/100 g).

Effect of zinc administration on DNA content in femur. The effect of zinc administration on DNA content in the femur is shown in Fig. 4. DNA content in the femoral diaphysis, but not in the epiphysis, was increased markedly by administration of zinc (1.0 mg/100 g). Administration of cycloheximide (150 μg/100 g) or actinomycin D (20 μg/100 g) did not inhibit the increase in DNA content by zinc administration (data not shown).

Changes in alkaline phosphatase activity and DNA content in the diaphysis after administration of various metals. The effects of the administration of various metals on alkaline phosphatase activity and DNA content in the femoral diaphysis were examined after oral administration of metals (15.3 μmoles/100 g) for 3 days in rats as shown in Table 3. Alkaline phosphatase activity decreased markedly after administration of copper sulfate, manganese chloride, cobalt chloride, nickel chloride, and chromium(III) chloride, whereas this enzyme activity increased significantly after administration of zinc sulfate. DNA content rose markedly after administration of zinc, nickel and chromium(III), although administration

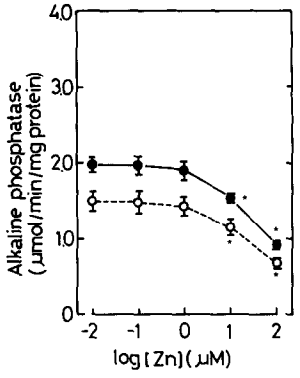

Fig. 2. Effect of zinc addition on alkaline phosphatase activity in the femoral diaphysis and epiphysis of weanling rats. Zn^{2+} was added in the incubation mixture to concentrations of 10^{-2}–10^{2} μM. The femoral tissue homogenate was prepared from normal rats. The addition of 0.01 μM Zn^{2+} per incubation mixture had no effect on the enzyme activity in comparison with the values of no addition. Each point is the mean of five animals. Vertical lines represent the S.E.M. Key: (*) $P < 0.01$, compared with the values of no addition; (●) diaphysis; and (○) epiphysis.

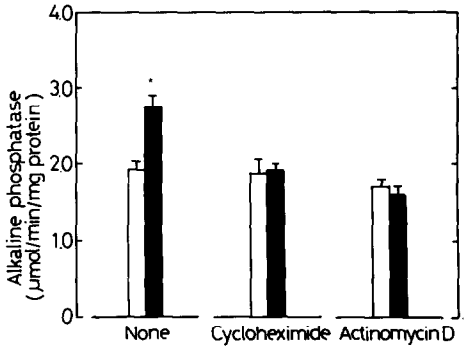

Fig. 3. Effects of cycloheximide and actinomycin D on alkaline phosphatase activity in the femoral diaphysis of weanling rats administered zinc sulfate orally. The rats were administered zinc sulfate (1.0 mg Zn^{2+}/100 g) orally for 3 days and were killed 24 hr after the last administration of zinc. Cycloheximide (150 μg/100 g) or actinomycin D (20 μg/100 g) was injected intraperitoneally 1 hr before zinc administration, and these drugs were administered three times at 24-hr intervals. Each bar is the mean of five animals. Vertical lines represent the S.E.M. Key: (*) $P < 0.01$, compared with the control; (□) control; and (■) zinc.

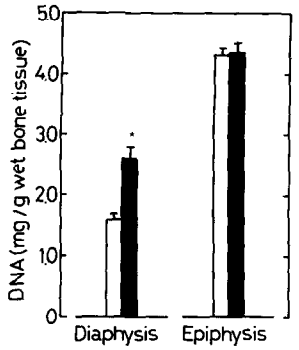

Fig. 4. Change of DNA content in the femoral diaphysis and epiphysis of weanling rats after zinc administration. The rats were administered zinc sulfate (1.0 mg Zn^{2+}/100 g) orally for 3 days and were killed 24 hr after the last administration of zinc. Each bar is the mean of five animals. Vertical lines represent the S.E.M. Key: (*) $P < 0.01$, compared with the control; (□) control; and (■) zinc.

of copper, manganese and cobalt did not cause a significant alteration of DNA content. Thus, only zinc administration produced an increase in both alkaline phosphatase activity and DNA content in the femoral diaphysis.

DISCUSSION

It was found recently that the calcium and collagen contents in the femoral diaphysis of weanling rats increase markedly in the rats after giving them zinc sulfate orally for 3 days, and that zinc administration stimulates the growth of the femur [5, 6]. However, the mechanism of zinc action in stimulating bone growth remains to be established. In the present study, we examined the effects of zinc administered orally on the activities of various enzymes (alkaline phosphatase, acid phosphatase, succinate dehydrogenase, ATPase, pyrophosphatase, 5′-nucleotidase, and β-*N*-acetylglucosaminidase) in the femoral homogenate of weanling rats. Of these enzymes, the activities of alkaline phosphatase and acid phosphatase were clearly increased by zinc administration. The effect of zinc, however, was greater on alkaline phosphatase in the femoral diaphysis. Thus, zinc administration largely caused an increase in alkaline phosphatase activity, related to bone calcification. The increase in alkaline phosphatase activity may be ascribed to stimulation of bone cell metabolism by zinc, since the metal increased bone tissue. The increase of zinc in bone tissue may result from a rapid elevation of the metal in serum following zinc administration.

Zinc accumulation in the diaphyseal tissue of femur was increased after administration of zinc. However, the metal itself did not have an effect on the activation of alkaline phosphatase, suggesting that zinc-increased enzyme activity may be based on the induction of the enzyme. Then, we examined the effects of cycloheximide and actinomycin D on alkaline phosphatase activity in the diaphyseal tissue. It is well known that cycloheximide is an inhibitor of protein synthesis and that actinomycin D is an inhibitor of RNA synthesis. Both inhibitors produced a remarkable reduction of alkaline phosphatase activity increased by zinc administration, although the enzyme activity of control rats was not reduced significantly by both inhibitors. From these results, it was suggested that zinc stimulates the synthesis of protein and RNA in the diaphyseal cells and, as a result, it can induce the increase in alkaline phosphatase activity.

Administration of zinc produced a remarkable elevation of DNA content in the diaphyseal tissue. This fact suggested the possibility of stimulation of DNA synthesis by administration of zinc. If zinc causes this, the increase in alkaline phosphatase following zinc administration may be related to not only the synthesis of protein and RNA but also the enhancement of DNA synthesis due to the metal. Presently, the mechanism of zinc action to increase DNA content in the diaphyseal tissue is unknown. It is possible that zinc may stimulate DNA synthesis, since DNA polymerase is a zinc-enzyme [15]. However, the increase in DNA resulting from zinc is suggestive of proliferation by diaphyseal bone cells. This remains to be elucidated.

Of various metals, only the effects of zinc on bone

Table 3. Effects of the administration of various metals on alkaline phosphatase activity and DNA content in the femoral-diaphyseal tissue of weanling rats

Treatment	Alkaline phosphatase (μmoles/min/mg protein)	DNA content (mg/g wet bone tissue)
Control	1.890 ± 0.090	1.622 ± 0.088
Zinc	2.680 ± 0.140*	2.601 ± 0.183*
Copper	0.875 ± 0.052*	1.436 ± 0.088
Manganese	1.226 ± 0.106*	1.431 ± 0.008
Cobalt	1.005 ± 0.051	1.842 ± 0.104
Nickel	0.967 ± 0.062*	2.215 ± 0.239*
Chromium(III)	0.889 ± 0.026*	2.497 ± 0.102*

Metals (15.3 μmoles/100 g) were administered orally for 3 days, and the rats were killed 24 hr after the last administration of metals. Each value is the mean ± S.E.M. of five animals.

* $P < 0.01$, compared with the control.

metabolism was specific. Administration of copper, manganese, cobalt, nickel or chromium(III) caused a remarkable reduction of alkaline phosphatase activity in the diaphyseal tissue, although nickel and chromium(III) produced a significant increase in DNA content in the diaphyseal tissue. Thus, only zinc had an effect on two components of the diaphyseal tissue. Zinc may play an important role, as an activator, in bone cell metabolism.

Additionally, administration of zinc produced significant increases in both alkaline phosphatase and acid phosphatase activities of femoral diaphysis. Alkaline phosphatase relates to the function of osteoblasts of bone cells, and acid phosphatase relates to that of the osteoclasts. Zinc, accumulated in the diaphyseal tissue, may stimulate the bone cell metabolism of two types. The osteoblasts, however, respond more sensitively to zinc, because the effect of the metal is greater on alkaline phosphatase than acid phosphatase.

In conclusion, the present findings, that oral administration of zinc sulfate in rats produced an increase in alkaline phosphatase activity and DNA content in the bone cells, suggest a physiological significance of zinc in the regulation of bone growth.

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