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Role of endogenous zinc in the enhancement of bone protein synthesis associated with bone growth of newborn rats

Zhong Jie Ma and Masayoshi Yamaguchi

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan

Abstract The role of endogenous zinc in protein synthesis in the bone tissues of newborn rats was investigated in the present study. Femoral–diaphyseal and metaphyseal tissues were obtained at 1, 7, 14, 21, and 28 days after birth. Many protein molecules were found to be present in the diaphyseal and metaphyseal tissues using sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. Bone protein synthesis activity was enhanced by increasing age, and reached a plateau 21 days after birth. Protein synthesis in the diaphyseal and metaphyseal tissues obtained from 7- or 14-day-old rats was significantly decreased by the addition of dipicolinate (10^{-3}M), a chelator of zinc ion, into the reaction mixture, while it was significantly enhanced by zinc sulfate (10^{-4}M). When the diaphyseal and metaphyseal tissues obtained from 7- or 14-day-old rats were cultured for 48h in a medium containing dipicolinate (10^{-3}M), bone protein synthesis was significantly reduced. This decrease was blocked completely by culture with the addition of zinc (10^{-4}M). Culture with zinc (10^{-5} and 10^{-4}M) alone had a stimulatory effect on the bone protein synthesis. Zinc (10^{-4}M)-induced increases in bone protein synthesis were completely blocked by culture with cycloheximide (10^{-6}M) or actinomycin D (10^{-7}M). The present study suggests that bone protein synthesis is enhanced with increasing age of newborn rats, and that endogenous zinc in bone tissues has a stimulatory role in the enhancement of protein synthesis with bone growth.

Key words zinc · protein synthesis · bone growth · newborn rats

Introduction

Zinc is known to be an essential element for the growth of human and other animals [1,2]. Bone growth retardation is a common finding in various conditions associated with zinc deficiency [3,4]. Zinc may be required for the growth, development, and maintenance of healthy bones. In recent years, growing evidence has accumulated indicating that zinc plays a physiologic role in the stimulation of bone formation and in the inhibition of bone resorption [5–10].

A role for zinc in the prevention of bone loss due to osteoporosis has been suggested [11–13]. Zinc supplementation has a preventative effect on bone loss in ovariectomized rats [11,12] and in skeletal-unloaded rats [13]. Osteoporosis patients have been shown to have lower levels of skeletal zinc than normal individuals [14]. Urinary zinc in postmenopausal women may be important as a marker of bone resorption, because women with osteoporosis excrete a large amount of zinc in their urine [15]. Zinc supplementation could have benefical effects on the bone density [16].

Zinc has been demonstrated to have a physiologic and pathophysiologic role in the regulation of bone metabolism. The intake of zinc-containing nutrients may have a role in the maintenance of healthy bone. More recently, zinc supplementation has been shown to have a stimulatory effect on the bone growth of newborn rats following lactation of maternal animals that were administered zinc sulfate orally [17]. However, this mechanism has not been fully clarified. The present study was undertaken to determine the role of endogenous zinc in the enhancement of bone protein synthesis of newborn rats with increasing age. We found that endogenous zinc in bone tissues has a role in the enhancement of bone protein synthesis associated with the bone growth of newborn rats.

Materials and methods

Chemicals

L-[4,5-^{3}H]-Leucine (5.29TBq/mmol) was obtained from New England Nuclear (Boston, MA, USA). Dulbecco's modified Eagle's medium (DMEM; high glucose:

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4.5 g/dl) and a penicillin–streptomycin solution (5000 units/mg penicillin; 5000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY, USA). Bovine serum albumin (BSA; fraction V), dipicolinate (2,6-pyridinedicarboxylic acid; neutralized with sodium hydroxide prior to use), cycloheximide, and actinomycin D were obtained from Sigma Chemical (St. Louis, MO, USA). Zinc sulfate and other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan). All water used was glass distilled.

Animals and bone tissues

Female Wistar rats, purchased from Japan SLC Inc. (Hamamatsu, Japan) were fed commercial laboratory chow (solid; Oriental Yeast Co. Ltd, Tokyo, Japan) containing 57.5% carbohydrate, 1.1% calcium, and 1.1% phosphorus, and were allowed distilled water ad libitum. Rats were pregnant. Newborn rats were divided into male and female groups, and bred separately. The newborn or weanling male rats were killed by bleeding between 1 and 28 days after birth. Femurs were removed aseptically after bleeding and were soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated. Marrow cells were completely removed by the washing of diaphyseal and metaphyseal tissues.

The femoral–diaphyseal and metaphyseal tissues obtained at 1, 7, 14, 21, and 28 days after birth were 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, centrifuged at 5500 *g* for 10 min, was used for gel electrophoresis and protein synthesis assays. Bone protein concentration was determined by the method of Lowry et al. [18] using BSA as the stardard.

Gel electrophoreses

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli [19] with minor modification. The electrophoresis was performed using 12% polyacrylamide resolving gel and the discontinuous Tris–glycine buffer system. Twenty-five microlitres (containing 20 μg protein) of the bone protein sample was dissolved in SDS gel loading buffer containing 4% SDS, 10% β-mercaptoethanol and bromophenol blue (BPB) marker. The protein mixture was denatured by heating at 90°C for 2 min and applied to individual wells. Electrophoresis was applied to the gel at 35 mA for 3 h at room temperature. After separation, proteins were simultaneously fixed with methanol–acetic acid and stored in water containing 20% glycerol.

Bone protein synthesis

The incubation mixture to assay in vitro protein synthesis contained, in a total of 1.0 ml, 25 mM Tris-chloride (pH 7.5), 5 mM $MgCl_2$, 5 mM KCl, 2.5 mM potassium ATP (pH 7.5), 1 mM [^{3}H]-leucine (48.5 kBq/ml), and the 5500 *g* supernatant fraction (260–400 μg protein/ml) [20]. In separate experiments, the reaction mixture contained either vehicle (distilled water), dipicolinate (10^{-3} M) or zinc sulfate (10^{-4} M). After incubation at 37°C for 30 min, the reaction was stopped by the addition of 10% trichloroacetic acid (TCA; 1.0 ml), and then the mixture was heated for 10 min at 100°C to deacylate [^{3}H]-leucine-labeled aminoacyl-tRNA, and an aliquot was withdrawn and applied to a Millipore (Bedford, MA, USA) filter disk (2.5 cm diameter); the latter was washed with an excess of cold aqueous 5% TCA. After three changes of 5% TCA solution, the disk was washed with an excess of cold ethanol–ether (1:1 v/v) and, finally, with cold ether. The radioactivity of the disks was counted for 5 min. Protein synthesis was determined after subtracting the incorporation of radioactivity in the absence of the 5500 *g* supernatant to take away the acylation of tRNAs other than leucine tRNA, and is expressed as disintegrations per minute (d.p.m.) per mg protein.

Bone tissue culture

The femoral–diaphyseal and metaphyseal tissues were aseptically obtained 7 and 14 days after birth from newborn rats. Bone tissue fragments were cultured for 48 h in a 35-mm dish in 2.0 ml medium consisting of DMEM (high glucose) supplemented with antibiotics (100 units penicillin and 100 μg streptomycin/ml medium) [5]. This medium contained 0.25% bovine serum albumin but not serum, and did not detect zinc. Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air. In experiments, bone tissues were cultured for 42 h in a medium containing either vehicle or dipicolinate (10^{-3} M) in the absence or presence of zinc sulfate (10^{-6}–10^{-4} M), cycloheximide (10^{-6} M), or actinomycin D (10^{-7} M). After culture, bone tissues were pooled and cut into small pieces. The 5500 *g* supernatant fractions of bone tissue homogenate were used for the assay of in vitro protein synthesis.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical differences were analyzed using Student's *t*-test. $P < 0.05$ was considered significant. In addition, we used a multiway ANOVA and Turky–Kramer multiple test to compare treatment groups.

Results

Alteration in bone protein synthesis of newborn rats with increasing age

The characterization of protein components in the femoral–diaphyseal and metaphyseal tissues of newborn rats with increasing age using SDS-PAGE analysis is shown in Fig. 1. Many protein molecules were present in the diaphyseal tissues, the bone protein components were elevated 14 days after birth, and this was also seen at 28 days. Such an elevation was not observed in the metaphyseal tissues. Strong bands were seen for protein molecules of molecular weights of approximately 14.3, 18, and 46 kDa in both diaphyseal and metaphyseal tissues. A 220 kDa band was seen in metaphyseal but not diaphyseal tissues.

The alteration in in vitro protein synthesis activity in the femoral–diaphyseal and metaphyseal tissues of newborn rats with increasing age is shown in Fig. 2. Protein synthesis activity was determined by using the 5500 *g* supernatant fraction (containing microsomes and cytoplasm) from diaphyseal and metaphyseal tissues. Bone protein synthesis activity was enhanced with increasing

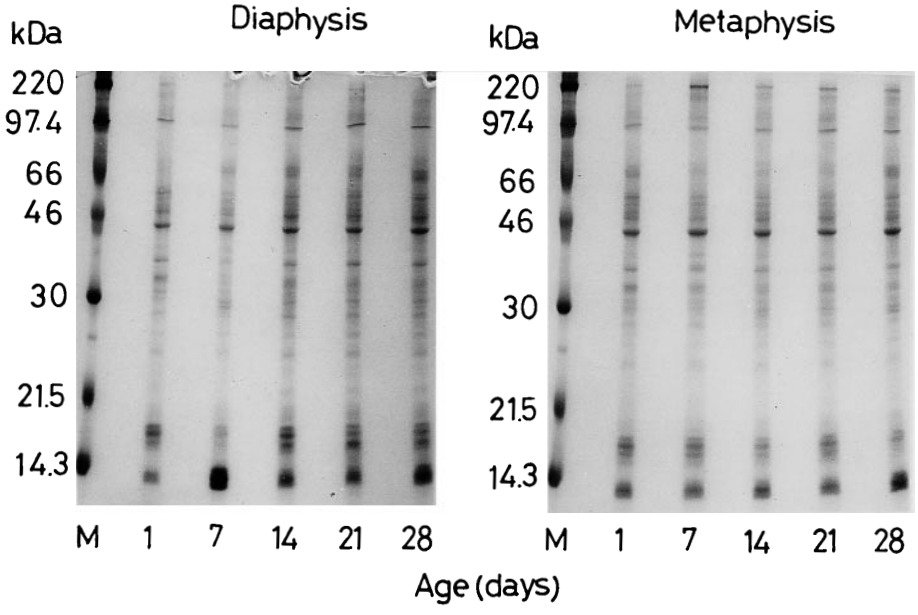

Fig. 1. Characterization of protein components in femoral–diaphyseal and metaphyseal tissues of newborn rats with increasing age using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Rats were killed 1, 7, 14, 21, and 28 days after birth. Samples for SDS-PAGE (containing 20 μg protein), which were the 5500 *g* centrifugation supernatant of bone homogenate, were pretreated with SDS in the presence of β-mercaptoethanol. Protein bands were visualized by Coomassie blue staining. The results of one of three experiments with separate rats are shown. *M*, molecular weight marker

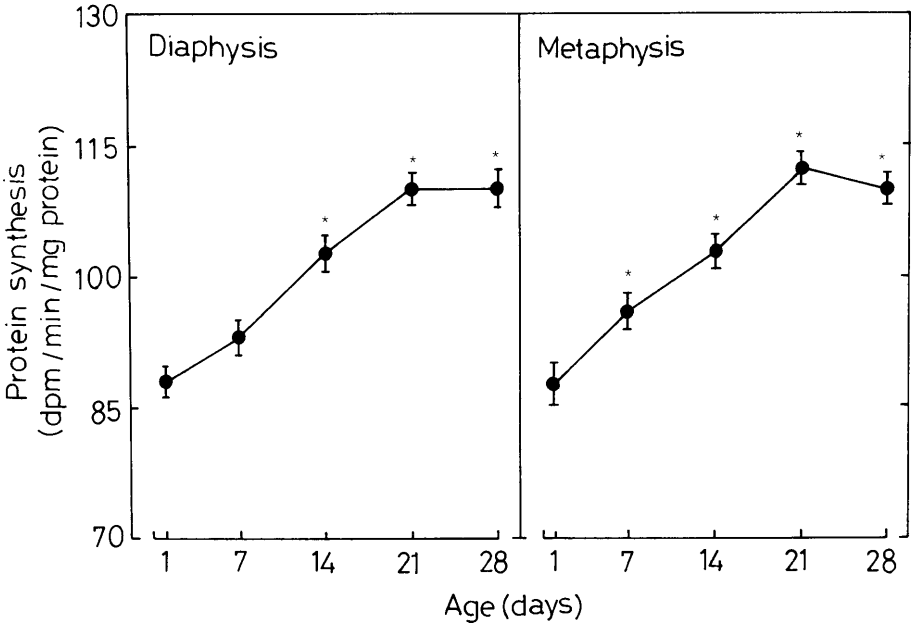

Fig. 2. Changes in in vitro protein synthesis activity in femoral–diaphyseal and metaphyseal tissues of newborn rats with increasing age. Protein synthesis was measured by using the 5500 *g* centrifugation supernatant of bone homogenate obtained at 1, 7, 14, 21, and 28 days after birth. Data are the mean ± SEM of five rats. $^{*}P < 0.01$ compared with values obtained at 1 day after birth

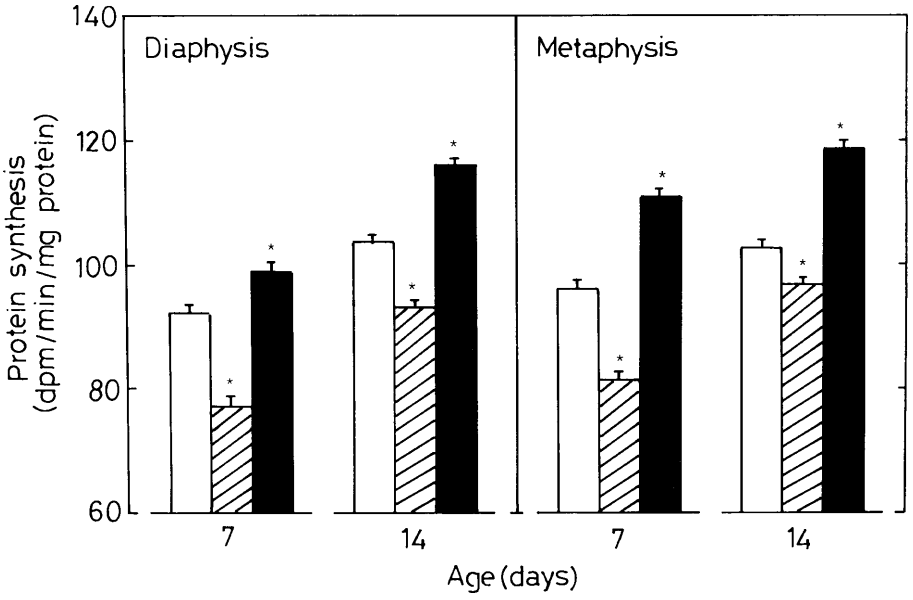

Fig. 3. Effect of dipicolinate, a chelator of zinc, on in vitro protein synthesis activity in femoral–diaphyseal and metaphyseal tissues of newborn rats with increasing age. Bone tissues were obtained at 7 and 14 days after birth. Protein synthesis was measured as described in Fig. 2. The reaction mixture contained either vehicle (□), 10^{-3}M dipicolinate (▨) or 10^{-4}M zinc sulfate (■). Data are the mean ± SEM of five rats. $*P < 0.01$ compared with the control (vehicle) values. *White bars*, control; *hatched bars*, dipicolinate addition; *black bars*, zinc addition

age. This increase reached a plateau at 21 days after birth. At 7 days after birth, a significant elevation of protein synthesis was seen in the metaphyseal but not diaphyseal tissues.

The effect of dipicolinate, a chelator of zinc [21,22], on in vitro protein synthesis activity in the femoral–diaphyseal and metaphyseal tissues of newborn rats with increasing age is shown in Fig. 3. The reaction mixture of protein synthesis contained either vehicle, dipicolinate (10^{-3}M) or zinc sulfate (10^{-4}M). Bone tissues were obtained at 7 and 14 days after birth. The presence of dipicolinate in the reaction mixture caused a significant decrease in protein synthesis in diaphyseal and metaphyseal tissues from 7- or 14-day-old rats. Meanwhile, protein synthesis in the diaphyseal and metaphyseal tissues of 7- and 14-day-old rats was raised significantly by the addition of zinc into the reaction mixture. At 7 days after birth, the effect of zinc in increasing protein synthesis in the metaphyseal tissues was approximately two-fold ($P < 0.01$) compared with that of diaphyseal tissues. Such an increase was not seen in bone tissues at 14 days after birth. The effect of dipicolinate in decreasing protein synthesis was not significantly different between diaphysis and metaphysis.

Alteration in protein synthesis in bone tissues cultured with zinc

Femoral–diaphyseal and metaphyseal tissues of newborn rats were obtained at 7 and 14 days after birth. Bone tissues were cultured for 48h in a medium containing either vehicle, dipicolinate (10^{-3}M) or dipicolinate (10^{-3}M) plus zinc sulfate (10^{-4}M; Fig. 4). Protein synthesis activity in the diaphyseal or metaphyseal tissues from 7- or 14-day-old rats was significantly decreased by culture with dipicolinate (Fig. 4). Such a decrease was not seen in cultures with dipicolinate plus zinc. The culture with dipicolinate (10^{-6} or 10^{-5}M) did not have an appreciable effect on bone protein synthesis (data not shown).

Changes in protein synthesis activity in femoral–diaphyseal and metaphyseal tissues of 7-day-old rats cultured with zinc sulfate are shown in Fig. 5. Bone tissues were cultured for 48h in a medium containing either vehicle or zinc sulfate (10^{-6}–10^{-4}M). Protein synthesis in diaphyseal and metaphyseal tissues was significantly elevated by culture with zinc (10^{-5} or 10^{-4}M). The stimulatory effect of zinc on bone protein synthesis was not seen at 10^{-6}M zinc.

The effect of cycloheximide or actinomycin D on the zinc-induced increase in bone protein synthesis activity is shown in Table 1. Femoral–diaphyseal and metaphyseal tissues were obtained from 7- or 14-day-old rats. Bone tissues were cultured for 48h in a medium containing either vehicle or zinc sulfate (10^{-4}M) in the absence or presence of cycloheximide (10^{-6}M) or actinomycin D (10^{-7}M). Zinc-induced increases in protein synthesis in the diaphyseal and metaphyseal tissues were completely blocked by culture with cycloximide or actinomycin D.

Discussion

Changes in the bone components of newborn rats with increasing age has been demonstrated in previous study [17]. Increasing age produces a remarkable elevation of zinc and calcium content and alkaline phosphatase ac-

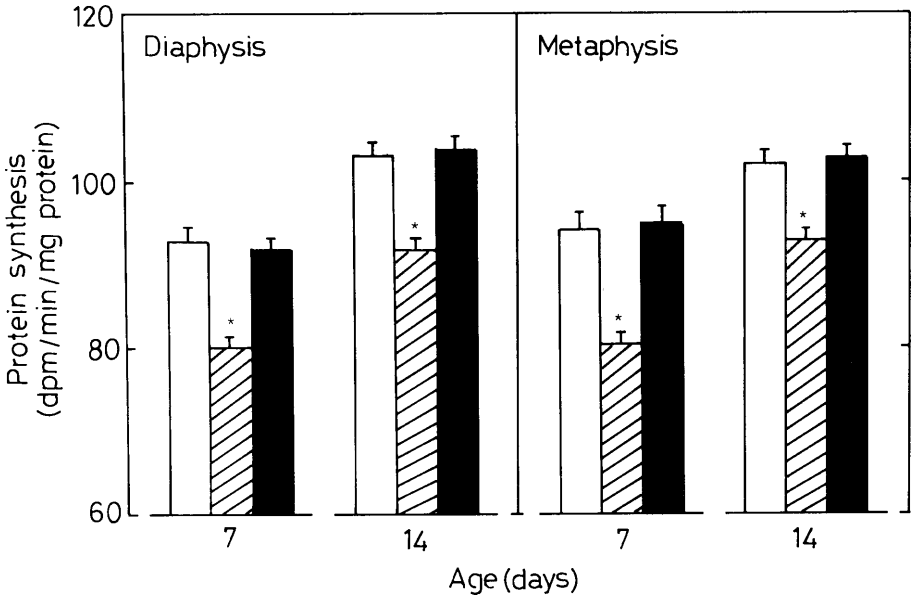

Fig. 4. Changes in in vitro protein synthesis activity in femoral–diaphyseal and metaphyseal tissues of newborn rats cultured with dipicolinate, a chelator of zinc. Bone tissues were obtained at 7 and 14 days after birth, and were cultured for 48 h in a medium containing either vehicle, 10^{-3} M dipicolinate or 10^{-3} M dipicolonate plus 10^{-4} M zinc sulfate. Protein synthesis was measured as described in Fig. 2. Data are the mean ± SEM of five rats. $^{*}P < 0.01$ compared with control (vehicle) values. *White bars*, control; *hatched bars*, dipicolinate addition; *black bars*, dipicolinate plus zinc addition

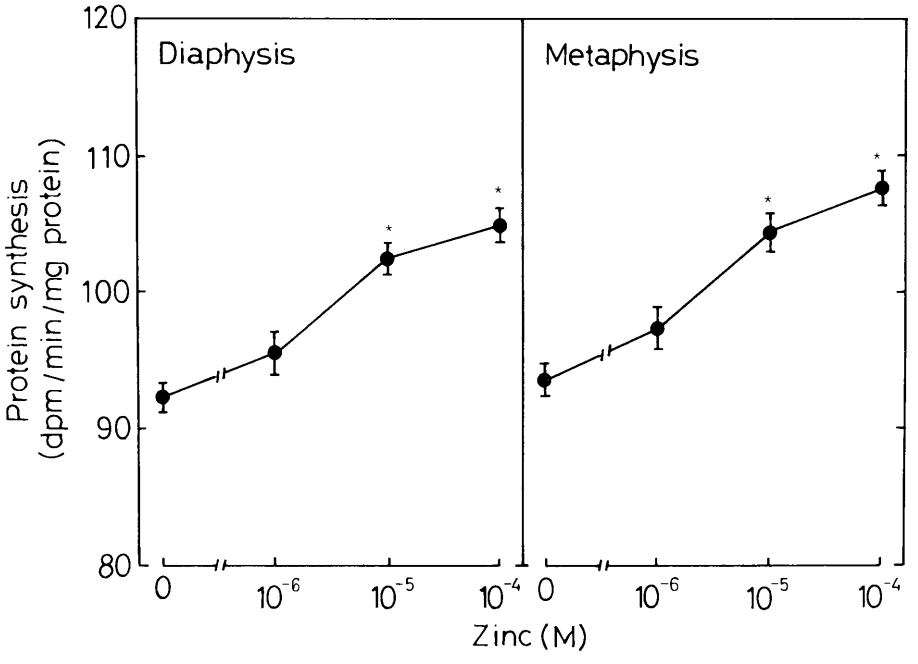

Fig. 5. Changes in in vitro protein synthesis activity in femoral–diaphyseal and metaphyseal tissues of newborn rats cultured with zinc. Bone tissues were obtained at 7 days after birth, and were cultured for 48 h in a medium containing either vehicle or zinc sulfate (10^{-6}–10^{-4} M). Protein synthesis was measured as described in Fig. 2. Data are the mean ± SEM of five rats. $^{*}P < 0.01$ compared with the control (none) value

tivity in femoral–diaphyseal and metaphyseal tissues of newborn rats, suggesting that bone formation and mineralization are associated with bone growth in the newborn rat [17]. However, changes in bone protein components with increasing age in newborn rats have not been fully clarified.

Protein molecules in femoral–diaphyseal and metaphyseal tissues of newborn rats with increasing age were characterized using SDS-PAGE. The results show that many protein molecules are present in the femoral–diaphyseal and metaphyseal tissues. There was a slight increase in protein molecules in diaphyseal tissues from 14-day-old newborn rats. Such an elevation was not seen in metaphyseal tissues. On the basis of these observations, it is assumed that constitutive protein molecules in the femoral tissues are not qualitatively altered by bone growth with increasing age in newborn rats. However, protein synthesis activity in femoral tissues was demonstrated to be enhanced by increasing age in newborn rats.

Protein synthesis activity was rapidly enhanced in femoral–diaphyseal and metaphyseal tissues until 21

Table 1. Effect of cycloheximide or actinomycin D on in vitro protein synthesis in femoral–diaphyseal and metaphyseal tissues of newborn rats cultured with zinc

Age	Treatment	Protein synthesis (d.p.m./min/mg protein)	
		Diaphysis	Metaphysis
7 days	None		
	Control	93.1 ± 2.5	94.5 ± 2.8
	Zinc	104.9 ± 2.5*	107.2 ± 2.0*
	Cyloheximide		
	Control	90.0 ± 1.8	91.2 ± 2.4
	Zinc	94.6 ± 2.3	95.1 ± 2.9
	Actinomycin D		
	Control	91.5 ± 2.5	92.0 ± 1.9
	Zinc	95.0 ± 2.3*	96.1 ± 2.8
14 days	None		
	Control	103.8 ± 2.1	102.9 ± 2.3
	Zinc	115.2 ± 2.0*	112.9 ± 2.4*
	Cyloheximide		
	Control	99.1 ± 3.2	98.4 ± 2.9
	Zinc	104.9 ± 2.0	104.3 ± 2.6
	Actinomycin D		
	Control	101.0 ± 2.4	99.5 ± 3.1
	Zinc	105.2 ± 2.3	104.6 ± 2.7

Data are the mean ± SEM of five rats. *$P < 0.01$ compared with the control (none) values. Bone tissues were obtained 7 or 14 days after birth, and were cultured for 48 h in a medium containing either vehicle or zinc sulfate (10^{-4} M) in the absence or presence of cycloheximide (10^{-6} M) or actinomycin D (10^{-7} M). Protein synthesis was measured as described in Fig. 2

days after birth, suggesting that a large amount of constitutive proteins in bone tissue is required for bone growth with increasing age in newborn rats. At 7 days after birth, protein synthesis in metaphyseal tissues was significantly elevated compared with 1 day after birth. Such an increase was not seen in diaphyseal tissues. The enhancement of protein synthesis in metaphyseal tissues may be involved in the growth of femoral length, which is stimulated by various bone growth factors.

Bone protein synthesis activity was significantly decreased by dipicolinate, a chelator of zinc ion [21,22], when it was added into the reaction mixture. Meanwhile, the addition of zinc to the mixture increased protein synthesis. These findings show that endogenous zinc in the bone tissues has a direct stimulatory effect on protein synthesis at the level of translational processes. Increasing age has been demonstrated to produce a marked increase in zinc content in femoral–diaphyseal and metaphyseal tissues obtained from 14- and 21-day-old newborn rats [17]. The increase in bone zinc content results from lactation of maternal rats fed dietary containing zinc [17]. Presumably, zinc in bone tissues is needed to enhance protein synthesis in growing bone with increasing age of newborn rats. In particular, the effect of zinc in increasing protein synthesis in femoral–metaphyseal tissues at 7 days after birth was approximately two-fold compared with that of diaphyseal tissues. It appears that zinc is needed to stimulate protein synthesis in femoral–metaphyseal tissues. The effect of zinc on protein synthesis in osteoblasts and chondrocytes of femoral–metaphyseal tissues remains to be elucidated.

When bone tissues obtained from 7- or 14-day-old newborn rats were cultured for 48 h in a medium containing dipicolinate, bone protein synthesis activity was significantly decreased. This reduction was completely restored by culture with zinc. This finding further supports the view that endogenous zinc in femoral–diaphyseal and metaphyseal tissues is required to enhance protein synthesis. In addition, culture with added zinc caused a significant increase in protein synthesis in femoral–diaphyseal and metaphyseal tissues of newborn rats.

Zinc-induced increases in bone protein synthesis were completely blocked by culture with cycloheximide, an inhibitor of protein synthesis at the level of the translational process, or actinomycin D, an inhibitor of transcription. These results suggest that zinc has a stimulatory effect on the translational and transcriptional processes of protein synthesis in the femoral–diaphyseal and metaphyseal tissues. The gene sequence for the receptor for steroid hormones that act on bone tissues has been shown to have two zinc fingers at the site of interaction with DNA [23]. In addition, zinc is a cofactor of DNA polymerase and RNA polymerase [24]. However, it is possible that zinc may stimulate bone protein synthesis through mechanisms other than the gene expression of proteins that are involved in the development of bone growth of newborn rats. This remains to be elucidated.

In conclusion, it has been demonstrated that protein synthesis in femoral–diaphyseal and metaphyseal tissues is enhanced by increasing age of newborn rats, and that endogenous zinc in bone tissues plays a stimulatory role in the enhancement of protein synthesis with bone growth.

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