Synergistic Effect of β-Cryptoxanthin and Zinc Sulfate on the Bone Component in Rat Femoral Tissues in Vitro: The Unique Anabolic Effect with Zinc

Satoshi UCHIYAMA, a Kaori ISHIYAMA, b Ken HASHIMOTO, b and Masayoshi YAMAGUCHI* a

Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka; 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan; and a Institute for Health Science, Yamada Apiculture Center, Inc.; 194 Ichiba, Kaggamino-cho, Tomata-gun, Okayama 708–0393, Japan.

Received May 24, 2005; accepted July 22, 2005

The effect of the combination of β-cryptoxanthin and zinc sulfate (zinc) on bone components in the femoral-diaphyseal and -metaphyseal tissues of young rats in vitro was investigated. Bone tissues were cultured for 48 h in a serum-free Dulbecco’s modified Eagle’s medium containing either vehicle, β-cryptoxanthin (10−9—10−7 M) or zinc sulfate (10−8—10−4 M). The presence of β-cryptoxanthin (10−9 M) or zinc (10−4 M) did not have a significant effect on calcium content in the femoral-diaphyseal or -metaphyseal tissues. However, culture which combined β-cryptoxanthin (10−7 M) and zinc (10−4 M) caused a significant increase in calcium content in the femoral-diaphyseal and -metaphyseal tissues. Such an effect was not observed by the combination of β-cryptoxanthin (10−7 M) plus genistein (10−5 M) or menaquinone-7 (10−4 M), or zinc (10−4 M) plus genistein (10−4 M) or menaquinone-7 (10−4 M). Also, the combination of β-cryptoxanthin (10−9 M) plus zinc (10−4 M) caused a remarkable increase in alkaline phosphatase activity and deoxyribonucleic acid (DNA) in the femoral-diaphyseal and -metaphyseal tissues, whereas their application alone did not have an effect on the enzyme activity or DNA content in the femoral tissues. The effect of the combination of β-cryptoxanthin (10−7 M) plus zinc (10−4 M) in increasing calcium content, alkaline phosphatase activity, and DNA content in the femoral-diaphyseal and -metaphyseal tissues was completely prevented in the presence of cycloheximide (10−3 M), an inhibitor of protein synthesis, or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DBR), an inhibitor of transcriptional activity. This study demonstrates that the combination of β-cryptoxanthin and zinc at a lower concentration has a synergistic effect on bone components in vitro.

Key words β-cryptoxanthin; zinc; bone formation; rat femur

Aging induces a decrease in bone mass. Osteoporosis with its accompanying decrease in bone mass is widely recognized as a major public health problem. The most dramatic expression of the disease is represented by fractures of the proximal femurs. A decrease in bone mass may be due to decreased bone formation and increased bone resorption. Pharmacological and nutritional factors may prevent bone loss with increasing age. Chemical compounds in food that act on bone metabolism, however, are poorly understood.

Carotenoids are present in fruit and vegetables. The effects of carotenoids on bone metabolism, however, have not been fully clarified. Of various carotenoids (including retinol, β-cryptoxanthin, lutein, β-carotene, and lycopene), β-cryptoxanthin has been found to have a unique anabolic effect on bone calcification in vitro.

β-Cryptoxanthin has been shown to stimulate osteoblastic bone formation and to inhibit osteoclastic bone resorption in vitro. Oral administration of β-cryptoxanthin may have a preventive effect on bone loss with increasing age and osteoporosis in vivo.

Zinc, an essential trace element, has been shown to have a stimulatory effect on osteoblastic bone formation and an inhibitory effect on osteoclastic bone resorption in vitro. Oral administration of zinc compounds has been demonstrated to prevent bone loss in animal models with osteoporosis in vivo.

Whether zinc can enhance the anabolic effect of β-cryptoxanthin on bone components has not yet been clarified. This study was undertaken to determine whether the combination of β-cryptoxanthin and zinc sulfate at a comparatively lower concentration reveals an anabolic effect on bone components. We found that the combination of β-cryptoxanthin and zinc sulfate has a synergistic effect in increasing bone components in bone tissue culture in vitro.

MATERIALS AND METHODS

Chemicals Dulbecco’s modified Eagle’s medium (MEM) (high glucose) and a penicillin–streptomycin solution (5000 units/mg penicillin and 5000 μg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Bovine serum albumin (BSA), genistein, cycloheximide, and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DBR), an inhibitor of transcriptional activity. All chemicals were reagent grade from Wako Pure Chemical Industries (Osaka, Japan). All water used was glass-distilled.

Animals Male Wistar rats (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 57.4% Ca and 1.1% P for 7 d at a room temperature of 25°C, and were given distilled water freely.

Bone Culture The femurs were removed aseptically after exsanguination and soaked in ice-cold 0.25 M sucrose solution. The femur was cleaned of soft tissue and marrow, and the diaphysis and metaphysis (not containing epiphyseal tissue) were separated. The femoral-diaphyseal and femoral-metaphyseal tissues were cut into small pieces. Femoral-di-
physeal or -metaphyseal fragments were cultured for 48 h in 35 mm dishes in 2.0 ml medium consisting of Dulbecco’s MEM (high glucose, 4.5 g/dl) supplemented with 0.25% BSA plus antibiotics (100 units of penicillin and 100 μg of streptomycin/ml of medium). The culture medium did not contain zinc. In experiments, bone tissues were cultured for 48 h in a medium containing either vehicle or compound (including 0.1% ethanol). Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO2 and 95% air.

Analytical Procedures The diaphyseal and metaphyseal tissues were dried for 16 h at 110°C. Calcium was determined by atomic absorption spectrophotometry. Calcium content in bone tissues is expressed as milligrams per gram of dry bone. To assay alkaline phosphatase activity, the diaphyseal and metaphyseal tissues were immersed in 3.0 ml of ice-cold 6.5 mM barbital (pH 7.4), cut into small pieces, homogenized with a physcotron homogenizer, and disrupted for 60 s with a water-saturated atmosphere containing 5% CO2 and 95% air.

Protein concentration was determined by the method of Lowry et al. To measure bone DNA content, the diaphyseal and metaphyseal tissues were shaken with 4.0 ml of ice-cold 0.1 M NaOH solution for 24 h after homogenization of the bone tissues. After alkali extraction, the samples were centrifuged at 10000 × g for 5 min, and the supernatant was collected. DNA content in the supernatant was determined using the method of Ceriotti, and expressed as the amount of DNA (mg)/g wet weight of bone tissue.

Statistical Analysis The significance of the difference between values was estimated by Student’s t-test or by analysis of variance (ANOVA) for comparing multiple groups. A p value of less than 0.05 was considered to indicate statistically significant differences.

RESULTS

The effect of β-cryptoxanthin and zinc sulfate (zinc) on calcium content in femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues of rats in vitro is shown in Fig. 1. Bone tissues were cultured for 48 h in a serum-free medium containing either vehicle, β-cryptoxanthin (10⁻⁵—10⁻⁷ M) or zinc sulfate (10⁻⁶—10⁻⁸ M). The presence of β-cryptoxanthin (10⁻⁷ M) or zinc (10⁻⁴ M) caused a significant increase in calcium content in the femoral-diaphyseal and -metaphyseal tissues. Culture with zinc (10⁻⁵ M) caused a significant increase in calcium content in the metaphyseal tissues but not diaphyseal tissues. β-Cryptoxanthin (10⁻⁸ or 10⁻⁷ M) or zinc (10⁻⁵ M) did not have a significant effect on the diaphyseal or metaphyseal calcium content. The combination of β-cryptoxanthin (10⁻⁷ M) and zinc (10⁻⁵ M), while their concentration did not have a significant effect on bone calcium content, caused a remarkable elevation in calcium content in the femoral-diaphyseal and -metaphyseal tissues. The synergistic effect with the two compounds on bone calcium content was also observed in the combination of β-cryptoxanthin (10⁻⁸ M) and zinc (10⁻⁷ M).

The effect of combination with β-cryptoxanthin and other factors on calcium content in the femoral-diaphyseal and -metaphyseal tissues of rats in vitro is shown in Table 1. Bone calcium content was not significantly changed by culture with β-cryptoxanthin (10⁻⁹ M), zinc (10⁻⁶ M), genistein (10⁻⁶ M), or menaquinone-7 (MK-7; 10⁻⁸ M) for 48 h. The combinations of genistein (10⁻⁶ M) plus zinc (10⁻⁶ M), MK-7 (10⁻⁶ M) plus zinc (10⁻⁶ M), β-cryptoxanthin (10⁻⁹ M) plus genistein (10⁻⁶ M), or β-cryptoxanthin (10⁻⁹ M) plus MK-7 (10⁻⁶ M) did not cause a significant change in bone calcium content. Thus, the combination of β-cryptoxanthin (10⁻⁹ M) plus zinc (10⁻⁶ M) revealed the most effective synergistic effect on bone calcium content. The effect of β-cryptoxanthin (10⁻⁹ M) plus zinc (10⁻⁶ M)
in increasing the calcium content in femoral-diaphyseal and -metaphyseal tissues in 
vitro was completely prevented in the presence of cycloheximide (10^{-6} M) or DRB (10^{-6} M) (Fig. 2).

The combination of β-cryptoxanthin (10^{-9} M) plus zinc (10^{-6} M) caused a remarkable increase in alkaline phosphatase activity (Fig. 3) or DNA content (Fig. 4) in femoral-diaphyseal and -metaphyseal tissue in 
vitro. β-Cryptoxanthin (10^{-8} M) or zinc (10^{-7} M) alone did not have a significant effect on alkaline phosphatase activity or DNA content in femoral tissues. The synergistic effect of β-cryptoxanthin (10^{-9} M) plus zinc (10^{-6} M) in increasing alkaline phosphatase activity (Fig. 3) and DNA content (Fig. 4) in femoral-diaphyseal and -metaphyseal tissues was completely prevented in the presence of cycloheximide (10^{-8} M) or DRB (10^{-6} M).

**DISCUSSION**

Bone loss with increasing age induces osteoporosis, which is widely recognized as a major public health problem.1–3 Food factors may play a role in the prevention of bone loss with aging. Carotenoids are present in large quantities in food and plants. Of the various carotenoids, β-cryptoxanthin had a unique stimulatory effect on calcium content and alkaline phosphatase activity in rat femoral-diaphyseal (cortical bone) and -metaphyseal (trabecular bone) tissues in 
vitro.6 Meanwhile, zinc has a potent stimulatory effect on bone formation among various trace metals.25 It was found that the combination of β-cryptoxanthin and zinc of a comparatively lower concentration, at which their concentration did not have a significant effect on bone components, induces a remarkable elevation of calcium content, alkaline phosphatase activity, and DNA content in rat femoral-diaphyseal and -metaphyseal tissues in 
vitro. The combination of β-cryptoxanthin and zinc had a potent anabolic effect on bone components in the cortical bone and trabecular bone. Trabecular bone is easily resorbed by various bone-resorbing factors. This finding suggests that the combination of β-cryptoxanthin and zinc has potentiality in the prevention of bone loss with aging.

The synergistic effect of nutritional factors on bone components was not observed in the combination of β-cryptoxanthin and other food factors (including genistein or menaquinone-7) which have an anabolic effect on bone metabolism.26,29,30 Genistein or menaquinone-7 has a stimulatory effect on osteoblastic bone formation. The synergistic effect was also not seen in the combination of zinc plus genistein or menaquinone-7, with their lower concentrations in femoral tissues in 
vitro. Thus, the synergistic effect with the combination of β-cryptoxanthin and zinc at a lower concentration on bone calcium content was unique.

The effect of the combination of β-cryptoxanthin and zinc in increasing the calcium content, alkaline phosphatase activity, and DNA content in rat femoral tissues was completely prevented in the presence of cycloheximide, an inhibitor of protein synthesis, or DRB, an inhibitor of transcriptional activity. Alkaline phosphatase is an enzyme marker of osteoblasts, and the enzyme participates in bone mineralization.22 The DNA content in bone tissues is an index of the number of bone cells.31 The present results suggest that the synergistic effect of the combination of β-cryptoxanthin and zinc on bone components requires newly synthesized protein components.

β-Cryptoxanthin has been suggested to have an effect on nuclear factors to stimulate transcriptional activity in os-
teoblastic cells.8,9) Meanwhile, zinc has been demonstrated to activate aminoacyl-tRNA synthase, a rate limiting enzyme of protein synthesis of the translational process, in osteoblastic cells.34) It is speculated that the combination of β-cryptoxanthin and zinc has a stimulatory effect on both gene expression and protein synthesis in osteoblastic cells. This may induce a synergistic effect on bone components in bone tissues.

Chemical factors in food may help to prevent bone loss with aging. Supplemental intake of a combination of β-cryptoxanthin and zinc may be useful as a tool in the prevention of osteoporosis. This remains to be elucidated using an animal model for osteoporosis.

In conclusion, it has been demonstrated, for the first time to the best of our knowledge, that the combination of β-cryptoxanthin and zinc with lower concentrations has a synergistic-anabolic effect on bone components in the femoral-diaphyseal and -metaphyseal tissues of rats in vitro.

REFERENCES