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Stimulatory effect of menaquinone-7 (vitamin K_2) on osteoblastic bone formation *in vitro*

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Abstract

Menaquinone-7, which is vitamin K_2 (menatetrenone) with seven isoprene units, is highly contained in the fermented soybean. The effect of menaquinone-7 (MK-7) on osteoblastic bone formation was investigated. Femoral-diaphyseal and metaphyseal tissues of young male rats (4 weeks old) were cultured for 48 h in a medium containing either vehicle or MK-7 (10^{-7}–10^{-5} M). Calcium content, alkaline phosphatase activity, and deoxyribonuclic acid (DNA) content in the diaphyseal and metaphyseal tissues was significantly increased in the presence of MK-7 (10^{-6} and 10^{-5} M). The effect of MK-7 in increasing the diaphyseal and metaphyseal calcium content and alkaline phosphatase activity was completely prevented in the presence of cycloheximide (10^{-6} M), an inhibitor of protein synthesis. Moreover, osteoblastic MC3T3-E1 cells after subculture were cultured for 24 h in a serum-free medium containing MK-7 (10^{-7}–10^{-5} M). Protein content, alkaline phophatase activity, osteocalcin and DNA content in the cells was significantly increased in the presence of MK-7 (10^{-6} and 10^{-5} M). The effect of MK-7 in increasing protein content, alkaline phosphatase activity, and osteocalcin production in the cells was completely blocked by cycloheximide. This study demonstrates that MK-7 has an anabolic effect on bone tissue and osteoblastic MC3T3-E1 cells *in vitro*, suggesting that the compound can stimulate osteoblastic bone formation. (Mol Cell Biochem **223**: 131–137, 2001)

Key words: menaquinone-7, vitamin K_2, osteoblast, bone formation

Introduction

Bone mass decreases with increasing age. Pharmacological and nutritional factors are needed to prevent bone loss with increasing age. There is growing evidence that vitamin K, which is a nutritional factor, may play a role in the regulation of bone metabolism. Vitamin K_2 (menatetrenone, MK-4) is essential for the γ-carboxylation of osteocalcin, a calcified tissue protein containing γ_2-carboxyglutamic acids that is synthesized in osteoblasts of bone tissue [1, 2]. Noncarboxylated osteocalcin cannot bind to hydroxyapatite in mineralized tissues [2, 3]. Much attention has been paid to the role of vitamin K in bone metabolism, because it supplementation may be important as a therapeutic tool for osteoporosis.

There are two types of vitamin K: vitamin K_1 and vitamin K_2. Vitamin K_1 is a single compound, but vitamin K_2 is a series of vitamers with multiisoprene units (1–4) at the 3-position of the naphthoquinone. Several reports have indicated the effects of vitamin K_1 on bone metabolism [4, 5]. In contrast, the effect of vitamin K_2 on bone metabolism has not attracted notice. Like vitamin K_1, vitamin K_2 (menatetrenone), with four isoprene units, not only enhances mineralization but also increases the amount of osteocalcin in cultured human osteoblasts [6]. Moreover, it has been reported that menatetrenone inhibits bone resorption, which may be released to its side chain [7], and that the compound inhibits

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bone loss in rat induced by ovariectomy [8]. However, the effect of menaquinone-7 (MK-7), with seven isoprene units, on bone metabolism has not been fully clarified.

It has been recently reported that menaquinone-7 (MK-7) can directly stimulate calcification in the femoral-metaphyseal tissues obtained from normal rats *in vitro* [9, 10]. The action of MK-7 on bone calcification has been shown to have same effect as menaquinone-4 (MK-4) [10]. Natural MK-7 is highly contained in the fermented soybean [10]. The intake of dietary MK-7 has been shown to prevent ovariectomy-induced bone loss in rats [11, 12], suggesting a role in the prevention of osteoporosis [13, 14].

The cellular mechanism by which MK-7 has an anabolic effect on bone metabolism, however, is unknown. The present study, therefore, was undertaken to determine whether MK-7 reveals a stimulatory effect on osteoblastic cells *in vitro*. We found that MK-7 can directly stimulate osteoblastic bone formation.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium (high glucose, 4.5 g/dl) and a penicillin-streptomycin solution (5000 units/ml penicillin; 5000 µg/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY, USA). α-Modification of Eagle's Minimal Essential Medium (α-MEM) was obtained from Flow Laboratories, Inc. (Mclean, VA, USA). Fetal bovine serum (FBS), bovine serum albumin (fraction V), and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Menaquinone-7 (vitamin K_2; 99.8% purity) was supplied by Honen Corporation (Tokyo, Japan), which was highly purified from the fermented soybean, and dissolved in ethanol solution. Other chemicals were reagent grade from Wako Pure Chemicals Industries (Osaka, Japan). Tissue culture plastic dishes were purchased from Falcon Plastics (Los Angeles, CA, USA). Other materials used were commercial products of the highest grade available.

Bone tissue culture

Male Wistar rats (4 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). Animals were fed commercial laboratory chow (solid) containing 57.4% carbohydrate, 1.1% calcium and phosphorus at a room temperature of 25°C and were given distilled water freely. Rats were bled by cardiac puncture under light anesthesia with ether. The femurs were removed aseptically after bleeding and 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 washing of metaphyseal tissues. The femoral-diaphyseal and metaphyseal tissues were cut into small pieces. Bone tissues were cultured for 48 h in a 35 mm dish in 2.0 ml mediun 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 µg streptomycin/ml of medium) [15]. Experimental medium contained either vehicle (1% ethanol) or menaquinone-7 (10^{-7}–10^{-5} M). This medium did not contain essential metals. Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air.

Determination of bone components

To assay alkaline phosphatase activity, the diaphyseal and metaphyseal tissues were immersed in 3.0 ml ice-cold 6.5 mM barbital buffer (pH 7.4), cut into small pieces, homogenized with a physcotron homogenizer, and disrupted for 60 sec with an ultrasonic device. The supernatant centrifuged at 600 × g for 5 min was used to measure enzyme activity. Enzyme assay was carried under optimal condition. Alkaline phosphatase activity was determined by the method of Walter and Schutt [16]. Enzyme activity was expressed as µmol of *p*-nitrophenol liberated/min/mg protein. Protein concentration was determined by the method of Lowry *et al.* [17].

To measure bone DNA content, the bone tissues were shaken with 4.0 ml ice-cold 0.1 N NaOH solution for 24 h after the homogenization of bone tissue [18]. After alkali extraction, the samples were centrifuged at 10,000 × g for 5 min, and the supernatant was collected. DNA content in the supernatant was collected. DNA content in the supernatant was determined by the method of Ceriotti [19] and expressed as the amount of DNA (mg)/g wet weight of bone tissue.

The diaphyseal or metaphyseal tissues were dried for 24 h at 110°C, weighted, and then digested in HNO_3 (2.0 ml) for 24 h at 120°C. Calcium was determined by atomic absorption spectrophotometry [15]. Calcium content was expressed as mg/g dry bone.

Cell culture

Osteoblastic MC3T3-E1 cells were cultured at 37°C in a CO_2 incubator in plastic dishes containing α-MEM supplemented with 10% FBS [20]. They were subcultured every 3 days using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS. For experiments, about 1×10^4 cells per dish were cultured for 3 days to obtain confluent monolayers in 35-mm plastic dishes containing 2 ml α-MEM with 10% FBS. After the cells were rinsed with PBS, the medium was exchanged for medium containing 0.1% BSA plus menaquinone-7 (10^{-7}–10^{-5}