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Inhibitory effect of menaquinone-7 (vitamin K_2) on the bone-resorbing factors-induced bone resorption in elderly female rat femoral tissues *in vitro*

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Abstract

The inhibitory effect of menaquinone-7 (MK-7; vitamin K_2) on osteoclast-like cell formation and osteoclastic bone resorption *in vitro* is found (Mol Cell Biochem 228: 39–47, 2001). This study, furthermore, was undertaken to determine the effect of MK-7 on the bone-resorbing factor-induced bone resorption using the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats *in vitro*. Femoral-diaphyseal and -metaphyseal tissues were cultured for 48 h in Dulbecco's modified Eagle's medium (high glucose, 4.5%) supplemented with antibiotics and bovine serum albumin. The experimental cultures contained MK-7 (10^{-7}–10^{-5} M). The bone-resorbing factors, parathyroid hormone (1–34) (PTH; 10^{-7} M) and prostaglandin E_2 (PGE_2; 10^{-5} M), caused a significant decrease in calcium content in the diaphyseal and metaphyseal tissues. The PTH or PGE_2-induced decrease in bone calcium content was completely inhibited in the presence of MK-7 (10^{-7}–10^{-5} M). In addition, MK-7 (10^{-7}–10^{-5} M) completely prevented the PTH (10^{-7} M)- or PGE_2 (10^{-5} M)-induced increase in medium glucose consumption and lactic acid production by bone tissues. These results support the view that MK-7 has a direct inhibitory effect on the bone-resorbing factor-induced bone resorption in bone culture using female aged femoral tissues *in vitro*. (Mol Cell Biochem **245**: 115–120, 2003)

Key words: menaquinone-7 (MK-7), vitamin K_2, bone resorption, aging, osteoporosis

Introduction

Bone loss with increasing age induces osteoporosis [1–3]. This loss may be due to induced bone resorption and decreased bone formation. A decrease in bone mass leads to bone fracture. Osteoporosis is widely recognized as a major public health problem [4]. Postmenopausal osteoporosis is resulted from estrogen deficiency. This is partly involved in the deterioration of bone metabolism with increasing age. Pharmacological and nutritional factors are important in preventing age-related bone loss.

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 (menaquinone) is essential for the γ-carboxylation of osteocalcin, a bone matrix protein containing γ-carboxyglutamic acids, which is synthesized in osteoblast of bone tissues [5–7]. MK-7 with seven isoprene units, one of analog of vitamin K_2, is abundant in fermented soybean (*natto*) [8]. It was recently demonstrated that the prolonged dietary intake of MK-7 has a preventive effect on bone loss induced by ovariectomy in rats [9, 10]. Moreover, it has been reported that the dietary intake MK-7 may enhance γ-carboxylation of osteocalcin in the serum of normal individuals [11, 12]. These observations support the view that dietary MK-7 may have a useful role in the prevention of osteoporosis on the basis of the direct promotion of γ-carboxylation of osteocalcin, which is important in the promotion of bone calcification [6, 7].

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Whether MK-7 has a direct anabolic effect on bone metabolism *in vitro* has not been fully clarified, however. It has been recently demonstrated that MK-7 has an anabolic effect on osteoblastic MC3T3-E1 cells and bone tissues *in vitro*, suggesting that the compound can stimulate osteoblastic bone formation [13]. Moreover, MK-7 has an inhibitory effect on osteoclast-like cell formation in bone marrow culture system *in vitro* and a suppressive effect on osteoclasts isolated from rat femoral tissues *in vitro* [14], suggesting that MK-7 can inhibit osteoclastic bone resorption *in vitro*.

The present study, furthermore, was undertaken to determine whether MK-7 can inhibit the bone-resorbing factors-induced bone resorption using the femoral-diaphyseal and -metaphyseal tissues obtained from female aged rats, which bone metabolism was deteriorated. We found that MK-7 could inhibit the PTH or PGE_2-induced bone resorption in bone culture of female aged rat femoral tissues *in vitro*, suggesting that MK-7 can inhibit bone resorption with increasing age.

Materials and methods

Chemicals

Dulbecco's modified Eagle's medium and penicillin-streptomycin solution (5000 units/ml penicillin; 5000 µg/ml streptomycin) were obtained from Gibco laboratories (Grand island, NY, USA). Bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). MK-7 (vitamin K_2; 96.8% purity) was supplied by Honen Corporation (Tokyo, Japan), which was highly purified from the fermented soybean (*natto*). MK-7 was dissolved in ethanol solution (20%). PGE_2 and synthetic human parathyroid hormone were purchased from Sigma. [PTH (1–34)]. Calcium chloride and other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan). All water used was glass-distilled.

Animals

Female Wistar rats (conventional), weighing 90–100 g (4 weeks old) or 220–250 g (50 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium and 1.1% phosphorus at room temperature of 25°C, with free access to distilled water.

Bone culture

Bone culture was carried out with the procedure as reported previously [15]. Femoral-diaphyseal and -metaphyseal tissues from 4- or 50-week-old female rats were removed aseptically. These tissues were then cultured in a 35-mm dish in 2.0 ml medium consisting of Dulbecco's modified Eagle's medium (high glucose; 4.5%) supplemented with 0.25% bovine serum albumin (fraction V) plus antibiotics, with either bone-resorbing factors (PTH or PGE_2) or vehicle (sterile distilled water) in the absence or presence of MK-7 (10^{-7}–10^{-5} M). Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air for 48 h.

Bone calcium

The bone tissues were dried for 16 h at 120°C, weighed, and then dissolved in nitric acid solution [15]. Calcium was determined by atomic absorption spectrophotometry [16]. The bone calcium content was expressed as milligrams of calcium per gram of dry bone.

Determination of medium glucose and lactic acid

The concentration of glucose in the medium cultured with bone for 48 h was determined by the colorimetric method using *o*-toludine [17]. Dry weight of the bone tissue was measured after extraction with 5.0% trichloroacetic acid, acetone, and ether. The medium glucose consumed by bone culture in 48 h was expressed as milligrams of glucose per gram of dry bone tissue. Likewise, the medium lactic acid was measured by the enzymatic method previously described [18]. Data were expressed as milligrams of lactic acid per gram of dry bone tissue.

Statistical methods

Data are expressed as means ± S.E.M. Statistical differences were analyzed using Student's paired *t*-test; p values of less than 0.05 were considered to indicate statistically significant difference.

Results

Change in calcium content, glucose consumption and lactic acid production in bone tissues with aging

Femoral-diaphyseal and -metaphyseal tissues obtained from young (4 weeks old) or elderly (50 weeks old) female rats were cultured for 48 h in a serum-free medium without MK-7. Bone calcium content was significantly decreased in the femoral-diaphyseal and -metaphyseal tissues from elderly rats as compared with those of young rats (Fig. 1). Medium

glucose consumption and lactic acid production by the diaphyseal and metaphyseal tissues were significantly lowered in the bone tissues from elderly rats as compared with those of young rats (Fig. 1). Increasing age caused a significant decrease in calcium content, glucose consumption, and lactic acid consumption in the femoral tissues of female rats.

Effect of MK-7 on the bone-resorbing factor-decreased bone calcium content in vitro

The effect of MK-7 on the bone-resorbing factors-induced decrease in calcium content in the femoral tissues obtained from elderly female rats was examined *in vitro*. Femoral-diaphyseal or -metaphyseal tissues were cultured for 48 h in a medium containing either vehicle or PTH (10^{-7} M), or PGE_2 (10^{-5} M) in the absence or presence of MK-7 (10^{-7}–10^{-5} M), as shown in Figs 2 and 3, respectively. Diaphyseal and metaphyseal calcium content was significantly decreased in the presence of PTH (Fig. 2) or PGE_2 (Fig. 3). These decreases were completely prevented by MK-7 (10^{-7}–10^{-5} M), as shown in Figs 2 and 3, respectively.

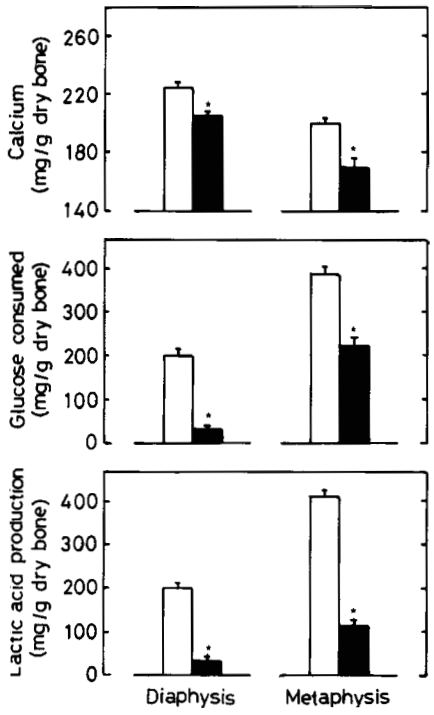

Fig. 1. Change in calcium content, glucose consumption and lactic acid production in the femoral-diaphyseal and -metaphyseal tissues of elderly female rats. Bone tissues obtained from young (4 weeks old) or elderly (50 weeks old) rats were cultured for 48 h in a serum-free medium without MK-7. Each value is the mean ± S.E.M. of 6 rats. *$p < 0.01$, compared with the value for young rats. White bars, young rats; black bars, elderly rats.

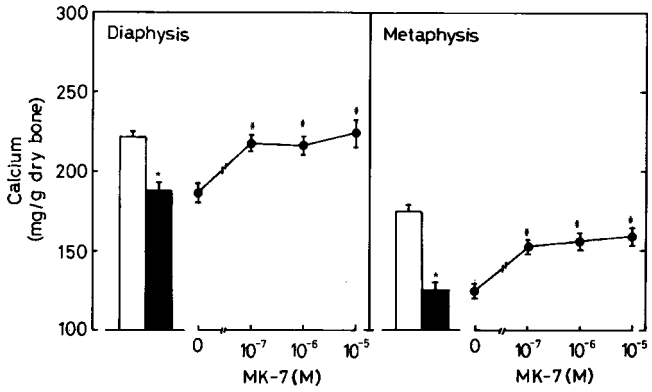

Fig. 2. Effect of MK-7 on PTH-decreased bone calcium content in the femoral-diaphyseal and -metaphyseal tissues of elderly female rats *in vitro*. Femoral-diaphyseal and -metaphyseal tissues obtained from elderly (50 weeks old) rats were cultured for 48 h in a medium containing either vehicle or PTH (10^{-7} M) in the absence or presence of MK-7 (10^{-7}–10^{-5} M). Each value is the mean ± S.E.M. of 6 rats. *$p < 0.01$, compared with the control (none) value. #$p < 0.01$, compared with the value for PTH alone. White bars, control (none); black bars, PTH alone.

Effect of MK-7 on the bone-resorbing factor-stimulated glucose consumption by bone tissues in vitro

The effect of MK-7 on the PTH or PGE_2-induced stimulation of medium glucose consumption in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats is shown in Figs 4 and 5. The presence of PTH (10^{-7} M) or PGE_2 (10^{-5} M) caused a significant increase in medium glucose consumption by the femoral-diaphyseal or -metaphy-

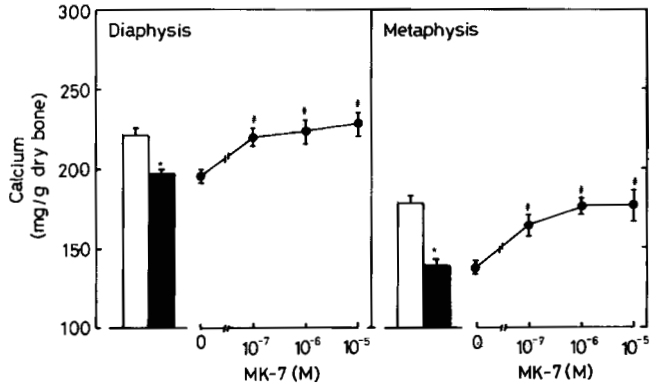

Fig. 3. Effect of MK-7 on PGE_2-decreased bone calcium content in the femoral-diaphyseal and -metaphyseal tissues of elderly female rats *in vitro*. Femoral-diaphyseal or -metaphyseal tissues obtained from elderly (50 weeks old) rats were cultured for 48 h in a medium containing either vehicle or PGE_2 (10^{-5} M) in the absence or presence of MK-7 (10^{-7}–10^{-5} M). Each value is the mean ± S.E.M. of 6 rats. *$p < 0.01$, compared with the control (none) value. #$p < 0.01$, compared with the value for PGE_2 alone. White bars, control (none); black bars, PGE_2 alone.

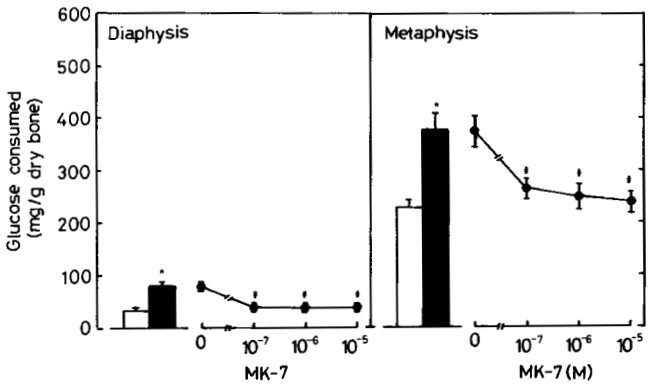

Fig. 4. Effect of MK-7 on PTH-stimulated glucose consumption by the femoral-diaphyseal and -metaphyseal tissues of elderly female rats *in vitro*. Femoral-diaphyseal and -metaphyseal tissues obtained from elderly (50 weeks old) rats were cultured for 48 h in a medium containing either vehicle or PTH (10^{-7} M) in the absence or presence of MK-7 (10^{-7}–10^{-5} M). Each value is the mean ± S.E.M. of 6 rats. *$p < 0.01$, compared with the control (none) value. #$p < 0.01$, compared with the value for PTH alone. White bars, control (none); black bars, PTH alone.

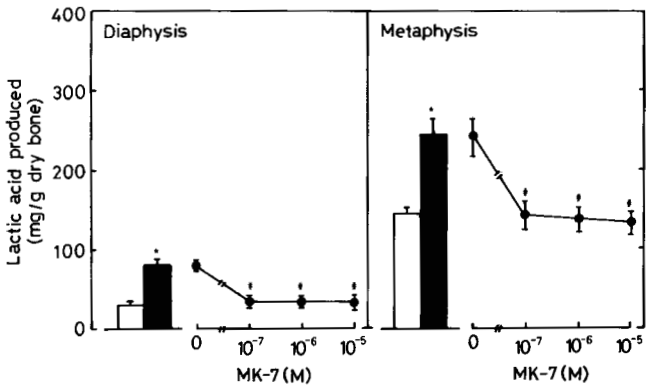

Fig. 6. Effect of MK-7 on PTH-stimulated lactic acid production by the femoral-diaphyseal and -metaphyseal tissues of elderly female rats in *vitro*. Femoral-diaphyseal and -metaphyseal tissues obtained from elderly (50 weeks old) rats were cultured for 48 h in a medium containing either vehicle or PTH (10^{-7} M) in the absence or presence of MK-7 (10^{-7}–10^{-5} M). Each value is the mean ± S.E.M. of 6 rats. *$p < 0.01$, compared with the control (none) value. #$p < 0.01$, compared with the value for PTH alone. White bars, control (none); black bars, PTH alone.

seal tissues, when the bone tissues were cultured for 48 h. These increases were completely prevented in the presence of MK-7 (10^{-7}–10^{-5} M).

Effect of MK-7 on the bone-resorbing factor-stimulated lactic acid production by bone tissues in vitro

The effect of MK-7 on the PTH- or PGE_2-induced increase in lactic acid production in the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats is shown in Figs 6 and 7. Bone tissues were cultured for 48 h. The production of lactic acid by the femoral-diaphyseal or -metaphyseal tissues was significantly increased in the presence of PTH (10^{-7} M) or PGE_2 (10^{-5} M), as shown in Figs 6 and 7, respectively. PTH- or PGE_2-induced increase in bone lactic acid production was completely prevented in the presence of MK-7 (10^{-7}–10^{-5} M) (Figs 6 and 7).

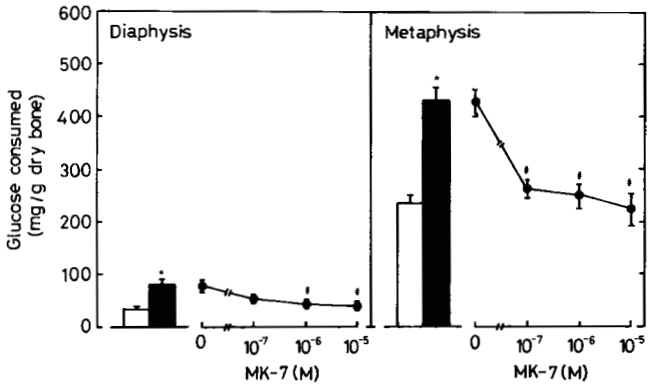

Fig. 5. Effect of MK-7 on PGE_2-stimulated glucose consumption by the femoral-diaphyseal and -metaphyseal tissues of elderly female rats *in vitro*. Femoral-diaphyseal and -metaphyseal tissues obtained from elderly (50 weeks old) rats were cultured for 48 h in a medium containing either vehicle or PGE_2 (10^{-5} M) in the absence or presence of MK-7 (10^{-7}–10^{-5} M). Each value is the mean ± S.E.M. of 6 rats. *$p < 0.01$, compared with the control (none) value. #$p < 0.01$, compared with the value for PGE_2 alone. White bars, control (none); black bars, PGE_2 alone.

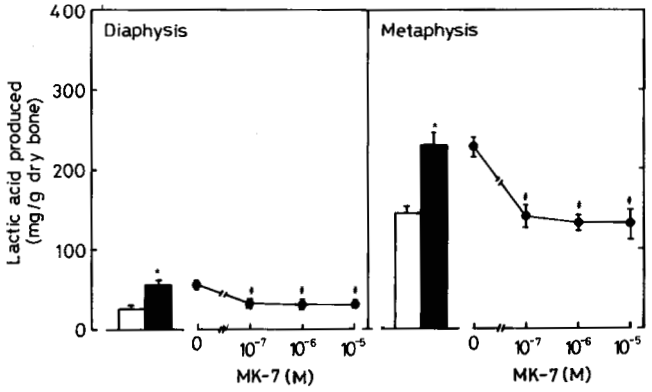

Fig. 7. Effect of MK-7 on PGE_2-stimulated lactic acid production by the femoral-diaphyseal and -metaphyseal tissues of elderly female rats *in vitro*. Femoral-diaphyseal and -metaphyseal tissues obtained from elderly (50 weeks old) rats were cultured for 48 h in a medium containing either vehicle or PGE_2 (10^{-5} M) in the absence or presence of MK-7 (10^{-7}–10^{-5} M). Each value is the mean ± S.E.M. of 6 rats. *$p < 0.01$, compared with the control (none) value. #$p < 0.01$, compared with the value for PTH alone. White bars, control (none); black bars, PTH alone.

Discussion

Nutritional and pharmacological factors can help to prevent bone loss with aging. Much attention has been paid to the role of vitamin K in bone metabolism, because its supplementation may be important as a therapeutic tool for osteoporosis [19–21]. 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 vitamins 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 [19, 20]. The effect of vitamin K_2 on bone metabolism has not attracted notice, however. Vitamin K_2 (menaquinone-4) has been shown to stimulate bone mineralization [22] and to inhibit bone resorption [23]. Natural menaquinone-7 (MK-7) is highly contained in the fermented soybean [24], suggesting a role of nutritional factor in preventing bone loss with aging. The anabolic effect of MK-7 on bone metabolism in aged rats *in vitro* has not been clarified so far.

PTH and PGE_2 are physiological agonists which stimulate bone cells to resorb bone, a process by which the mineralized extracellular bone matrix is dissolved. Physiological bone resorption has a key role in the maintenance of plasma calcium levels [25]. The present study clearly demonstrates that MK-7 can directly inhibit the bone-resorbing factors (PTH or PGE_2)-induced bone resorption using the femoral-diaphyseal and -metaphyseal tissues obtained from female elderly rats *in vitro*. PTH or PGE_2, with an effective concentration in inhibiting bone resorption [26, 27], markedly stimulated medium glucose consumption and lactic acid production by femoral-diaphyseal and -metaphyseal tissues *in vitro*. The promotion of bone resorption by PTH stimulation is partly related to release of lactic acid from osteoclastic cells [28]. MK-7 was found to prevent completely the PTH- or PGE_2-induced increase in medium glucose consumption and lactic acid production by femoral-diaphyseal and -metaphyseal tissues *in vitro*. This finding further supports the view that MK-7 has a potent effect on bone resorption in bone culture using young rat femoral tissues *in vitro*. MK-7 has been shown to inhibit the formation of osteoclast-like cells from marrow cells [14]. Moreover, the present results suggest that MK-7 can suppress the function of osteoclastic cells in aged rat femoral tissues. It is speculated that MK-7 acts on the activity of enzymes which are related to metabolic pathway from glucose consumption to lactic acid production in osteoclastic cells of aged rat femoral tissues.

The bone-resorbing factors (PTH and PGE_2)-induced decrease in calcium content in the femoral-diaphyseal and -metaphyseal tissues *in vitro* was completely blocked in the presence of MK-7. The preventive effect of MK-7 may be partly resulted from the inhibition of the PTH- or PGE_2-stimulated medium glucose consumption and lactic acid production by bone tissues *in vitro*. PTH can stimulate extracellular release of lactic acid from osteoclastic cells. The PTH-increased lactic acid production is partly based on the consumption of glucose by osteoclastic cells. This acid production is important as a cellular mechanism of bone resorption [28].

Previous studies have shown that vitamin K_2 inhibits osteoclastic bone resorption *in vitro* by targeting osteoclasts to undergo apoptosis [29]. This same mechanism of action of vitamin K_2 has also been reported *in vivo* [30]. The vitamin K_2 analog menaterenone, also able to inhibit bone resorption, has been shown to act in part by inhibiting PGE_2 synthesis *in vitro* [31]. Further investigation into the specific mechanism of action of MK-7 is required to evaluate the change in apoptotic activity or PGE_2 synthesis in the MK-7-treated bone tissue *in vitro.*

Increasing age caused a significant decrease in calcium content, glucose consumption and lactic acid production in the femoral-diaphyseal and -metaphyseal tissues, indicating that aging induces a deterioration of bone metabolism. Presumably, aging leads to decrease in cell number and their function of osteoblasts and osteoclasts. Nevertheless, the bone-resorbing factors (PTH and PGE_2) could induce the augmentation of glucose consumption and lactic acid production by femoral tissues obtained from elderly rats. The bone resorbing factor's effect was completely blocked by MK-7. The compound may be able to prevent the development of bone loss with increasing age. The present study with the femoral tissues of female elderly rats may support a role of dietary MK-7 in preventing postmenopausal osteoporosis with aging. Further study, however, is needed to determine whether MK-7 as a supplementation can interfere with the aging-induced bone resorption *in vivo.*

In conclusion, it has been demonstrated that MK-7 has an inhibitory effect on the bone-resorbing factors-induced decrease in bone calcium content and increase in glucose consumption and lactic acid production by the femoral-diaphyseal and -metaphyseal tissues obtained from elderly female rats *in vitro*, suggesting that MK-7 can inhibit bone resorption in bone tissues with increasing aging.

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