Effect of Low Dose Vitamin K2 (MK-4) Supplementation on Bio-Indices in Postmenopausal Japanese Women

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Summary It has been reported that treatment with a pharmacological dose (45 mg/d) of menaquinone-4 (MK-4) prevents bone loss in postmenopausal women. However, it is not known whether supplementation with low dose MK-4 has beneficial effects on bone metabolism in healthy women. The aim of this study is to examine the effects of the supplementation of 1.5 mg/d MK-4 for 4 wk on bone and lipid metabolism in healthy postmenopausal Japanese women. The study was performed as a randomized double blind placebo-controlled trial. The participants aged 53–65 y were randomly assigned to 2 groups and supplemented with 1.5 mg/d of MK-4 or a placebo for 4 wk (n=20 for each group). The most marked effects of MK-4 intake were observed on serum osteocalcin (OC) concentrations. Serum undercarboxylated OC (ucOC) concentration decreased, and the γ-carboxylated OC (GlaOC) and GlaOC/GlaOC+ucOC ratio that indicates the degree of OC γ-carboxylation increased significantly at 2 and 4 wk compared with that at baseline in the MK-4 group. The serum ucOC and GlaOC concentrations in the MK-4 group were significantly different from those in the placebo group at 2 wk. These results suggest that supplementation with 1.5 mg/d MK-4 accelerated the degree of OC γ-carboxylation. The concentrations of serum lipids and other indices were not different between the groups at either intervention period. Thus, the additional intake of MK-4 might be beneficial in the maintenance of bone health in postmenopausal Japanese women.

Key Words menaquinone-4, osteocalcin, bone metabolism, postmenopausal women, serum lipids

It is well-known that vitamin K is responsible for the γ-carboxylation of peptide-bound glutamate (Glu) residues to produce γ-carboxyglutamate (Gla) in target proteins that are synthesized in a limited number of tissues such as liver, bone and vascular tissues (*1*, *2*).

Dietary vitamin K exists in 2 major forms: phylloquinone (PK) and menaquinones (MKs). PK exists in green and leafy vegetables, whereas MK is either produced by bacteria or is found in animals. There is also evidence that MK-4 is synthesized either from dietary PK or menadione in certain animal tissues (*3*, *4*).

The adequate intake (AI) of vitamin K for women aged 30 y and more was set at 65 μg/d in the Dietary Reference Intakes (DRIs) for Japanese, 2005 (*5*). However, it has been suggested that the amount of vitamin K required to maintain bone health is higher than that required for blood coagulation (*6*). Furthermore, it has been reported that higher circulating concentrations of vitamin K are required for preventing osteoporosis in elderly people (*7*, *8*). Based on these evidences, intervention studies with fermented soy food or supplementation studies with MK-7 have been performed in healthy people (*9*, *10*).

Few studies on the effect of MK-4 supplementation on bone metabolism in healthy postmenopausal women are currently available. To date, these studies are the only published trials that have assessed the effects of MK-4 on postmenopausal bone loss using super dietary daily doses of 45 mg/d MK-4 (*11–14*). In women with kidney disease, the daily coadministration of 15 mg of MK-4 with glucocorticoid significantly reduced bone loss at the femoral neck compared with that in the placebo group (*15*). However, it is not well-known whether supplementation of low doses of MK-4 benefits bone health in healthy postmenopausal women at a high risk for bone loss. Furthermore, there is little evidence concerning the health effects of MK-4 supplementation other than bone metabolism in postmenopausal women.

Thus, the aim of the present study is to examine the effects of an additional daily intake of 1.5 mg MK-4 for 4 wk on various markers of bone turnover and other bio-indices in postmenopausal Japanese women.

MATERIALS AND METHODS

Subjects. Fifty-four postmenopausal women aged

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53–65 y (mean age, 59.5±3.4 y) were recruited from Shinjuku wards through advertisements in local newspapers. Forty subjects were selected using exclusion criteria and participated in the study till its completion (4 wk). None of the subjects took dietary supplements other than those prescribed by the study.

The exclusion criteria were as follows: a history of metabolic bone diseases, cancer, ovariectomy, or hysterectomy or daily intake of vitamin K-containing vitamin concentrates.

The protocol was approved by the institutional review board of the National Institute of Health and Nutrition, Japan, and the study was carried out according to the guidelines of the Declaration of Helsinki. Informed consent was obtained from all subjects.

Experimental design. The study was performed as an intervention study in free-living subjects and consisted of a randomized double-blind placebo control trial with 2 successive intervention periods. The total duration of the study was 4 wk from October to November 2006. Participants were identified by a single randomization number using a computer-generated random permutation procedure in SPSS software version 15.0J. Participants were randomly assigned to 2 groups: (1) the "MK-4 group," intake of 1.5 mg/d of MK-4 (Kyowa Hakko Kogyo Co., Tokyo, Japan) (n=20) and (2) the "control group," placebo intake (n=20). The subjects were instructed to take 2 capsules after breakfast and return the bag of capsules after the experiment, and treatment compliance was determined by counting the number of remaining capsules. The subjects in both groups were under restricted natto intake during the study period and almost all the subjects refrained from taking natto 1 wk before the study; natto is fermented soybean containing MK-7. The participant randomization codes were allocated sequentially in the order in which the participants were enrolled. After completion of all the analyses, the randomization code was disclosed to the investigators.

Blood and urine samples. Blood and urine samples were collected at baseline and after 2 and 4 wk. Fasting (>12 h) blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes by venipuncture, refrigerated immediately, and centrifuged at 1,500 rpm for 30 min at 4°C within 2 h. Serum samples from each participant were stored at −80°C.

Serum concentrations of total cholesterol (TC) and triacylglycerol (TG) were determined using commercial kits (cholesterol C-test and triglyceride G-test, respectively; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The serum high-density lipoprotein (HDL) cholesterol levels were measured by an enzymatic method (HDL-cholesterol test; Wako Pure Chemical Industries). The serum low-density lipoprotein (LDL) cholesterol levels were calculated as follows:

LDL-cholesterol (mg/dL)=total cholesterol (mg/dL)−HDL-cholesterol (mg/dL)−TG (mg/dL)×0.2.

17β-Estradiol levels (E2) were assessed by a radioimmunoassay (Amersham Biosciences, Piscateway, NJ, USA).

Serum concentrations of PK and MK-4 were measured by high-performance liquid chromatography (HPLC) (*16*). A bone formation marker—serum bone-specific alkaline phosphatase (BAP)—was analyzed by an enzyme immunoassay (Alkphase-B: Metra Biosystems, Mountain View, CA, USA).

Serum under carboxylated osteocalcin (ucOC) and γ-carboxylated OC (GlaOC) levels were determined by separate immunoassays using the respective ELISA kits from Takara Shuzo (Otsu, Shiga, Japan). The vitamin K status of bone was also evaluated by expressing the GlaOC fractions as a ratio of GlaOC plus ucOC (GlaOC/GlaOC+ucOC).

Urine samples were collected from the second void of the day at the same time as collection of blood samples, and the samples stored at −80°C. The levels of bone resorption marker—urine free deoxypiridinoline (DPD)—was determined by a sandwich EIA (Pyrilinks-D Assay; Metra Biosystems) and corrected for the urinary creatinine concentration. Urinary cross-linked N-telopeptides of type I collagen (NTx) were measured using the NTX ELISA kit Osteomark (Ostex, Seattle, WA, USA). Serum 25-hydroxyvitamin D {25(OH)D} was analyzed by a radioimmunoassay (25-hydroxyvitamin D ^{125}I RIA KIT (100); DiaSorin, Ltd., Stillwater, MN, USA).

Blood pressure measurement. Chronic levels of arterial blood pressure at rest were measured with a semi-automated device (Form PWV/ABI, Colin Medical Technology) in the brachial and dorsalis arteries. Recordings were made in triplicate while the subjects were in the supine position.

Questionnaire interview. Individual information was collected by trained interviewers through face-to-face interviews based on the structure of a previously validated questionnaire that included the following: socio-demographic data: years since menopause, physical activities, medications, smoking and drinking alcohol, and other factors that may have possible confounding effects on the relationship between dietary vitamin K consumption and bone and lipid metabolism.

Dietary analysis. At baseline and after 4 wk, the usual dietary intakes of energy, protein, vitamin K, calcium, etc., were estimated by a 3-d dietary food record and calculated on the basis of the Fifth Revision of the Standard Tables of Food Composition in Japan (*17*). Each food record was confirmed by trained dietitians through face-to-face interviews.

Statistical analysis. Descriptive statistics were performed for all variables at baseline and at each 2-wk visit. Data were transformed to normality and parametric tests were used, with statistical significance taken as $p<0.05$. Comparisons between the variables of the 2 groups at baseline and after 2 and 4 wk were performed using an unpaired Student's t-test. The changes in serum PK, MK-4, 25(OH)D, estradiol, osteocalcin, bone metabolic markers, and serum lipids over time were evaluated by repeated-measures analysis of variance (ANOVA) and Tukey's post hoc test. Components measured only at baseline and after 4 wk were compared

Table 1. Characteristics of subjects of different study groups at baseline and 4 wk.

		Placebo $n=20$		MK-4 $n=20$		p value vs. placebo group
		Mean (SD)		Mean (SD)		
Age (y)	Baseline	59.3	(3.7)	59.8	(3.1)	0.714
Years since menopause (y)	Baseline	7.9	(3.6)	8.6	(4.5)	0.589
Height (cm)	Baseline	154.4	(6.3)	154.4	(5.3)	0.972
	After 4 wk	54.4	(6.4)	154.2	(5.6)	0.925
Weight (kg)	Baseline	54.2	(7.8)	50.2	(5.8)	0.073
	After 4 wk	53.7	(7.5)**	50.0	(5.8)	0.092
BMI (kg/m^2)	Baseline	22.7	(2.6)	21.1	(2.2)	0.040
	After 4 wk	22.5	(2.5)*	21.0	(2.2)	0.061
Energy (kcal/d)	Baseline	1,750	(302)	1,868	(681)	0.487
	After 4 wk	1,727	(324)	1,678	(244)	0.594
Protein (g/d)	Baseline	68.4	(13.2)	72.6	(22.6)	0.480
	After 4 wk	70.3	(16.8)	65.5	(13.1)	0.321
Fat (g/d)	Baseline	47.2	(11.1)	55.3	(27.3)	0.229
	After 4 wk	49.7	(11.5)	50.3	(10.0)	0.858
Cholesterol (mg/d)	Baseline	269	(124)	277	(117)	0.827
	After 4 wk	307	(128)	315	(130)	0.845
Calcium (mg/d)	Baseline	600	(179)	641	(212)	0.514
	After 4 wk	649	(270)	557	(206)	0.238
Iron (mg/d)	Baseline	9.5	(3.0)	9.1	(2.6)	0.682
	After 4 wk	9.3	(3.9)	8.4	(2.4)	0.381
Phosphorus (mg/d)	Baseline	1,104	(250)	1,145	(343)	0.666
	After 4 wk	1,131	(274)	1,032	(221)	0.216
Potassium (mg/d)	Baseline	3,080	(825)	3,107	(974)	0.924
	After 4 wk	3,033	(868)	2,808	(816)	0.404
Magnesium (mg/d)	Baseline	316	(100)	303	(88)	0.675
	After 4 wk	296	(91)	277	(81)	0.491
Sodium (mg/d)	Baseline	3,752	(947)	4,211	(1,318)	0.214
	After 4 wk	3,733	(1,268)	4,273	(3,418)	0.501
Vitamin K (μg/d)	Baseline	285	(223)	233	(114)	0.367
	After 4 wk	337	(446)	208	(112)	0.221
Vitamin D (μg/d)	Baseline	6.3	(4.7)	9.4	(6.2)	0.080
	After 4 wk	9.7	(4.8)*	7.1	(4.1)	0.072

*$p<0.05$, **$p<0.01$ vs. baseline (Repeated-measures ANOVA).

using a paired t-test.

All analyses and calculations were performed using SPSS 15.0J for Windows.

RESULTS

The baseline characteristics and daily nutrient intake of the subjects are shown in Table 1. The characteristics of the subjects in the control and MK-4 groups were not significantly different, with the exception that the subjects in the MK-4 group had a lower body mass index (BMI) than those in the control group ($p<0.05$) (Table1). Further, among the groups, the mean body weight and BMI at 4 wk in the control group were slightly lower than those of the baseline; however, this change was statistically significant.

There were no significant differences between the baseline and 4-wk values of the daily intake of nutrients with the exception of a higher intake of vitamin D at 4 wk than at baseline in the control group. Differences in the daily intake of nutrients were not observed between the control and MK-4 groups at baseline or 4 wk intervention.

Effect of MK-4 supplementation on serum vitamin K and vitamin D concentrations

The time course of changes in the serum PK, MK-4, and 25(OH)D concentrations for the control and MK-4 groups are shown in Fig. 1. The serum concentrations of PK and MK-4 in the MK-4 group at 2 wk were significantly higher than those at baseline. No significant difference in the mean serum PK concentration was observed between the control and MK-4 groups throughout the study period. However, the mean serum MK-4 concentration in the MK-4 group at 4 wk was significantly higher than that in the control group. The serum 25(OH)D concentration was unaffected by MK-4 supplementation.

The time course of changes in the serum OC levels for the control and MK-4 groups are shown in Fig. 2. A significant time-dependent decrease in the serum concentration of ucOC was observed in the MK-4 group but

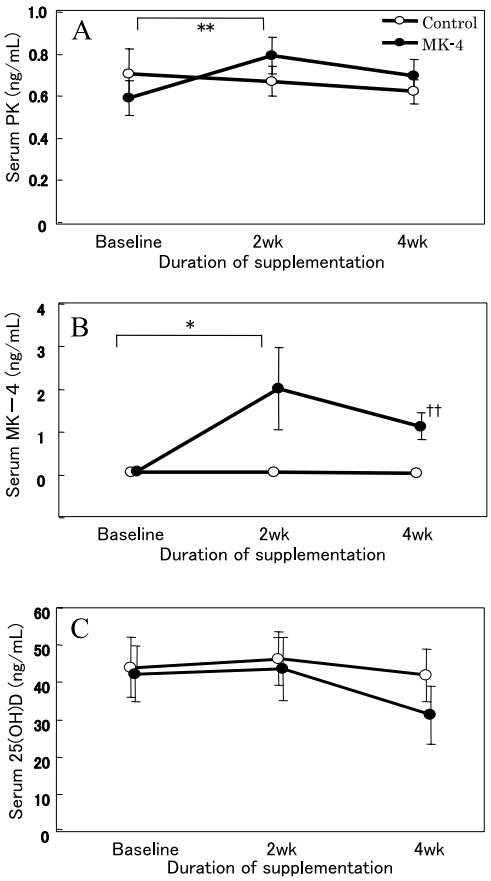

Fig. 1. Time course of changes in serum vitamin K and D concentrations of the subjects in control and MK-4 groups. (A) PK, (B) MK-4, and (C) 25(OH)D. Control (○) and MK-4 groups (●). *Significantly different from baseline ($p<0.05$). **($p<0.01$). ††Significantly different from the control group ($p<0.01$).

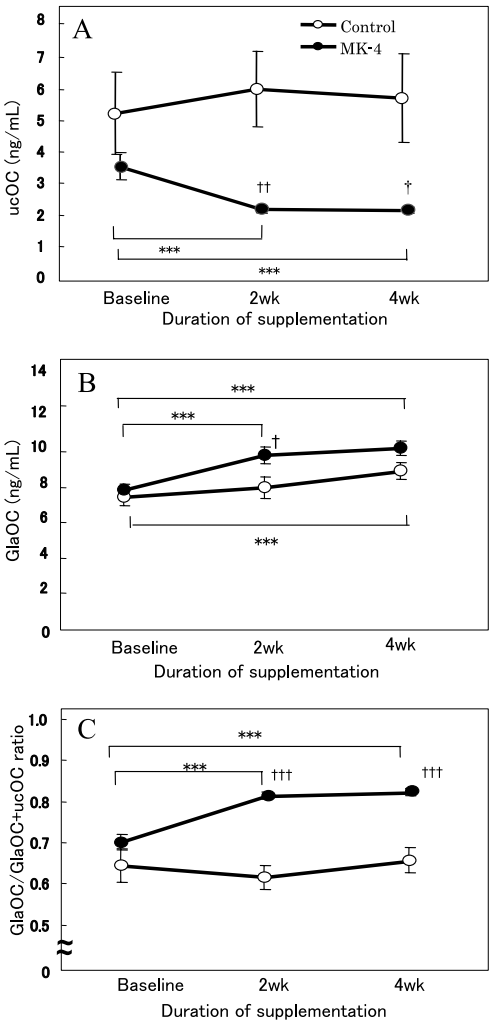

Fig. 2. Time course of changes in osteocalcin concentrations of the subjects in the control and MK-4 groups. (A) ucOC, (B) GlaOC, and (C) GlaOC/GlaOC+ucOC ratio. Control (○) and MK-4 groups (●). *Significantly different from baseline ($p<0.05$), ***($p<0.001$). †Significantly different from the control group ($p<0.05$), ††($p<0.01$), †††($p<0.001$).

not in the control group (Fig. 2A). After 2 and 4 wk of supplementation, ucOC in the MK-4 group was significantly lower than that in the control group. A significant corresponding increase in the serum GlaOC levels was observed at 4 wk of intervention in both groups when compared with the serum GlaOC levels at baseline. At 2 wk, GlaOC in the MK-4 group was significantly higher than that in the control group (Fig. 2B).

The GlaOC/GlaOC+ucOC ratio was significantly increased at 2 and 4 wk when compared with that at baseline in the MK-4 group (Fig. 2C). The GlaOC/GlaOC+ucOC ratio of the MK-4 group at 2 and 4 wk was significantly higher than those of the control group, although there was no significant difference between the 2 groups at baseline (Fig. 2C).

Table 2 summarizes the serum biomarkers of bone and lipid metabolism at baseline and after 2 and 4 wk of intervention. The bone formation marker bone-specific alkaline phosphatase (BS-ALP) was decreased in both the control and MK-4 groups at 4 wk. The levels of bone resorption markers—urine-free deoxypyridinoline (DPD) and NTx—showed no significant changes in either group. There was no significant difference between the levels of the bone metabolic markers of the control and MK-4 groups at either intervention period.

No significant differences were observed between the serum estradiol and lipid levels of the 2 groups throughout the study period (Table 2). However, significant decreases in the total cholesterol levels in both groups and in the levels of low-density lipoprotein (LDL)-cholesterol and high-density lipoprotein (HDL)-cholesterol in the MK-4 group were observed after 4 wk of intervention (Table 2).

There was a decrease in the systolic blood pressure (SBP) and the diastolic blood pressure (DBP) in both groups; however, especially in the MK-4 group, the blood pressure at 4 wk (SBP: 113.4±13.8 mmHg, DBP: 67.0±8.7 mmHg) was significantly lower than that at baseline (SBP: 118.5±16.4 mmHg, DBP: 70.6±9.5 mmHg) (data not shown).

DISCUSSION

Previous studies have reported that treatment with 45 mg/d of MK-4 prevents osteoporotic fracture as well as postmenopausal bone loss (*11–14*, *18*); therefore,

Table 2. Serum estradiol, biomarkers of bone turnover and lipids of different study groups at baseline, 2 wk and 4 wk.

			Placebo $n=20$	MK-4 $n=20$	p value vs. placebo group
			Mean (SD)	Mean (SD)	
Hormone	Estradiol (pg/mL)	Baseline	11.7 (5.1)	11.6 (2.8)	0.909
		After 2 wk	11.1 (2.1)	11.2 (1.6)	0.870
		After 4 wk	11.1 (2.2)	11.4 (2.5)	0.741
Bone markers	BSALP (U/L)	Baseline	31.9 (13.8)	30.9 (7.4)	0.780
		After 2 wk	31.1 (14.2)	30.1 (6.5)	0.775
		After 4 wk	28.4 (12.6)***	27.0 (6.6)***	0.658
	DPD/Creatinine	Baseline	9.0 (5.9)	7.5 (1.5)	0.285
	(nmol/mmol CRE)	After 2 wk	10.0 (8.1)	8.2 (1.9)	0.327
		After 4 wk	9.9 (7.2)	7.9 (2.3)	0.250
	NTx/Creatinine	Baseline	71.2 (56.5)	60.9 (22.8)	0.455
	(nmol BCE/mmol CRE)	After 2 wk	71.8 (61.9)	60.2 (21.9)	0.439
		After 4 wk	70.3 (58.6)	59.3 (24.6)	0.444
Serum lipids	Total-cholesterol (mg/dL)	Baseline	231.4 (43.1)	229.1 (36.3)	0.859
		After 2 wk	225.3 (38.9)	226.0 (31.6)	0.951
		After 4 wk	214.6 (37.4)**	210.4 (30.4)***	0.699
	HDL-cholesterol (mg/dL)	Baseline	74.1 (14.9)	75.7 (17.0)	0.754
		After 2 wk	74.3 (15.0)	77.4 (16.4)	0.530
		After 4 wk	70.9 (17.1)	71.3 (17.2)**	0.949
	LDL-cholesterol (mg/dL)	Baseline	137.1 (40.6)	136.7 (38.7)	0.975
		After 2 wk	137.3 (39.0)	135.6 (36.6)	0.888
		After 4 wk	129.1 (36.6)	125.2 (34.7)***	0.728
	TG (mg/dL)	Baseline	87.0 (45.4)	79.4 (33.9)	0.550
		After 2 wk	83.5 (32.7)	86.5 (31.3)	0.768
		After 4 wk	72.2 (33.5)	76.3 (28.1)	0.674

*$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. baseline (Repeated-measures ANOVA).

this dose has been used for the treatment of patients with osteoporosis in Japan. In this study, we examined the effects of the administration of low-dose of MK-4 on markers of bone turnover and other bio-indices in healthy postmenopausal Japanese women between 53 and 65 y of age.

The recorded mean dietary intake of energy and nutrients in the subjects of the present study were sufficient for the Adequate Intake (AI) levels of the DRIs for Japanese, 2005 (*5*), in both the MK-4 and control groups. Booth et al. reported that biochemical markers of bone turnover that reflected the vitamin K status remained below normal levels following the short-term supplementation of approximately 450 μg of PK, although the γ-carboxylation of prothrombin was restored to its normal levels (*6*). Furthermore, Binkley et al. reported that a daily PK intake of approximately 1,000 μg was required to maximally γ-carboxylate the circulating OC in premenopausal women (*19*). Similar to the results of these reports, the daily intake of vitamin K in our study participants was sufficient for the AI levels of the DRIs for Japanese, 2005 (*5*) from the γ-carboxylation of blood coagulation factors; however, it seems to be insufficient for the carboxylation of OC because the ucOC level in our subjects at baseline was relatively high compared with those in premenopausal women (*20*).

There are several reports indicating the pharmacological amount of MK-4 supplementation that is beneficial for OC carboxylation in healthy aged people (*11*, *12*) as well as in osteoporotic postmenopausal women (*21*). However, in case of insufficient daily intake of vitamin K for bone metabolism, the exact amount of MK-4 supplementation required to accelerate the carboxylation of OC in healthy postmenopausal women is unclear.

Vitamin K is thought to maintain bone strength via the γ-carboxylation of OC (*2*). It has been reported that incompletely carboxylated serum OC is an indicator of poor bone vitamin K status of a healthy population (*22*). In vitamin K insufficiency or deficiency, a small amount of ucOC is released from osteoblasts into the circulation. Thus, the serum concentration of ucOC has been considered to be a sensitive marker of vitamin K deficiency status in bone (*23*, *24*). A high serum ucOC concentration has been associated with skeletal turnover (*25*), low bone mineral density (*26*), and an increased risk of osteoporotic fractures (*24*, *26*, *27*).

In this study, supplementation with 1.5 mg/d of MK-4 for 4 wk significantly increased the serum MK-4 concentration, and the most marked effects of MK-4 intake were observed on the serum concentration of OC. The most significant response was observed at 2 wk of the intervention period, and the response was sustained

until week 4. The serum GlaOC concentration increased and the ucOC concentration decreased at 2 wk compared with that at baseline in the MK-4 group. Further, the GlaOC/GlaOC+ucOC ratio that indicates the degree of the γ-carboxylation of OC increased at 2 wk. These results suggest that in healthy postmenopausal women, the supplementation of 1.5 mg/d of MK-4 accelerates the γ-carboxylation of OC with an increase in the serum MK-4 concentration. Recently, Tsugawa et al. indicated that the circulating vitamin K concentration in elderly people should be maintained at a higher level than that in young people (*20*). In fact, the serum ucOC level in our subjects at baseline was higher than that in premenopausal women (*20*) even though the daily intake of vitamin K in the subjects was higher than the AI level for Japanese DRI, 2005. Since there is evidence indicating the correlation between high serum concentrations of ucOC and bone fracture (*24*, *26*, *27*), the supplementation of vitamin K might be beneficial in maintaining the bone health of postmenopausal women.

In the present study, serum concentration of BS-ALP, a bone formation marker, was slightly but significantly low at 4 wk compared with baseline in both groups. There is a possibility that the change is due to restriction of natto intake during the study, effects of some ingredients other than MK-4 in the capsule, or decrease in bone turnover by aging (*28*).

The bone resorption markers urinary NTx and DPD did not respond to MK-4 supplementation over the 4-wk intervention period. Previous studies have reported results similar to that those of our study, suggesting that MK-4 does not affect bone resorption in postmenopausal women (*21*).

Blood lipid levels are also known to be influenced by the serum concentrations of vitamin K (*29–31*). In this study, the HDL and LDL cholesterol levels significantly decreased only in the MK-4 group. Although, the relationship between MK-4 intake and the serum cholesterol levels is unclear, it has been suggested that an adequate intake of MK may have an important role for coronary heart disease prevention (*32*). On the other hand, TG levels were not significantly changed in either group. Several previous reports have indicated the relationship between vitamin K and TG (*29*). Since triacylglycerol-rich lipoproteins act as PK carriers, it can be concluded that the plasma concentrations of PK and TG are positively correlated (*29*). In a recent human trial, PK was almost exclusively incorporated into the TG-rich lipoprotein fraction following intestinal absorption, whereas a substantial portion of MKs was recovered from the LDL fraction (*31*). Since the relationship between vitamin K and serum lipid levels is unclear, further studies are required to determine whether vitamin K has a beneficial effect on the concentration of serum lipids.

There is a report that vitamin K dependent γ-carboxylation of matrix Gla protein in blood vessels is essential for its inhibitory effects on calcification (2). Although the SBP and the DBP were not different between the groups at any experimental periods in this study, the effects of vitamin K on blood pressure might be confirmed in near future.

The limitations of this study are as follows: (1) the intake of MK-4 from daily meals was not restricted and (2) it was unclear if the amount of MK-4 supplemented in this study was appropriate for postmenopausal women. Further studies are required to investigate the effects of supplementation with less than 45 mg/d of MK-4.

In conclusion, our study clearly shows that the vitamin K status of postmenopausal women taking an extra dose of 1.5 mg MK-4 daily substantially improved after 4 wk. This improved status was evidenced by the more than 1 ng/mL of serum MK-4 concentration. This suggests that increasing MK-4 intake by 1.5 mg/d led to an increase in the degree of the γ-carboxylation of OC. Thus, the supplementation of low doses of vitamin K2 may favorably affect bone health in healthy postmenopausal women. It is desirable that the required amount of vitamin K be taken with daily meals.

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