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Recent Topics in the Studies of Laboratory of Nutrition, Tohoku University: Newly Clarified Function of Vitamin K

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Summary

Vitamin K (VK) is essential for blood coagulation and bone metabolism in mammals as a Gla-protein activating factor, i.e., VK acts as a cofactor in the posttranslational synthesis of γ-carboxyglutamic acid (Gla) from glutamic acid (Glu) residues in the nascent proteins. Menaquinone-4 (MK-4) is one of the VK_2 analogues, and is well known to have bioactivity in the suppression of bone resorption through apoptosis of osteoclast cells, thus MK-4 is now also used clinically as a therapeutic drug for the osteoporosis. Besides of these well-known functions, MK-4 is strongly presumed to have other novel functions because we have gradually recognized that MK-4 accumulates in various tissues of germfree animals fed an MK-4-free diet. Accordingly, we have focused on clarification of the mechanism of MK-4 formation in several tissues, using both *in vitro* tissue homogenates (bovine, rats, mice, chicken) and *in vivo* experiments with rats and mice. To elucidate the biological role of MK-4 production, we used germfree rats to eliminate MK-n synthesized by intestinal flora. Our *in vivo* results indicate that MK-4 is produced in diverse tissues from ingested vitamin K analogues, including vitamin K_1, MK-n (MK-6, MK-7, and MK-10); and VK_3 without enzymatic participation of microorganisms in the intestine. In addition to the liver and bone, VK is found in the brain, heart, testis, kidney, pancreas and salivary glands mainly as menaquinone-4 (MK-4). However, the physiological role of MK-4 in these various organs has not been fully understood yet. In the present study we identified genes of which expression is changed in testis under vitamin K deficient condition using DNA microarray. The genes involved in the biosynthesis pathway of cholesterol and steroid hormone were decreased in vitamin K deficient group. The amount of Cyp11a (P450scc) mRNA, rate-limiting enzyme for testosterone synthesis, was positively correlated with the concentration of MK-4 in testis. Moreover, the concentration of testosterone in plasma and testis was decreased in vitamin K deficient group compared with the control and vitamin K supplemented

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groups. These results suggests that vitamin K is involved in steroid production in testis through the regulation of Cyp11a.

Key words : vitamin K_1 (VK_1), menaquinone-4 (MK-4 = VK_2), testis, testosterone, steroidogenesis, Cyp11a (P450scc)

Introduction

Vitamin K (VK) is essential for both blood coagulation and bone metabolism in mammals. VK acts as a cofactor for microsomal γ-glutamyl-carboxylase (GGCX) in the posttranslational synthesis of γ-carboxyglutamic acid (Gla) from glutamic acid (Glu) residues contained in the K-dependent precursor proteins (Suttie, 1993, Wu, et al., 1997). There are two types of naturally occurring VK : phylloquinone (VK_1), synthesized in plants, and menaquinone (VK_2, or MK-n), produced mainly by microorganisms (Fig. 1). Significant amounts of menaquinones are synthesized by intestinal microorganisms in the distal intestine and partly absorbed. Microbiologically-synthesized menaquinones (MKs) in the intestine can contribute to the maintenance of VK requirements in the host, but their relative contribution to the VK nutritional status remains a subject of debate (Yamamoto, et al., 1997). After absorption from the intestine, VK is transported to the liver by triglyceride-rich lipoprotein chylomicrons to activate VK-dependent proteins. Although a significant portion of VK is redistributed to extrahepatic organs, including heart, kidney, brain, salivary glands, pancreas and gonadal tissue (Ronden, et al., 1998 ; Huber, et al., 1999 ; Akiyama, et al., 1994), it has not been known whether this redistributed VK functions as a cofactor for GGCX (γ-glutamyl carboxylase) in these tissues.

VK_1	**Phylloquinone** 2-methyl-3-phytyl-1,4-naphthoquinone (Plant origin)	
VK_2 (MK-n)	**Menaquinone-n** 2-methyl-3-multiprenyl-1,4-naphthoquinone (Bacterial origin, except for MK-4)	
VK_3	**Menadione** 2-methyl-1,4-naphthoquinone (Synthetic, or estimated intermediate of tissue MK-4 formation)	

FIG. 1. The chemical structure of vitamin K analogues.

Menaquinone-4 (MK-4) is one of the vitamin VK2 analogues, and is well known to have bioactivity in the suppression of bone resorption (=acceleration of bone formation) through apoptosis (Nishimaki, et al., 1999) and suppression of RANKL of osteoclast cells (Takeuchi, et al., 2000). MK-4 is now widely used as a therapeutic drug for the osteoporosis. Besides of these well-known action of MK-4, we have gradually recognized that MK-4 accumulates in various tissues of germfree animals fed a MK-4-free diet (Kimura, et al., 1992). Accordingly, we have focused on clarification of the mechanism of MK-4 formation in several tissues, using both *in vitro* tissue homogenates (bovine, rats, mice, chicken) and *in vivo* experiments with rats and mice. Our *in vivo* results indicate that MK-4 is produced in diverse tissues other than gastrointestinal mucosa (stomach, small intestine, and colon) from ingested VK analogues, including VK_1, MK-n (MK-6, MK-7, and MK-10), and VK_3 without enzymatic participation of intestinal microorganisms (Fig. 2). Its occurrence is possibly due to the conversion of side-chain replacement to geranylgeranyl group at 3-position of naphthoquinone ring. To elucidate the biological role of MK-4 production in various tissues, we used germfree rats to eliminate MK-n synthesized by intestinal flora (Komai, et al., 1988), and employed DNA microarray techniques to identify patterns of gene expression.

As far as the testis is concerned, of many organs, we identified genes of which expression is changed in testis under VK deficient condition using DNA microarray. The genes involved in the biosynthesis pathway of cholesterol and steroid hormone were decreased in VK deficient group. The amount of Cyp11a (P450scc) mRNA, rate-limiting enzyme for testosterone synthesis, was positively correlated

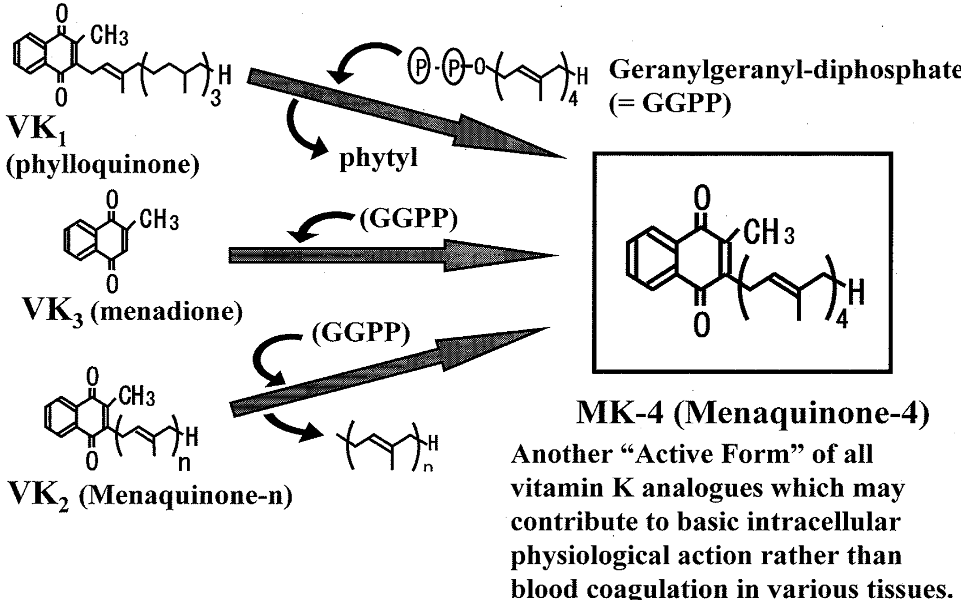


FIG. 2. Conversion of vitamin K analogues to MK-4 in the rat tissues.

with the concentration of MK-4 in testis. Moreover, the concentration of testosterone in plasma and testis was decreased in VK deficient group compared to the Control and VK supplemented groups. The present study speculates the action of VK in controlling hormone levels for the first time. These results have been published in the BBA journal in 2006 (Shirakawa, et al., 2006, accepted).

Materials and Method

Animals and diets

VK deficient (K-Def) diet was obtained from Harlan-Teklad (Wisconsin, USA, CAT #TD97053). Control or VK1 supplemented (K1-sup) diets were prepared by adding VK1 (Eisai. Co., Ltd., Japan) to the K-def diet (final concentrations of 0.75 and 75 mg/Kg diet, respectively). MK-4 supplemented (MK-4-sup) diet was also prepared by adding MK-4 (Eisai. Co., Ltd., Japan) to the K-def diet (final concentration of 75 mg/Kg diet). All diets were sterilized by irradiation with gamma-ray (50 KGy).

Germfree rats were originally obtained from ALA Research Center (Yokohama, Japan) and self-bred. Animals, male 10–12 weeks of age, were housed at 23 +/− 2°C on a 12 : 12 light : dark cycle (lights on at 8 : 00 am) in sterilized vinyl isolator, and fed experimental diets for 9 days.

RNA preparation and DNA microarray experiment

Total RNA was isolated from testis with the guanidine-isothiocyanate based reagent Isogen (Nippon Gene, Japan), as detailed in the instruction manual. In each dietary group, equivalent amounts of RNA from individual animals were pooled, and 10 μg of pooled RNA were used as a template to synthesize cDNA hybridized with probes on DNA microarray. RNA was denatured with oligo-dT (2 μg, Amersham Biosciences) at 65°C for 5 min, then incubated in 50 mM Tris-HCl (pH8.3), 75 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol (DTT) containing 1 mM dATP, dGTP, TTP, 200 units of SuperscriptII reverse transcriptase (Invitrogen), 60 units of RNase OUT RNase inhibitor (Invitrogen), [α-^{33}P] dCTP (1.85 MBq, 110 TBq/mmol, Amersham Biosciences) at 42°C for 50 min. After cDNA synthesis, free nucleotides were removed using a BioRad gel filtration Spin column. Labeled cDNAs were hybridized with GeneFilter (Research Genetics) in ExpressHyb hybridization solution (Clonetech) containing mouse Cot-1 DNA (1 μg/ml, Invitrogen) for 16 h at 65°C. Following hybridization, filters were washed according to the instructions in GeneFilter's manual and exposed to Fuji Imaging Plate (Fuji Photo Film, Japan), and then analyzed using a BioImage analyzer FLA-2000 (Fuji Photo Film, Japan).

Quantitative RT-PCR

Two micrograms of total RNA were used as a template for cDNA synthesis. RNA was incubated in RT buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 5 mM DTT) containing 50 units of Superscript III reverse transcriptase, 40 units of RNaseOUT RNase inhibitor (Invitrogen), 1 mM dNTP, 1 mg of oligo-dT at 50°C for 50 min. An aliquot of cDNA was used as a template for quantitative PCR using Applied Biosystems Sequence Detection System 7000. The gene specific primers were used for amplification of certain cDNA in Syber Premix Ex Taq solution (Takara Bio Inc., Jpana). The amplified DNA fragment by each primer set was confirmed by DNA sequencing. Relative expression level of each mRNA was normalized by the amount of glyceraldehydes-3-phosphate dehydrogenase mRNA.

Western blot analysis

Testis was homogenized in 5 volume of phosphate buffered saline and centrifuged at 12,000 $\times$ *g*, 4°C for 5 min. Obtained supernatant was denatured in sodium dodecyl sulfated (SDS) gel loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 50 mM dithiothreitol, 6% glycerol, final), and eletrophoresed in 12.5% SDS-polyacrylamide gel. The protein bands were transferred to Immobilon-P membranes (Millipore). After blocking in TBS-T (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 5% dried milk, the membranes were reacted with anti-rat Cyp11a antibody (Chemicon International Inc., 1/5000 dilution in TBS-T containing 5% dried milk) for 1 hr at room temperature, washed, and incubated with anti-rabbit IgG peroxidase conjugate (Pierce, 0.16 μg.ml in TBS-T containing 5% dried mil) for another 1 hr. The bound antibodies were detected with ECL Plus Western blotting detection reagent (Amersham Bioscience).

Statistical analysis

Results are expressed as means$\pm$SD or means$\pm$SEM. The data were analyzed statistically by means of a one-way ANOVA, and multiple comparisons were made with the Scheffé's test, and with the Tukey's test. The StatcelQC program (OMS publishing Inc., Saitama, Japan) was used for the analysis in each case.

Results and Discussion

Influence of VK-deficient diet on testis VK levels

To analyze the influence of dietary VK content on VK level in testis, we measured testicular VK content with fluorescent HPLC. As a result, menaquinone analogues except for MK-4 were not detected in testis because all rats were lacking intestinal flora and fed semi-purified experimental diets in the present study. Any menaquinone analogues were not detected in commercially

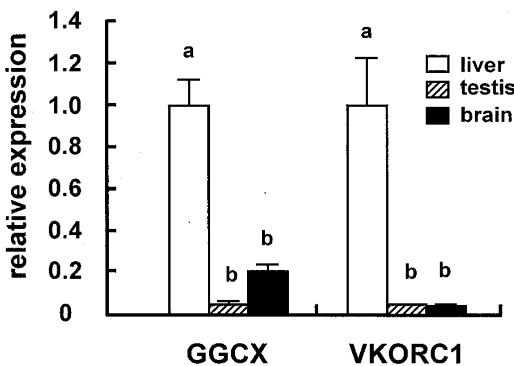


FIG. 3. Comparison of mRNA level of γ-glutamyl carboxylase and vitamin K epoxide reductase complex 1 among tissues. Total RNA was isolated from the liver, testis, and brain of conventional Wistar male rats that were fed commercial chow diet. Messenger RNAs of γ-glutamyl carboxylase (GGCX) and vitamin K epoxide reductase complex 1 (VKORC1) were measured by quantitative RT-PCR method described in Materials and methods and expressed as a fold relative to liver values. The data are expressed as mean$\pm$SEM, $n=3$. The values with different superscript letters are significantly different at $P<0.05$.

perchased VK-def diet (TD97053). Since total amount of VK (K1 and MK-4) in K-def group was lowest, it was obvious that dietary VK content strongly influenced testicular VK level. To presume whether plenty of VK may function as cofactor for GGCX in testis, we measured mRNA of GGCX and VK epoxide reductase complex subunit 1 (VKORC1), one of the VK cycle enzymes, in conventional Wistar rats fed commercial VK-supplemented pellet diet. Both mRNA were detected but expression levels in testis, as well as brain, were much lower than in liver (Fig. 3). It has been reported that enzyme activities of GGCX and VKORC1 in brain are extremely lower than in liver, even though the brain contains high level of VK (especially MK-4) almost the same as in the liver (Li, et al., 2003). Therefore, testicular VK may be involved in some other cellular function on testis, for instance regulation of gene expression (Tabb, et al., 2003) or stimulation of cAMP dependent kinase (Otsuka, et al., 2004), rather than protein γ-glutamyl carboxylation.

Although VK1 was the major source of dietary VK in K-deficient, Control and VK1 supplemented diets, most of VK included in testis was MK-4 (K-def group, 99.4 ; Cont group, 99.5 ; and K1-sup group, 94.7%, respectively). Several papers including our previous research demonstrated that orally administered VK1 is a source of MK-4 which is distributed in the testis, brain, pancreas, and kidney. Conversion of VK1 into MK-4 was observed not only in experimental animals but also in human (Yamamoto, et al. ; Kimura, et al., 1992 ; Thijssen and Drittij-Reijnders, 1994 ; Davidson, et al., 1998 ; Thijssen, et al., 2002). MK-4 seems to be a tissue-activated form of VK such as found in other lipids-soluble

vitamins (VA and VD), though physiological significance of MK-4 conversion has not been elucidated yet.

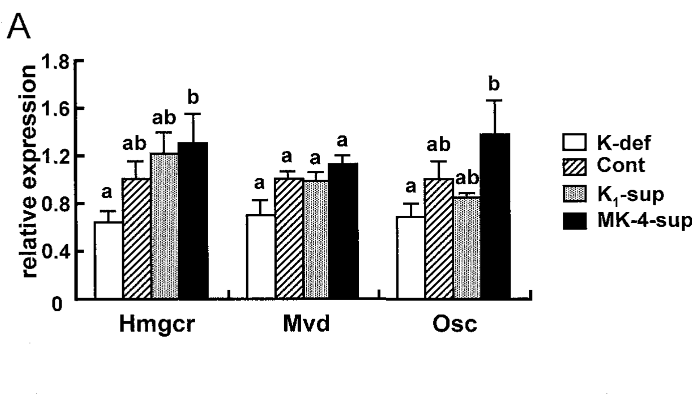
Reduced gene expression involved in cholesterol synthesis and steroid synthesis in testis under VK deficency

We conducted the gene expression analysis in the testis using the DNA microarray to clarify the physiological role of the MK-4 synthesis in the testis. In comparison of gene expression profiles between K-def and K1-sup groups, the expression of genes involved in the cholesterol/steroid synthesis and the isoprenoid metabolism was decreased in K-def group compared to K1-sup group (Table 1). To confirm their expression difference, we measured mRNA level of those genes in all experimental groups with quantitative RT-PCR method. The amount of mRNA encoding mevalonate diphosphate decarboxylase (Mvd), oxidosqualene cyclase (OSC), farnesyltransferase α-subunit (Fnta), and Rab geranylgeranytransferase α-subunit (Rabggta) was decreased in K-def group compared with Control and both K1 and MK-4 supplemented groups (Fig. 4A and 4B) which was in accordance with the result of the DNA microarray. Besides these, mRNA level of HMG-CoA reductase (HMGCR), a rate limiting enzyme in cholesterol synthesis, was also decreased in K-def group. Therefore, VK deficient condition is likely to affect the cholesterol synthesis and isoprenoid metabolism in testis.

A mitochondrial enzyme Cyp11a, alternatively named as cholesterol side chain cleavage enzyme (P450scc), is a rate limiting enzyme in testosterone synthesis and catalyzes the production of pregnenolone from cholesterol. The mRNA level of Cyp11a was significantly decreased in K-def group compared with both K1 and MK-4 supplemented groups (Fig. 4C). The relative expression of Cyp11a mRNA in K-def and Cont groups was positively correlated with MK-4 levels in testis ($r=0.8610$, $p<0.01$, Fig. 5). Furthermore, Cyp11a protein was also de-

Table 1. Down-regulated genes in the K-def group compared with the K_1-sup group

Accession no.	Gene name	K_1-sup/K-def
AA998854	Acyl-coenzyme A : Cholesterol acyltransferase	3.73
AA818329	Cyp11a (cholesterol side-chain cleavage enzyme)	1.94
AI070588	Farnesyltransferase α-subunit	1.77
AI058569	Mevalonate diphosphate decarboxylase	3.08
AA997956	Oxidosqualene cyclase (Lanosterol synthase)	1.76
AI071807	Rab geranylgeranyl transferase α-subunit	17.65
AI029123	Rab geranylgeranyl transferase β-subunit	2.53
AA957963	Rab GDIβ	5.17
AI072276	Ras-related protein (rad)	9.17
AA964948	Steroid 3α dehydrogenase	1.94



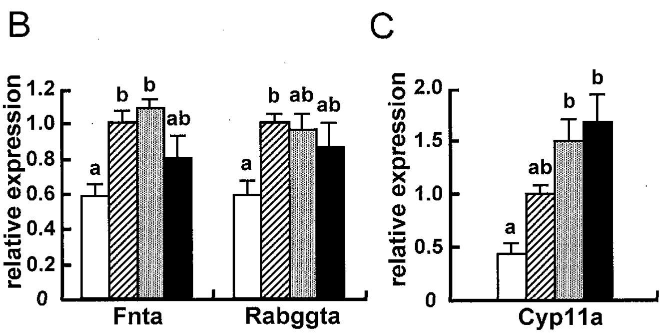


FIG. 4. Decreasing amount of mRNA encoding steroidogenesis and isoprenoid metabolism enzyme in testis of K-deficient group. Each mRNA was measured by quantitative RT-PCR method described in Materials and methods and expressed as a fold relative to Cont diet group values. Panel A; HMG-CoA reductase (Hmgcr), mevalonate diphosphate decarboxylase (Mvd), oxidosqualene cyclase (Osc); B, farnesyltransferase α-subunit (Fnta), Rab geranylgeranyltransferase α-subunit (Rabggta); and C, Cyp11a (cholesterol side chain cleavage enzyme). The data are expressed as mean$\pm$SE, $n=3$-5. Values with different superscript letters are significantly different at $P<0.05$.

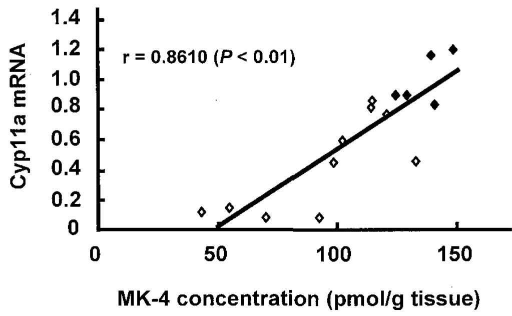
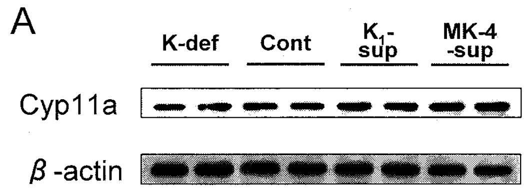


FIG. 5. Positive correlation between the testis menaquinone-4 concentrations and the relative expression levels of Cyp11a mRNA in K-def and Cont groups.

creased significantly in K-Def group compared with Control, and both K1 and MK-4 supplemented groups (Fig. 6). The expression of Cyp11a in testis is stimulated by nuclear receptor steroidogenic factor-1 (SF-1), Nur77 and cAMP signal cascade via luteinizing hormone receptor (LHR). SF-1 is an essential



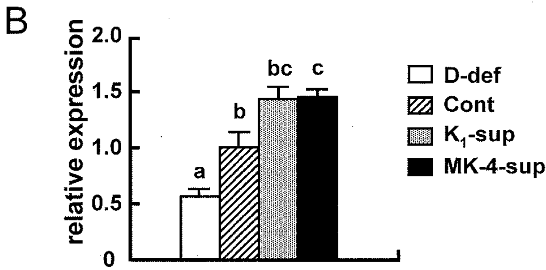


FIG. 6. Decrease in the Cyp11a protein level in the testis seen in the vitamin K-deficient group. Testis Cyp11a protein was measured by Western blot analysis described in Materials and methods. The whole testis lysate (10 μg) was separated in 12.5% SDS-polyacrylamide gel, blotted on PVDF membrane, then Cyp11a was detected with anti-rat Cyp11a antibody (panel A). β-Actin was used as loading control. Panel B; the data are expressed as mean$\pm$SEM, $n=3$–5. Values with different superscript letters are significantly different at $P<0.05$.

factor for steroid hormone synthesis (Parker, et al., 1996; Luo, et al., 1999; Jeyasuria, et al., 2004). On the other hand, proinflammatory cytokine tumor necrosis factor α (TNFα) repress the expression of Cyp11a mRNA through the activation of NFκB signal cascade (Hong, et al., 2004). The amount of mRNA encoding SF-1, Nur77, LHR and TNF receptor in testis was not different among the groups (data are not shown). The inhibition of Nur77 transactivation by activated NFκB was likely due to the competition of NFκB subunit p65 for Nur77 binding with coactivators in rat Leydig cells (Hong, et al., 2004). The signal transduction pathway toward the activation of NFκB is inhibited by protein kinase A. Otsuka et al. showed that MK-4 inhibits proliferation and invasiveness of hepatocellular carcinoma via the stimulation of protein kinase A (Otsuka, et al., 2004). Therefore, MK-4 may contribute to maintain the level of Cyp11a mRNA through the inhibition of NFκB pathway in testis. Similar inhibitory effect could be found in our study (macrophage and the rat model (Ohsaki, et al., 2006)) and other researcher's study that VK can suppress inflammatory cytokine interleukine-6 (IL-6) expression induced by lipopolysaccharide (LPS) administration in human fibroblast (Reddi, et al., 1995). Stimulation of IL-6 expression in LPS treated cells is said to be due to the activation of NFκB.

Reduced testosterone concentration in testis and plasma in vitamin K deficient condition

We measured plasma and testis testosterone concentration because Cyp11a mRNA and protein levels were significantly decreased in K-def group compared with Cont and MK-4-sup groups. Testosterone concentration in both testis and plasma was also significantly decreased in K-def group compared with MK-4-sup group (Fig. 7). These results suggest that testicular MK-4 can influence testosterone production through participation in the regulation of Cyp11a gene expression.

Osteoporosis was widely seen in aged women in most cases, however, the decreased testosterone level in blood is frequently observed in elderly men and thought to be one of the pathogenic markers of osteoporosis (Weber, et al., 2004 ; Finkelstein, et al., 1987). On the other hand, it has been reported that one population of elderly persons are under subclinical state of VK deficiency when their VK condition was evaluated by serum undercarboxylated osteocalcin (Binkley, et al., 2000). MK-4 is prescribed as the therapeutic and preventive agent

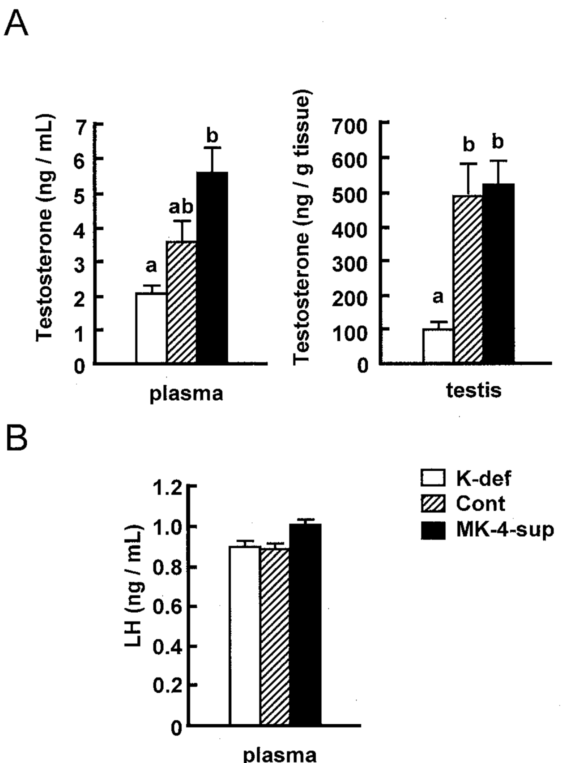


FIG. 7. Reduction of plasma and testis testosterone level in vitamin K deficient rats.
Testosterone in plasma and testis were extracted by chloroform and measured by ELISA (panel A). Plasma luteinizing hormone was measured by ELISA (panel B). The data are expressed as mean±SEM, $n=5$-12, plasma ; $n=5$, testis. Values with different superscript letters are significantly different at $P<0.05$.

against osteoporosis because MK-4 increased the bone mass by acting directly on osteoblast and osteoclast. In addition to this fact, MK-4 could maintain normal testosterone production in the testis to prevent the occurrence of osteoporosis in elder men.

In conclusion, we found the decrease of testosterone production in the testis of vitamin K deficient condition through decreased expression of Cyp11a. Vitamin K, especially MK-4, one of the VK2 analogues abundantly detected in testis, contributes to maintain normal testosterone production in rat testis. The mechanism is still unclear, but knowledge provided from further experiments would increase understanding of the relationship between VK condition and disease prevention.

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