Changes in serum osteocalcin, plasma phylloquinone, and urinary γ-carboxyglutamatic acid in response to altered intakes of dietary phylloquinone in human subjects¹⁻⁴

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ABSTRACT  The response of osteocalcin and other biochemical markers of vitamin K status to diets formulated to contain different amounts of phylloquinone was assessed in nine healthy subjects aged 20–33 y. Subjects resided in a metabolic ward for two 15-d cycles with a minimum of 6 wk between cycles. A mixed diet containing 100 μg phylloquinone/d was fed throughout both cycles; however, the phylloquinone content of one of the cycles was increased to a total of 420 μg/d on days 6 through 10 by fortifying corn oil in the diet with phylloquinone (supplemented diet). Total serum osteocalcin concentrations were not affected by either of the dietary treatments. The percentage of undercarboxylated osteocalcin increased an average of 28% over the 15-d cycle with the mixed diet (P < 0.05) and declined significantly an average of 41% with 5 d of the supplemented diet (day 6: 21.9 ± 1.3%, day 11: 12.8 ± 1.1%; P = 0.0001) with a rise after the return to the mixed diet (16.7 ± 1.3%, P < 0.001). Plasma phylloquinone concentrations increased significantly with supplementation (day 6: 0.95 ± 0.16 nmol/L, day 11: 1.40 ± 0.29 nmol/L, P < 0.001) and then rapidly returned to presupplementation concentrations on returning to the mixed diet. Twenty-four-hour ratios of urinary γ-carboxyglutamic acid to creatinine were unchanged with the supplemented diet; however, excretion declined to 91 ± 2% of baseline after 10 d on the mixed diet (P = 0.01). These results show that undercarboxylated osteocalcin, plasma phylloquinone, and urinary γ-carboxyglutamic acid excretion appear to be sensitive measures of vitamin K nutritional status because all of these variables were responsive to changes in dietary intake. Am J Clin Nutr 1997;65:779–84.

KEY WORDS  Dietary phylloquinone, serum osteocalcin, undercarboxylated osteocalcin, urinary γ-carboxyglutamic acid, plasma phylloquinone, human subjects

INTRODUCTION

Vitamin K functions as a cofactor for the posttranslational synthesis of the amino acid γ-carboxyglutamic acid (Gla) from glutamic acid. Prothrombin; factors VII, IX, and X; and proteins C and S are vitamin K–dependent proteins whose roles are well established as essential components in the blood clotting cascade (1, 2). Recent discoveries of vitamin K–dependent proteins, such as osteocalcin, matrix Gla protein (MGP), and protein S in tissues other than the liver, the synthesis site of the coagulation factors, as well as widespread distribution of MGP mRNA and the vitamin K–dependent carboxylase, suggest that potential new functions exist for vitamin K (2–7). One such potential function is in skeletal metabolism because of the numerous vitamin K–dependent proteins found in bone (8). Although the specific roles of these proteins are unclear, there is evidence linking vitamin K status to bone mineral density and to the risk of hip fracture (9, 10).

Osteocalcin, a 5700-Da protein synthesized in bone and dentin, has been the most widely studied link between vitamin K metabolism and bone metabolism (2). Assessment of the carboxylation state of this protein has shown promise as a sensitive marker of vitamin K nutritional status. Undercarboxylated osteocalcin may respond to alterations in vitamin K nutritional status, increasing with oral anticoagulant therapy and decreasing with phylloquinone supplementation in subclinically deficient populations (11–21). Undercarboxylated osteocalcin has also been reported to have a negative association with plasma phylloquinone concentrations (22).

Previous research has examined undercarboxylated osteocalcin, and other vitamin K indexes, in the context of vitamin K deficiency induced by dietary restriction or by oral anticoagulant therapy, as well as in relation to short-term vitamin K supplementation (11–21, 23, 24). The response of biochemical markers of vitamin K status to a diet formulated to contain variable amounts of phylloquinone has not been specifically explored. In the past, lack of food-composition data for vitamin K has limited research efforts. However, a recently published

¹ From the Jean Mayer United States Department of Agriculture Human Nutrition Research Center on Aging at Tufts University, Boston.
² Supported by the United States Department of Agriculture Research Service (contract no. 53–3K06–5–10).
³ The contents of this publication do not necessarily reflect the views or policies of the US Department of Agriculture nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government.
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database, which has expanded the knowledge of the vitamin K content in foods, has allowed the formulation of defined diets as well as estimations of usual intakes of vitamin K (25).

The purpose of the present study was to evaluate undercarboxylated osteocalcin as well as other biochemical markers of vitamin K nutritional status in response to dietary supplementation with phylloquinone. Specifically, a mixed diet containing 100 μg phylloquinone/d was compared with a mixed diet supplemented with phylloquinone to concentrations of 420 μg/d. The dietary supplementation was achieved by fortifying corn oil sources for mixed dishes in the diet.

SUBJECTS AND METHODS

Subjects and study design

The 10 healthy subjects (five males, five females) who participated in this study ranged in age from 20 to 33 y (x ± SD: 28.7 ± 4.6 y), with an average body mass index (kg/m²) of 25.1 ± 3.1. The research protocol was approved by the Tufts University Human Investigation Review Committee and writer informed consent was obtained from each subject. All subjects accepted into this study had normal physical examinations and laboratory test results [standard clinical chemistry and hematologic profiles, urinalysis, prothrombin time, and activated partial thromboplastin time (APTT)]. Subjects who were smokers or used oral contraceptive agents were excluded. None of the subjects had any diseases or were taking any medications, including oral anticoagulants, known to affect vitamin K or bone metabolism. In addition, no subjects used antiinflammatory medications, including aspirin, on a regular basis; used anticonvulsants, barbiturics, or phenobarbital within the past 6 mo; or took vitamins or antibiotics within 1 mo of or during the study periods.

This study was part of a larger study examining the bioavailability of phylloquinone from different food sources. Changes in serum carotenoid profiles in response to the study protocol were reported previously (26). Subjects resided in a metabolic ward for two 15-d cycles with a minimum of 6 wk of washout between cycles. The order of the two cycles was randomized. Throughout the entire study the subjects were fed a weight-maintaining, mixed diet (3-d rotating menu) based on foods that are commonly consumed by Americans (27), which contained ~100 μg phylloquinone/d. Energy balance was maintained by using non-phylloquinone-containing foods such as bread, rice, and carbonated beverages; thus, phylloquinone intake was kept uniform among participants. The phylloquinone-rich sources included green peas and beans, tomato sauce, vegetable juice, and pumpkin muffins, and were distributed between lunch and dinner. A more detailed description of the diet was reported elsewhere (28). During one of the cycles, the phylloquinone content of the diet was increased to 420 μg phylloquinone/d on days 6–10 by fortifying the corn oil in the mixed diet with phylloquinone (Sigma Chemicals, Inc, St Louis). Corn oil, with little endogenous phylloquinone, was fortified to maintain the integrity of the diet. The fortified oil was stored in opaque containers as were the foods or dishes containing phylloquinone to prevent photodegradation of phylloquinone (29). Duplicate meals from one day of the study for each cycle and subject were prepared, homogenized, and analyzed for phylloquinone to verify the phylloquinone content.

The phylloquinone in the diet was split equally between the lunch and dinner meals and the diet contained a mean (± SD) of 25.6 ± 4.3% of energy from fat and met the recommended dietary allowance (RDA) for all other macro- and micronutrients (30). Subjects were allowed one cup of instant coffee per meal if they so chose. No other supplemental food or beverages were allowed during the entire study with the exception of deionized water.

Twenty-four–hour urine specimens were collected daily throughout the study for the measurement of urinary Gla and creatinine, and fasting blood specimens were obtained on days 1, 2, 4, 6, 7, 9, 11, 12, 14, and 16. Serum total and undercarboxylated osteocalcin, and plasma phylloquinone measurements were assessed on all days on which blood specimens were drawn whereas prothrombin time and APTT were only assessed on days 1, 6, 11, and 16. If subjects fell outside the safety criterion of an abnormal clotting time, they were not allowed to continue the study.

Methods

Prothrombin time and APTT were determined by photooptical detection with an MLA Electra 800 automated clot timer (Medical Laboratory Automation, Inc, Pleasantville, NY) with reagents from Dade Diagnostics (Miami). Serum osteocalcin and undercarboxylated osteocalcin were quantitated before and after treatment with BaSO₄ by radioimmunoassay using a rabbit polyclonal antibody raised against purified bovine bone osteocalcin that recognized both native and undercarboxylated osteocalcin (11). Phylloquinone in food composites and in plasma with EDTA was analyzed by reversed-phase HPLC using post-column solid-phase chemical reduction of phylloquinone to its hydroquinone followed by fluorometric detection (31). Sample preparation for both plasma and the food composites was described previously (31, 32). Urinary Gla was determined by ortho-phthalaldehyde derivatization followed by reversed-phase HPLC with fluorometric detection (33). Urinary creatinine was analyzed by a colorimetric method on a Cobas Mira (Roche Instruments, Belleville, NJ). The urinary Gla data are expressed as a percentage of baseline values and presented as means of 3-d moving averages from each subject.

Statistics

The analysis group for this study consisted of only nine subjects (five males, four females) because data from one subject was excluded because of the adverse effect of hemoysis on the osteocalcin assay (34). Time and treatment effects were assessed for all variables by using repeated-measures analysis of variance comparing the completion of the mixed diet (day 6), the end of the supplementation period (day 11), and the end of the study (day 16) after the return to the mixed-diet-only phase. If the time-by-treatment interaction was significant (P < 0.05), paired Student’s t tests were performed to establish differences within and between treatments. Statistics were performed on logarithmically transformed values for plasma phylloquinone because of its non-Gaussian distribution. Analyses were performed by using SPSS (version 4.1 for VAX/VMS; SPSS Inc, Chicago).
RESULTS

Prothrombin time and APTT

Clotting times, prothrombin time, and APTT were measured in the nine young subjects in response to the mixed diet and the mixed diet supplemented with phylloquinone. Clotting times did not change with the mixed diet [day 1 (mean ± SEM): 12.4 ± 0.1 s]. For the supplementation period, prothrombin time decreased by 1.5% after the 5-d supplementation, from 12.6 ± 0.1 to 12.4 ± 0.1 s (P < 0.05), with an average difference of 0.25 s. The subsequent return to the mixed diet did not affect the prothrombin time (12.4 ± 0.1 s). The only effect of the increased dietary vitamin K on APTT was a decline from the beginning of the supplementation phase to the end of the study (day 6: 32.2 ± 0.9 s, day 16: 30.8 ± 0.6 s; P < 0.01).

Serum osteocalcin

Serum total osteocalcin concentrations (Figure 1) were not affected by either of the dietary treatments. The percentage of undercarboxylated osteocalcin did not change from day 6 to the end of the study when the subjects were on the mixed diet, although over the entire 15-d period there was a significant 28% increase (P < 0.05, Figure 2). Concentrations declined on average of 41% over the 5-d phylloquinone supplementation period (day 6: 21.9 ± 1.3%, day 11: 12.8 ± 1.1%; P < 0.0001) and then rose significantly after the return to the mixed diet (day 16: 16.7 ± 1.3%, P < 0.001 compared with day 11). At the end of the study cycle, concentrations had not yet returned to presupplementation values (P < 0.01) and were also lower than those of the mixed diet cycle (day 16: 26.7 ± 2.0%; P < 0.001).

Plasma phylloquinone

Plasma phylloquinone concentrations (Figure 3) did not change during the mixed diet treatment cycle after the baseline period, although there was a significant decline from days 1 to 6 (day 1: 0.95 ± 0.16 nmol/L, day 6: 0.52 ± 0.08 nmol/L; P < 0.01). In support of the undercarboxylated osteocalcin results, plasma phylloquinone concentrations increased with the fortification.

FIGURE 1. Serum total osteocalcin concentrations (mean ± SEM) in nine young subjects fed a mixed diet (●) and a supplemented diet (○). Vertical lines delineate the supplementation phase. Concentrations in the five males were significantly greater than those of the four females (P < 0.05); however, there were no treatment or time differences.

FIGURE 2. Percentage of undercarboxylated osteocalcin (mean ± SEM) in nine young subjects fed a mixed diet (●) and a supplemented diet (○). Vertical lines delineate the supplementation phase. Concentrations in the supplemented cycle were significantly different on days 6 and 11 (P = 0.0001), days 11 and 16 (P < 0.001), and days 6 and 16 (P < 0.01). Concentrations differed between the two cycles on days 11 (P = 0.0001) and 16 (P < 0.001).

FIGURE 3. Plasma phylloquinone concentrations (mean ± SEM) in nine young subjects fed a mixed diet (●) and a supplemented diet (○). Vertical lines delineate the supplementation phase. Concentrations in the supplemented cycle were significantly different on days 6 and 11 (P < 0.001), and days 11 and 16 (P = 0.01). Concentrations differed significantly between the two cycles on day 11 (P < 0.01).

Ratio of urinary Gla to creatinine

The initial, baseline ratio of urinary Gla to creatinine concentrations (means of days 1–3) were 2.84 ± 0.12 μmol Gla/mmol creatinine and 2.85 ± 0.11 μmol Gla/mmol creatinine for the mixed diet and supplemented groups, respectively. Urinary Gla was normalized to creatinine to account for differences in lean body mass between subjects. Presented in Figure 4 are ratios of urinary Gla to creatinine concentrations expressed as a percentage of baseline values. With dietary
supplementation to 420 µg phylloquinone/d, urinary Gla excretion did not significantly vary. However, on the mixed diet, Gla excretion declined significantly to 91 ± 2% of baseline values after 10 d on the diet (P < 0.01) compared with the first 5 d with no subsequent changes at the end of the study cycle compared with days 5 or 10.

Sex effects

There were no sex differences in treatment effects or changes with time for any of the variables studied. Total osteocalcin concentrations were greater on average for the males than for females, however (males: 12.1 ± 0.7 µg/L, females: 10.1 ± 0.5 µg/L; P < 0.05).

DISCUSSION

Traditionally, vitamin K status was assessed with prothrombin and other clotting times. These measures are insensitive to small changes in vitamin K nutritional status because prothrombin times do not change until the concentration of prothrombin has decreased 50% (35). With the potential for new functions for vitamin K and the vitamin K–dependent proteins, new and more sensitive measures are warranted. The ability to detect subclinical vitamin K deficiency is of interest because frank deficiencies of vitamin K, with corresponding bleeding complications, are a rare occurrence in the healthy general population (35). More current indicators of vitamin K status include plasma phylloquinone and prothrombin induced by vitamin K absence (PIVKA)-II concentrations, urinary Gla excretion, as well as the carboxylation state of osteocalcin (36). The utility of prothrombin time and undercarboxylated osteocalcin as markers of vitamin K status were compared in a previous study in which subjects received minidose warfarin for 7 d and then were repleted with 5 mg phylloquinone (11). The percentage of undercarboxylated osteocalcin increased an average of 170% with the warfarin therapy whereas concentrations dropped 17% below baseline values with phylloquinone repletion. In contrast, the prothrombin time did not leave the reference range. Additional studies have also shown the effectiveness of undercarboxylated osteocalcin as an indicator of vitamin K nutritional status in situations of vitamin K antagonism with varying doses of warfarin and with supplementation (10–17, 19–21). In addition, undercarboxylated osteocalcin was also shown to positively correlate with plasma PIVKA-II concentrations and to negatively correlate with plasma phylloquinone concentrations (22).

During one cycle of this study vitamin K nutritional status was altered by dietary supplementation with phylloquinone. After the 5-d supplementation period, the percentage of undercarboxylated osteocalcin declined an average of 41%, which can be attributed to the undercarboxylated portion because total concentrations were not altered. In comparison, the prothrombin time declined only 1.5% whereas the APTT decline was not significant. These results provide further evidence that undercarboxylated osteocalcin is a reliable marker of vitamin K nutritional status and more sensitive to small alterations than are clotting times. Despite the very small decrease in prothrombin time, this change appeared to be significant in the nine people reported on here. However, this significant difference was not substantiated in the larger study from which these subjects were chosen. Dietary factors alone may be capable of affecting the prothrombin time, although it is unlikely that these changes would be observed in the general population not studied under controlled conditions or on similar diets. The average clotting times for the nine young subjects in this study did not change during the 15 d when they consumed the mixed diet. These data agree with the reasoning behind the current RDA for vitamin K that 1.0 µg vitamin K · kg body wt⁻¹ · d⁻¹ is sufficient to maintain normal blood clotting (30). Unlike the clotting results, carboxylation of osteocalcin is not maximized on a mixed diet containing 100 µg phylloquinone. The percentage of undercarboxylated osteocalcin increased significantly by 28% after the 15 d on the mixed diet and there was a 41% decrease with 5 d of increased phylloquinone intake. The degree of carboxylation then increased again after the return to the lower intake of phylloquinone. A recent report of the vitamin K status in 18 healthy adult men concluded similarly that their intakes of one to two times the RDA were not sufficient to maximize the carboxylation of osteocalcin (21). The relation between the carboxylation state of osteocalcin and bone health in healthy subjects remains to be elucidated. In a cohort of elderly institutionalized women however, the negative association between undercarboxylated osteocalcin and bone mineral density of the hip implies that greater carboxylation is advantageous (10). As a corollary, the risk of hip fracture was increased six times in these same women with elevated undercarboxylated osteocalcin (9).

The differences observed here between liver and bone requirements for vitamin K may reflect the different sensitivities of the biochemical measures discussed previously. Another potential explanation is the fact that the liver is the major organ for uptake of dietary vitamin K after absorption from the small intestine (37). Extrahepatic tissues may consequently have increased requirements. Hepatocyte and osteoblast metabolism of vitamin K may also differ. In fact, bone does not appear to be the only tissue with different vitamin K requirements. Degradation of osteocalcin and MGP contributes < 10% to the total daily urinary excretion, whereas the Gla component of the clotting factors contributes an estimated 60–80% (23, 38). Therefore, there appears to be Gla-containing proteins not yet
discovered or known proteins with more universal roles (23). In this study, urinary Gla excretion responded to changes in phylloquinone intake with a decline on the mixed diet (100 μg phylloquinone/d) and no change when intakes were increased to 420 μg phylloquinone/d. Little change in the clotting factors and the small contribution of osteocalcin to Gla excretion is further evidence for more widespread existence of vitamin K–dependent proteins.

The urinary Gla results from this study as well as the undercarboxylated osteocalcin results suggest that full carboxylation of bone and other vitamin K–dependent proteins may not be realized with the mixed diet. However, this study was not designed to determine requirements. In 5 d, the percentage of undercarboxylated osteocalcin dropped ~50% with a rapid decline that showed no signs of a plateau. It is unknown, but appears likely that there could be a continued decline with an extended supplementation period potentially achieving full carboxylation or to the minimum detectable dose of the assay. Intermediate phylloquinone intakes, realizing similar results, are also possible. Clearly, further research is required. Further study is also necessary to determine whether the responses to dietary supplementation in older subjects are similar to those observed here in young subjects. It is possible that they may differ because a previous report from this laboratory showed younger subjects to be more sensitive to dietary vitamin K depletion than older subjects (23).

Plasma phylloquinone concentrations were used to estimate usual dietary intakes of phylloquinone because concentrations are thought to reflect recent dietary intake (23) and plasma phylloquinone concentrations have been weakly correlated with intakes, assessed by using 3-d diet records in postmenopausal women (39). In this study, plasma phylloquinone concentrations declined significantly after the 5-d baseline period when subjects were consuming a diet containing 100 μg phylloquinone/d. Intakes before study entry were, therefore, presumably greater than the currently recommended intakes.

In summary, undercarboxylated osteocalcin appears to be a sensitive marker of vitamin K nutritional status based on changes observed with dietary supplementation. In addition, the nutritional status of young subjects was improved with increased dietary intakes of phylloquinone based on measures of undercarboxylated osteocalcin, plasma phylloquinone, and urinary Gla excretion. This study also suggests that a diet sufficient to maintain normal blood clotting may not be able to maximize carboxylation of osteocalcin and other vitamin K–dependent proteins. Further research is necessary to determine optimum intakes of phylloquinone and to further define the relation between vitamin K nutritional status and bone health.

We gratefully acknowledge Jacqueline M Charnley for developing the study diet, the Metabolic Research Unit staff of the Jean Mayer USDA HNRC at Tufts University, Robert Russell for his medical assistance in monitoring the volunteers, and Kristina Nordensten for her technical assistance. Finally, we thank the volunteers who participated in this study.

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