ABSTRACT Biological markers indicative of poor vitamin K status have been associated with a greater risk for hip fracture in older men and women. However, the dietary phylloquinone intake required to achieve maximal carboxylation of hepatic and extrahepatic vitamin K–dependent proteins is not known. In an 84-d study in a metabolic unit, 21 older (60–80 y) women were fed a phylloquinone-restricted diet (18 µg/d) for 28 d, followed by stepwise repletion of 86, 200 and 450 µg/d of phylloquinone. Plasma phylloquinone, urinary γ-carboxyglutamic acid excretion and γ-carboxylation of hepatic (prothrombin) and extrahepatic proteins (osteocalcin) decreased in response to phylloquinone restriction (P < 0.001), consistent with the production of subclinical vitamin K deficiency. The γ-carboxylation of prothrombin was restored to normal levels in response to phylloquinone supplementation at 200 µg/d. In contrast, all other biochemical markers of vitamin K status remained below normal levels after short-term supplementation of up to 450 µg/d of phylloquinone. These data support previous observations in rats that hepatic vitamin K–dependent proteins have preferential utilization of phylloquinone in response to phylloquinone dietary restriction. Moreover, our findings suggest that the current recommended Adequate Intake levels of vitamin K (90 µg/d) in women do not support maximal osteocalcin γ-carboxylation in older women.

KEY WORDS: vitamin K deficiency, undercarboxylated prothrombin, undercarboxylated osteocalcin, urinary γ-carboxyglutamic acid, adequate intake

Vitamin K is a cofactor for the conversion of specific glutamyl residues to γ-carboxyglutamyl (Gla) residues in certain proteins (1). On the basis of representative dietary intake data, the Adequate Intake (AI) for vitamin K was recently set at 120 and 90 µg/d for men and women, respectively (2). In previous metabolic studies of various age groups, dietary restriction of phylloquinone (vitamin K-1) to <35 µg/d caused rapid decreases in plasma phylloquinone (3–5) and urinary excretion of Gla residues, a measure of turnover of all vitamin K–dependent proteins (3–5), and increases in the under-γ-carboxylated forms of the vitamin K–dependent proteins, osteocalcin (OC) (5) and prothrombin (3–5). Collectively, these data are consistent with the development of subclinical vitamin K deficiency. However, the clinical implications of these changes in individual biological markers are not known because coagulation times, the classic measure of vitamin K status, consistently remained stable.

Low dietary intakes of vitamin K and biological markers indicative of poor vitamin K status have been associated with a greater risk for hip fracture (6–8) and age-related bone loss (9). Interpretation of these epidemiologic studies has been limited by an incomplete understanding of the physiologic importance of the biological markers used. Furthermore, the optimal amount of dietary phylloquinone intake required to achieve maximal carboxylation of hepatic vitamin K–dependent proteins, such as prothrombin, is not known (2). An intake of 1000 µg/d in the form of a supplement has been reported as the optimal dose to maximally γ-carboxylate OC (10). Plasma phylloquinone, undercarboxylated prothrombin (PIVKA-II) and under-γ-carboxylated osteocalcin (ucOC) respond to dietary supplementation (5,11). The response of urinary Gla excretion to dietary supplementation is not consistent across studies (3,4,12). However, there is a lack of comprehensive dose-response data, particularly in older subjects, for these vitamin K status measures within the same study.

The objective of this study was to determine the response of a battery of vitamin K status markers to a stepwise depletion-repletion of dietary phylloquinone among older women.

SUBJECTS AND METHODS

Subjects. Women, aged 60–80 y (n = 21), were recruited from the general population. All subjects were in good health, and fulfilled...
the following eligibility criteria: 1) no history of cardiovascular, hepatic, gastrointestinal or renal disease; 2) no alcoholism; 3) no antibiotic or supplemental vitamin and/or mineral use within 4 wk before the start of the study; 4) no smoking; and 5) no current use of exogenous hormones. The Institutional Review Board of Tufts University and the New England Medical Center approved the study protocol, and written informed consent was obtained from each subject.

**Experimental design.** Each subject resided in the Metabolic Research Unit at the Jean Mayer U.S. Department of Agriculture Human Nutrition Research Center on Aging at Tufts University for 84 consecutive days. The study consisted of five dietary phases: phase 1 (14 d), a baseline study period that supplied 90 μg phylloquinone/d, which is the current Adequate Intake (AI) recommendation for women; phase 2 (28 d), a vitamin K depletion period that supplied 18 μg phylloquinone/d; phases 3, 4, and 5 (all 14 d), vitamin K repletion phases supplying 86, 200 and 450 μg phylloquinone/d, respectively. Upon discharge from the study, all women participating in the study were given vitamin K (single dose of 5 mg; Mephyton, Merck, West Point, PA) to ensure adequate repletion of vitamin K stores.

To investigate the possible effects of vitamin K status on 1,25-dihydroxy vitamin D–induced changes in intestinal Ca absorption and bone turnover markers, subjects were randomized into two groups; one group (n = 11) received calcitriol (cholecalciferol) and the other group (n = 10) was not treated. Calcitriol (Rocaltrol, Hoffman-LaRoche, Nutley, NJ), 1 μg/d was given to the 11 women assigned to the calcitriol group only during the last 7 d of study phases 1, 2 and 5. The analyses of measures of calcium metabolism will be described in a separate report.

**Diet.** As described in greater detail elsewhere (5), a weight-maintaining diet (using 3-d rotating menus) was provided throughout the study, except fortified breakfast cereal was removed for the purpose of this study, and a daily multivitamin was provided to adjust all nutrients to recommended levels, with the exception of phylloquinone and calcium. The calcium content of the diet (mean ± SD) was calculated to be 598 ± 5.0 mg/d. For the baseline (90 μg phylloquinone/d) and repletion diets (86, 200 and 450 μg phylloquinone/d), additional purified sources of phylloquinone (Sigma Chemical, St. Louis, MO) were added to the corn oil used in the preparation of muffins, which were given to subjects as part of the breakfast meal. Otherwise, the composition of the repletion diets was identical to the depletion diet. All corn oil used in this study was purchased from a single lot and protected from light.

With the exception of the phylloquinone, the nutrient composition of the diets was calculated with the use of Minnesota Nutrient Data Systems Software (version 4.01, Nutrient Data System, University of Minnesota Nutrition Coordinating Center, Minneapolis, MN). For confirmation of phylloquinone content, replicates of each meal (based on a daily 8.368 MJ intake) were prepared as for consumption, and the entire contents of each single-day menu was homogenized in a stainless steel blender (Waring Products Division, New Hartford, CT). Aliquots were then frozen at −20°C and protected from light until the time of phylloquinone analysis. To confirm stability of the fortified corn oils used in the muffins, oil samples were routinely analyzed for phylloquinone throughout the study.

**Blood and urine sampling.** After a 12-h overnight fast, blood samples were collected between 0630 and 0830 h on d 1, 6, 14 (phase 1), 17, 20, 22, 26, 29, 32, 34, 36, 38, 42 (phase 2), 50, 56 (phase 3), 64, 70 (phase 4), 76, 78 and 84 (phase 5). Prothrombin time (PT) and activated partial thromboplastin time (APTT) were assessed for all days on which blood samples were collected. All other blood measures (plasma phylloquinone, PIVKA-II, and ucOC) and 24-h urine collections (Gla and creatinine) were assessed on d 1, 14 (phase 1), 42 (phase 2), 56 (phase 3), 70 (phase 4) and 84 (phase 5). In addition, on d 6 (phase 1), 34 (phase 2) and 76 (phase 5), blood samples were obtained 3 and 6 h after intake of the phylloquinone-fortified muffin and used to measure postprandial plasma phylloquinone concentrations. Aliquots of all samples were stored at −70°C and were protected from light and multiple freeze-thaw cycles until analysis.

**Laboratory analysis.** Plasma phylloquinone concentrations were determined by reverse-phase HPLC using postcolumn reduction and fluorometric detection (13). The lower limit of detection for phylloquinone using this assay is 0.02 nmol/L. The HPLC methodology used for the phylloquinone analysis of metabolic diets and the individual oils has been described elsewhere (13,14).

PT and APTT were determined by photometric detection with a MLA Electra 800 automated clot timer (Medical Laboratory Automation, Pleasantville, NY) using reagents from Dade Diagnostics (Miami, FL). PIVKA-II was assayed in citrated plasma with an ELISA from Diagnostica Stago (Parsippany, NJ). PIVKA-II is a functional measure of the biological activity of vitamin K in a hepatic vitamin K–dependent protein. The assumption is that PIVKA-II levels are inversely related to the functionality of prothrombin.

Serum total OC and ucOC were analyzed using procedures described by Gundberg et al. (15). A RIA for ucOC uses human OC for standard and tracer and a polyclonal antibody directed to intact human OC (16). The antibody recognizes intact OC and the large N-terminal-mid molecule OC fragment. ucOC is a marker of extrahepatic vitamin K status and is determined in this assay as plasma OC that does not bind in vitro to hydroxypatite; it is expressed as a percentage of total OC (%ucOC) (15).

Serum Gla was analyzed by an enzyme-linked immunosorbent assay (ELISA from Diagnostica Stago (Parsippany, NJ). PIVKA-II is a functional measure of the biological activity of vitamin K in a hepatic vitamin K–dependent protein. The assumption is that PIVKA-II levels are inversely related to the functionality of prothrombin.

Serum total OC and ucOC were analyzed using procedures described by Gundberg et al. (15). A RIA for ucOC uses human OC for standard and tracer and a polyclonal antibody directed to intact human OC (16). The antibody recognizes intact OC and the large N-terminal-mid molecule OC fragment. ucOC is a marker of extrahepatic vitamin K status and is determined in this assay as plasma OC that does not bind in vitro to hydroxypatite; it is expressed as a percentage of total OC (%ucOC) (15).

Statistical analysis. Values are means ± SEM, unless otherwise specified. Differences were considered significant if the observed, two-sided significance level (P-value) was <0.05. Because the within-group variance was heterogeneous, a logarithmic transformation was applied to the plasma phylloquinone and PIVKA-II data before all analyses.

There was no calcitriol effect on any of the outcome measures, nor did change over time depend on whether a subject was taking calcitriol or receiving no treatment. Also, those consuming calcitriol did not have any responses that were uniformly higher or lower than those not taking calcitriol for any outcome. Therefore, for the purpose of these analyses, data were combined for the calcitriol and untreated group.

The data were analyzed using the Mixed procedure in SAS for Windows, version 8.2 (SAS Institute, Cary, NC). Two different covariance structures were fitted: compound symmetry and unstructured. Both structures fit equally well, with the Akaike Information Criterion giving a slight advantage to compound symmetry, and led to the same conclusions concerning the significance of the effects. The P-values from compound symmetry are reported here because of their equivalence to the values from a standard univariate repeated-measures analysis. Tukey’s Honestly Significant Differences was used to compare time points for those responses that changed significantly during the study period.

**RESULTS**

The age of the subjects (n = 21) was 70.4 ± 1.1 yr (mean ± SD). Body weight was 70.0 ± 2.4 kg and BMI 26.8 ± 0.9 kg/m2. The phylloquinone content of the diet analyzed in triplicate was 18 ± 4.7 μg/d (mean ± SD). The total intakes of the diet plus the muffins with phylloquinone-fortified oil were (means ± SD): 90 ± 8.0 μg/d (phase 1); 18 ± 4.7 μg/d (phase 2); 86 ± 14 μg/d (phase 3); 200 ± 18 μg/d (phase 4); and 450 ± 28 μg/d (phase 5).

All of the vitamin K status biomarkers assessed (plasma phylloquinone and PIVKA-II, serum %ucOC and urinary Gla) were changed (P < 0.05) by the end of the vitamin K depletion period. Coagulation time, as measured by PT and APTT, did not change during the study period (data not shown).

As expected, the postprandial increases in plasma phylloquinone reflected the administered dose of dietary phylloquinone given at breakfast (Fig. 1). Compared with d 1 of the study, plasma phylloquinone concentrations of fasting women (Fig. 2A) decreased markedly (P < 0.0001) during phase 1 of
the study, when 90 μg phylloquinone/d was consumed. The phylloquinone concentration in our fasting elderly subjects consuming this level of dietary phylloquinone fell below the expected normal range for this age group [0.32–2.67 nmol/L (18)]. Consumption of the vitamin K depletion diet containing 18 μg phylloquinone/d for an additional 28 d did not further reduce plasma phylloquinone concentrations of fasting women. Increasing the dietary phylloquinone intake to 86 μg/d for 2 wk during the first repletion period had no effect on phylloquinone in fasting women, nor did an increase in vitamin K intake to 200 μg phylloquinone/d for an additional 2 wk during the second repletion phase of the study. However, increasing the vitamin K intake to 450 μg phylloquinone/d for 2 wk during the final repletion phase increased (P < 0.006) plasma phylloquinone in fasting women, although plasma phylloquinone concentration did not return to prestudy levels.

Plasma PIVKA-II, a measure of prothrombin undercarboxylation, reflected the different levels of vitamin K intake during the 84-d metabolic study (Fig. 2B). Plasma PIVKA-II increased (P < 0.001) from within the normal range (≤2 μg/L) on d 1 to the abnormal range at the end of the vitamin K depletion period (Fig. 2B). PIVKA-II concentrations then decreased in a stepwise fashion with each subsequent increased level of vitamin K intake during the three repletion phases of the study. By the end of the study, plasma PIVKA-II concentration was restored to the prestudy (d 1) level.

FIGURE 1  Postprandial plasma phylloquinone concentration in elderly women 3 and 6 h after consumption of a muffin fortified with 72, 0 or 432 μg vitamin K. The postprandial measures were made in blood obtained on study d 6 (phase 1: 90 μg/d), d 34 (phase 2: 18 μg/d) and d 76 (phase 5: 450 μg/d). Values are means ± SEM, n = 21. All 6-h postprandial measures were greater (P < 0.001) than 3-h postprandial measures.

FIGURE 2  Vitamin K status measures in elderly women fed various levels of dietary vitamin K. Plasma phylloquinone (A), plasma undercarboxylated prothrombin (PIVKA-II) (B), % plasma undercarboxylated osteocalcin (C) and 24-h urinary γ-carboxyglutamic acid (Gla) excretion, expressed per mmol of creatinine (Creat) (D) in response to different intakes of phylloquinone during an 84-d depletion and repletion study in elderly women. All measures of vitamin K status differed (P < 0.0001) from prestudy (d 1) measures after consumption of the vitamin K depletion diet. Values are means ± SEM, n = 21. Means without a common letter differ, P < 0.05 (Tukey's Honestly Significant Differences).
The proportion of the undercarboxylated form of serum osteocalcin (%ucOC) was increased ($P < 0.0001$) by the end of the first 14 d of the study after consumption of the 90 µg phylloquinone/d diet (Fig. 2C). No further change in %ucOC occurred during the 30-d vitamin K depletion period or during the three vitamin K repletion phases of the study.

Urinary Gla excretion was decreased ($P < 0.004$) by the end of the month-long vitamin K depletion phase of the study (Fig. 2D). Despite increased dietary vitamin K intake during the vitamin K repletion phases, urinary Gla excretion did not increase.

**DISCUSSION**

We achieved our primary study objective of creating subclinical vitamin K deficiency in these elderly women. In addition, on the basis of the response of plasma PIVKA-II, a measure of undercarboxylation of a hepatic vitamin K–dependent protein, we also succeeded in a subacute repletion of hepatic vitamin K status. The subclinical vitamin K deficiency state produced was strongly supported by the concurrent observation of low plasma phylloquinone, elevated serum ucOC and reduced urinary Gla excretion after the dietary vitamin K depletion phase of the study. In a previous study from this laboratory (3), a marked drop in plasma phylloquinone and a significant rise in plasma undercarboxylated prothrombin occurred in both young and elderly subjects after the consumption of a vitamin K depletion diet for 13 d. However, in that study (3), no effect of vitamin K depletion on urinary Gla excretion was evident in the elderly subjects, prompting the suggestion that older persons might be relatively resistant to dietary vitamin K depletion. In contrast, in our current study of elderly women, we found a significant reduction in urinary Gla at the end of the vitamin K depletion phase of the study. The difference in urinary Gla response to feeding a low dietary vitamin K diet in the two studies may have been due to the longer vitamin K depletion period in the current study.

The dietary vitamin K intake for 2 wk before and 2 wk after the vitamin K depletion period was ~90 µg phylloquinone/d, the current recommended dietary AI level of vitamin K for women (2). Our findings clearly demonstrate that the initial consumption of the AI for vitamin K by these elderly women caused a prompt and marked decrease in plasma phylloquinone. Similar responses of plasma phylloquinone to this level of vitamin K intake in controlled feeding studies have been reported (3,5). This initial drop in plasma phylloquinone during phase 1 of the study was anticipated and likely reflects the fact that the usual dietary vitamin K intake of these older subjects was >90 µg/d. Although we did not collect food intake information from these subjects before they entered the study, dietary surveys conducted in this age group (19) indicate usual vitamin K intakes of ~130–150 µg/d. Interestingly, a further reduction in dietary vitamin K intake in these women to 18 µg/d caused no additional decrease in plasma phylloquinone, perhaps reflecting a residual more slowly turning over pool of plasma phylloquinone. Nevertheless, these low concentrations of phylloquinone were not sufficient to sustain maximal γ-carboxylation of PIVKA-II in the liver or OC in the skeleton. Unexpectedly, plasma phylloquinone did not increase after consumption of 200 µg phylloquinone/d for 2 wk during the repletion phase of the study. Other vitamin K depletion-repletion studies have demonstrated that lower dietary vitamin K intakes, such as 50 µg/d, are insufficient to restore plasma phylloquinone concentrations to baseline after a period of vitamin K depletion in both younger (3,4) and older (3) adults. However, in a previous study from our laboratory (5), plasma phylloquinone increased and the %ucOC was restored to baseline levels in response to an intake of 200 µg phylloquinone/d. We do not have a ready explanation for this unexpected finding. Two factors that differed between this study and our previous one that may have affected the response to vitamin K repletion were a shorter period of vitamin K depletion (10 d) and a younger (20–40 y) group of subjects than in the earlier study. Additional research will be necessary to evaluate these factors.

Consuming a diet containing 450 µg phylloquinone/d for 2 wk significantly increased plasma phylloquinone to 0.48 ± 0.12 nmol/L. This plasma phylloquinone concentration is lower than expected on the basis of our recent report concerning the relation between plasma phylloquinone and usual dietary phylloquinone intakes from a cohort of older men and women from the Framingham Heart Study (20). In that study, we found that plasma phylloquinone was ~1 nmol/L at median vitamin K intakes > 150 µg/d, whereas plasma phylloquinone in the range of 0.5–1.0 nmol/L was observed at estimated usual vitamin K intakes ≥ 50 µg vitamin K/d. Furthermore, Suttie et al. (4) demonstrated in young men that a 12-d supplementation period with 500 µg vitamin K/d significantly increased plasma phylloquinone concentrations above baseline values despite a 21-d period of dietary phylloquinone depletion.

We cannot explain why in our current study plasma phylloquinone concentrations were about one half of those seen in similar aged Massachusetts people with high dietary vitamin K intakes, nor why the absolute or relative change in plasma phylloquinone was not as marked as observed by Suttie et al. (4). We suggest that the reason for the low plasma phylloquinone concentrations in fasting women may be due to the way we administered the additional phylloquinone. We gave the phylloquinone as a single bolus dose with breakfast ~24 h before collection of the fasting blood samples the following morning. Postprandial plasma phylloquinone data from this study, shown in Figure 1, confirm that the supplemented phylloquinone was absorbed from the meal, and fasting plasma PIVKA-II concentrations (Fig. 2B) confirm that the vitamin K utilized biochemically in the liver in proportion to the administered dose. Evidently, then, by 24 h after the vitamin K dose, the postprandial rise in plasma phylloquinone concentration had returned to predosing levels. This position is supported by additional evidence from our laboratory based on the plasma kinetics of ingested deuterium-labeled phylloquinone (21). We speculate that phylloquinone did not accumulate in the plasma at supplemental vitamin K doses up to 200 µg/d because the vitamin K absorbed at the higher intakes was first preferentially utilized by the liver to sustain carboxylation of hepatic vitamin K–dependent proteins, which are responsible for critical blood coagulation functioning. Thus, insufficient phylloquinone was available to maintain elevated plasma phylloquinone and to support vitamin K–dependent carboxylation in nonhepatic tissues, such as bone. This notion is supported by our observation that the hepatic protein PIVKA-II was reflected in the plasma phospholipid PIVKA-II concentrations (Fig. 2B) but ucOC, produced in skeletal osteoblasts, was related reciprocally to plasma phylloquinone (Fig. 2C and 2A). Preferential hepatic utilization of dietary vitamin K is supported by a previous study in rats by Kindberg and Suttie (22). These investigators found that liver phylloquinone concentrations were correlated with dietary phylloquinone intake, but serum phylloquinone did not increase with increased dietary vitamin K intake until the liver contained sufficient vitamin K to maintain optimal synthesis of vitamin K–dependent proteins.

In conclusion, we have shown that subclinical vitamin K deficiency can be induced in elderly women by consumption of...
low amounts of dietary vitamin K. This suboptimal vitamin K status is reflected by concomitant low plasma phylloquinone concentration and urinary Gla excretion and elevated plasma PIVKA-II and ucOC. Moreover, elevated blood levels of ucOC do not necessarily reflect suboptimal carboxylation status of hepatic vitamin K–dependent proteins, such as prothrombin, due to an apparent preferential utilization of available dietary vitamin K to support hepatic γ-carboxylation function. Finally, our current findings in elderly women support the observation (10) that vitamin K intakes greater than the recommended dietary vitamin K AI levels are required to support maximal osteocalcin γ-carboxylation.

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LITERATURE CITED