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Research Article

Dietary Induced Subclinical Vitamin K Deficiency in Normal Human Subjects

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Abstract

A subclinical vitamin K deficiency was induced in 32 healthy subjects (four groups of eight males and females) aged 20–40 and 60–80 yr residing in the Metabolic Research Unit of the Human Nutrition Research Center on Aging at Tufts University. Volunteers were initially fed (4 d) a baseline-period diet containing the recommended daily allowance for vitamin K which is equivalent to 80 μg/d of phylloquinone (vitamin K_1). During the baseline period various parameters of vitamin K nutritional status were monitored. The baseline period was followed by a 13-d depletion period during which the subjects were fed a very low vitamin K_1 diet (~ 10 μg/d). After depletion, the subjects entered a 16-d repletion period (four stages lasting 4 d each) during which time they were repleted with 5, 15, 25, and 45 μg of vitamin K_1 per day. Vitamin K_1 depletion dramatically and significantly decreased plasma vitamin K_1 levels ($P < 0.0001$) in both elderly and young groups to values 13–18% of day 1 (elderly 0.22 nM, young 0.14 nM). Repleting the subjects with up to 45 μg of vitamin K_1 per day failed, in the case of the young subjects, to bring plasma vitamin K_1 levels back into the normal range. Dietary vitamin K_1 restriction induced different responses in the urinary excretion of γ-carboxyglutamic acid between the young and the elderly subjects with values decreasing significantly ($P < 0.03$) in the young while remaining unchanged in the elderly. The vitamin K_1 depletion period had no significant effect on either prothrombin and activated partial thromboplastin times, or Factor VII and protein C (as determined by antigenic and functional assays). By using a monoclonal antibody, descarboxy prothrombin was found to increase slightly but significantly in both groups ($P < 0.05$) as a consequence of the low vitamin K_1 diet. This study clearly shows that a diet low in vitamin K_1 can result in a functional subclinical deficiency of vitamin K (decreased urinary γ-carboxyglutamic acid excretion) without affecting blood coagulation. (*J. Clin. Invest.* 1993. 91:1761–1768.) Key words: blood coagulation • γ-carboxyglutamic acid • descarboxy prothrombin • vitamin K • vitamin K deficiency • vitamin K-dependent proteins

Introduction

Vitamin K is required for the post-translational biosynthesis of γ-carboxyglutamic acid (gla)[1] from glutamic acid in several proteins involved in hemostasis (prothrombin; Factors VII, IX, and X; proteins C and S) and in other proteins not related to blood coagulation (osteocalcin or bone gla protein, and matrix gla protein) (1–4). There are two forms of vitamin K available for the synthesis of gla residues in humans. Phylloquinone or vitamin K_1 is found in plants and oils derived from plants and represents the predominant dietary source of the vitamin. The menaquinones or vitamins K_2 are synthesized by the intestinal bacterial flora. Despite frequent assumptions that part of the daily vitamin K requirement in humans is met by the bacterial synthesis of menaquinones, their relative contribution to vitamin K nutriture remains to be clarified (2, 3).

Studies that correlate vitamin K_1 intake with vitamin K status are scant and present limitations for the normal healthy population (5–12). Indeed, many of the studies published to date have involved sick, hospitalized people who suffered pathological conditions that may have altered their vitamin K requirements (6, 12). In other instances the relationship between dietary vitamin K intake and vitamin K status was studied in subjects taking antibiotics (5) or in subjects where metabolic conditions were not adequately controlled (7, 8, 11). While antibiotics are commonly used in hospital settings, estimates of dietary vitamin K requirements established under these conditions cannot be extrapolated to normal healthy individuals.

In a study involving healthy male adults not taking antibiotics, Suttie et al. (11) looked at the effects of a low dietary vitamin K_1 intake on vitamin K status by asking subjects to consume a self-selected diet that eliminated foods known to be high in vitamin K. Although the authors were able to induce a vitamin K deficiency in their volunteers, this experiment has the drawback of having been performed in free living conditions where dietary vitamin K intake was not stringently controlled. Except for the study of Allison et al. (9), which involved a limited number of male subjects, there has been no metabolically controlled study that has correlated vitamin K_1 intake with vitamin K status in normal healthy humans of both genders and varying age.

In the present study, we induced a subclinical vitamin K deficiency in 32 healthy subjects kept in a metabolically controlled environment by feeding them a diet containing ~ 10 μg of vitamin K_1 per day for 13 d. The effect of low dietary vitamin K_1 intake on the plasma levels of vitamin K_1, the synthesis of gla, prothrombin times (PT) and activated partial thromboplastin times (APTT), and functional and antigenic assays for Factor VII and protein C was studied. After the depletion period the subjects were supplemented for 16 d with increasing amounts of vitamin K_1 and their vitamin K status was evaluated. This study also examines the age-related differences in responses to dietary vitamin K_1 intakes.

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1. *Abbreviations used in this paper:* APTT, activated partial thromboplastin time; BGP, bone gla protein; gla, γ-carboxyglutamic acid; MGP, matrix gla protein; PIVKA-II, descarboxy prothrombin; PT, prothrombin time; RDA, recommended daily allowances.

Methods

This study was conducted with four groups of healthy volunteers (eight subjects per group) and stratified by gender (male or female) and age (young, aged 20–40 yr; or old, aged 60–80 yr). The duration of the study was 34 d and during that time course all of the study subjects were residents in the Metabolic Research Unit of the Human Nutrition Research Center on Aging (HNRCA) at Tufts University in Boston, MA. Volunteers for this study were recruited from the general population and most resided in the Boston metropolitan area. All subjects were in good health as determined by medical history, physical examination, and clinical laboratory analyses before enrolling in the study. Potential subjects were excluded from participation in this study if they presented with any of the following conditions at screening: history of a bleeding disorder; abnormal hematology; abnormal PT or APTT; abnormal level of protein C; history of either cardiovascular, hepatic, gastrointestinal, or renal disease; and chronic alcoholism. Smokers and pregnant or lactating women were not allowed to participate in the study. Subjects taking any of the following drugs were also excluded: anticoagulants (any history of treatment), antibiotics, anticonvulsants, barbiturates or phenobarbital (within 6 mo), aspirin or any aspirin containing medication, anti-inflammatory drugs (on a regular basis), and oral contraceptives. The study was approved by the Human Investigation Review Committee of Tufts University and the New England Medical Center. A signed informed consent was obtained from each subject. Subjects were paid a stipend for their participation in this study.

A schematic representation of the vitamin K depletion/repletion protocol is shown in Fig. 1. Upon arrival at the research center each volunteer entered a 4-d baseline period during which time they consumed a mixed diet containing ~ 100 μg of vitamin K_1 per day. Briefly, this diet consisted of a 2-d cycle menu and was designed to meet the recommended dietary allowances (RDA) for energy, protein, and all vitamins and minerals (13). The diet included food items regularly consumed by the general population (the four food groups were represented) but avoided foods known to contain large amounts of vitamin K_1 (green and leafy vegetables and vegetable oils and food products derived from these vegetables and oils). During this period of time, baseline values were determined for the various study parameters that would be monitored during the depletion and repletion stages. On the fifth day, each subject began a 13-d vitamin K_1 depletion period by consuming a 2-d cycle diet that contained ~ 10 μg of vitamin K_1 per day (actual analysis: 10.11±0.31 μg/d, n = 32). Briefly, the diet was designed to contain as little vitamin K_1 as possible while remaining palatable and providing nutrient adequacy by meeting the RDA (13) for energy, protein, minerals, and all vitamins except vitamin K_1. The diet included lean ground beef, skinless chicken breast, and skim milk in addition to food items from the other food groups with limited choices for fruits (orange, peeled apple, cranberry, and pear) and vegetables (potatoes and onions). A detailed description of this diet has been published (14). To verify that the vitamin K_1 intake did not exceed the 10 μg/d target value, a composite analysis for vitamin K_1 was determined for each subject's 2-d menu at the beginning of the depletion period. Each menu was prepared for analysis as follows: all foods and beverages contained in each day's menu were collected, transferred to a commercial stainless steel blender (Waring, New Hartford, CT) and blended for 3 min at maximum speed. Total weight was recorded and an aliquot was frozen for later analysis (see analytical section).

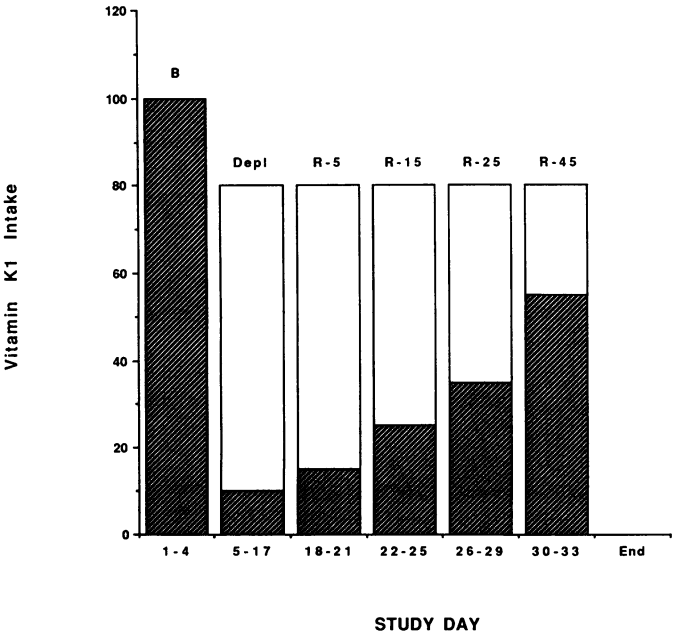

Figure 1. Study protocol. *Filled bars:* Dietary vitamin K_1 intake of corresponding study periods: (*B*) baseline (days 1–4), 100 μg/d; (*Depl*) depletion (days 5–17), ~ 10 μg/d. During the repletion period (*R-*) subjects were repleted with 5 μg (days 18–21), 15 μg (days 22–25), 25 μg (days 26–29), and 45 μg (days 30–33) of vitamin K_1 per day, for a total daily intake of 15, 25, 35, and 55 μg of vitamin K_1 respectively. *Open bars:* Vitamin K RDA.

At the end of the depletion period, volunteers entered a 16-d repletion period that consisted of four stages lasting 4 d each during which they were repleted with 5 μg (days 18–21), 15 μg (days 22–25), 25 μg (days 26–29), and 45 μg (days 30–33) of vitamin K_1 per day, for a total daily intake of 15, 25, 35, and 55 μg of vitamin K_1 respectively, as shown in Fig. 1. Total dietary vitamin K_1 for the repletion period was adjusted by oral administration of known volumes of Sustacal Liquid (Mead Johnson, Evansville, IN) containing 0.48 μg/ml of vitamin K_1. The vitamin K_1 concentration in the formula was determined by direct analysis in our laboratory by HPLC as described in the analytical section. Except for the addition of the vitamin given with the breakfast meal, the diet was identical to that used during the depletion period.

At the start of the depletion period, subjects were individually informed by a dietician of the importance to comply with the diet and to limit their food consumption to the food items that they received from the kitchen staff of the metabolic unit. Subjects were routinely visited by the investigators to assure proper conduct throughout the protocol period. The experimental diet was well tolerated by all volunteers and no subjects had to discontinue their participation in the study because of weight disturbances, clinical problems or discomfort.

Throughout the study 24-h urine collections were obtained daily and blood was drawn in the fasting state (12–14 h postprandial) every other day. Urinary gla excretion was determined daily, while plasma vitamin K_1 concentrations, PT, and APTT were determined every other day. Protein C, Factor VII (both antigenic and functional), and descarboxy prothrombin (PIVKA-II) assays were performed on days 1, 5, 18, and 34 which correspond respectively to the beginning of baseline, depletion, and repletion periods and to the last day of the study. A strict criteria was established for monitoring all subjects for vitamin K deficiency. For the purpose of this study, subclinical vitamin K deficiency was defined as any state where plasma vitamin K_1 concentration decreased below the established normal range (< 0.30 nM) and 24-h urinary gla excretion fell below baseline values for each individual subject as a result of the dietary intervention. Instances where the PT or APTT were outside of the normal laboratory range (11.0–13.7 and 22.8–33.1 s, respectively) for two consecutive determinations were defined as clinical vitamin K deficiency. Any patient presenting with a clinical deficiency would have been terminated from the study and given a 1.0-mg dose of vitamin K_1. None of the subjects had to be terminated from the study due to the development of clinical vitamin K deficiency as defined by these criteria.

Analytical procedures. Vitamin K_1 in plasma was determined by reverse-phase HPLC using postcolumn solid-phase reduction of vita-

min K_1 to its hydroquinone followed by fluorometric detection as previously described (15). The vitamin K_1 content of aliquots of the homogenized food composites was determined according to the same analytical methodology but with slightly different sample preparation. Briefly, 2.0 g of the homogenate was weighed directly into a 50-ml polystyrene screw-cap tube to which was added 2.0 ml of water, 8.0 ml of a mixture of isopropanol/hexane (3:2, vol/vol), and an appropriate amount of internal standard, dihydro-vitamin K_1. Lipids and lipophilic substances were extracted by sonicating 1 min (Sonifier Cell Disrupter 350, Branson Ultrasonics Corp., Danbury, CT). Samples were centrifuged at 3,500 *g* for 10 min, and the top layer was removed and evaporated to dryness under reduced pressure in a centrifugal evaporator (Savant Instruments Inc., Farmingdale, NY). The residue was then redissolved in 2 ml of hexane and applied to a 3.0-ml silica column (J. T. Baker Inc., Phillipsburg, NJ) which had been preconditioned by a wash of 8.0 ml of hexane. The silica column was washed with an additional 8.0 ml of hexane-diethyl ether (97:3, vol/vol). The eluant was collected and evaporated to dryness. The final residue was dissolved first in 0.045 ml of methylene chloride then with 0.255 ml of methanol containing 10 mM zinc chloride, 5 mM acetic acid, and 5 mM sodium acetate. 150 μl of sample was analyzed by HPLC.

The gla concentration in urine samples was determined by reverse-phase HPLC of the ortho-phthalaldehyde derivatives (16). PT and APTT were performed on an MLA Electra 800 automated clot timer (Medical Laboratory Automation, Inc., Pleasantville, NY) using thromboplastin C and actin reagents (Dade Diagnostic, Miami FL). Protein C antigen, Factor VII antigen, and prothrombin induced by vitamin K absence (PIVKA-II) antigenic levels in plasma were quantitatively determined by sandwich techniques using ELISA kits purchased from American Bioproducts Co. (Parsippany, NJ). Functional colorimetric assays for protein C and Factor VII were performed using STACHROM kits (American Bioproducts Co.). Functional protein C determination was conducted on a COBAS FARA centrifugal analyzer while Factor VII was measured using a spectrophotometer. Abnormal (descarboxy) forms of prothrombin and protein C were further analyzed by ELISA using the H-11 monoclonal antibody from American Bioproducts Co. In the presence of calcium, descarboxyprothrombin and descarboxyprotein C bind antibody H-11 while the corresponding fully carboxylated proteins do not (17).

Vitamins A and E and 25-hydroxyvitamin D were assessed by HPLC (18, 19). The data were examined for statistical significance using repeated-measures analysis of variance (SAS general linear procedure). A $P < 0.05$ was considered to be significant.

Results

All 32 subjects recruited for the study completed the protocol. The mean age, height, and weight were 29.0±1.2 yr, 171.7±2.5 cm, and 68.3±3.0 kg, respectively, for the young, and 70.1±1.4 yr, 168.1±3.7 cm, and 71.2±4.2 kg for the elderly.

Plasma vitamin K_1 concentrations are shown in Fig. 2. Subjects were divided according to age since the statistical analyses indicate a significant ($P < 0.01$) age difference in response to the changes in dietary vitamin K_1 intake during the study period. No gender differences were observed in plasma vitamin K_1 levels during the study period. Ingestion of the low vitamin K_1 diet induced a highly significant ($P < 0.0001$) decrease in plasma vitamin K_1 concentrations with values at the end of the depletion period (day 17) being only 13% of the values observed on day 1. Repleting subjects with 5, 15, or 25 μg of vitamin K_1 (days 18–21, 22–25, and 26–29 respectively) had virtually no effect on plasma vitamin K_1 concentrations. Only during the last stage of repletion (45 μg of vitamin K_1 per day) did plasma vitamin K_1 concentrations begin to increase slightly with values on day 33 corresponding to 30% of the values obtained for day 1. Although varying dietary vitamin K_1 intakes produced similar changes in plasma vitamin K_1 levels in both the young and elderly subjects over time, the absolute plasma vitamin K_1 concentrations were consistently higher (40%) in the elderly than in the young ($P < 0.01$). Consequently, plasma vitamin K_1 concentrations never dropped as low in the

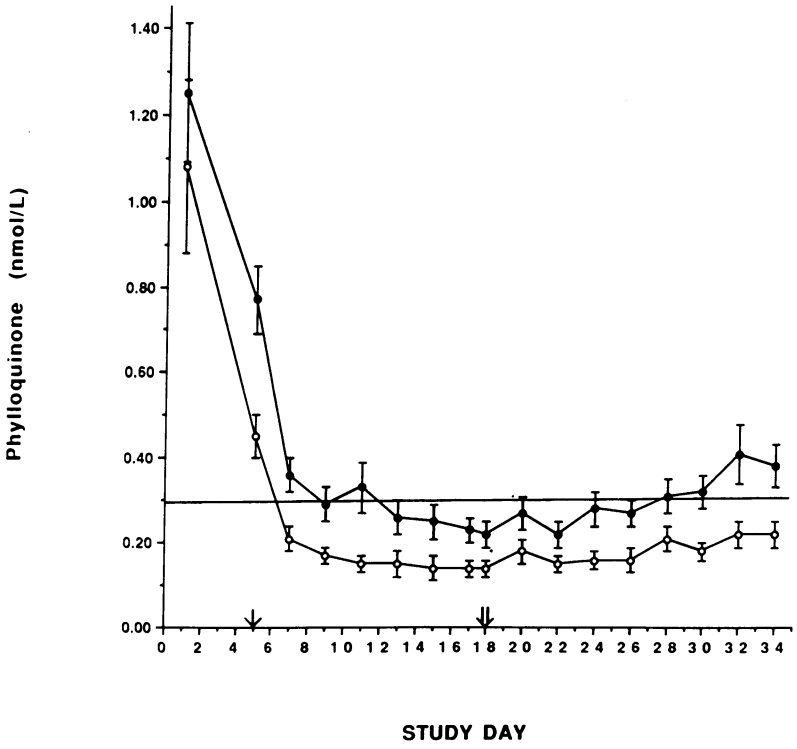

Figure 2. Fasting plasma vitamin K_1 concentrations (nmol/liter) in young (○) and elderly (●) subjects after varying dietary vitamin K_1 intakes. Values are mean±SEM, $n = 32$; arrows indicate beginning of depletion and repletion periods. Over the course of the study, vitamin K_1 levels were significantly affected by vitamin K_1 intake ($P < 0.0001$) and age ($P < 0.01$).

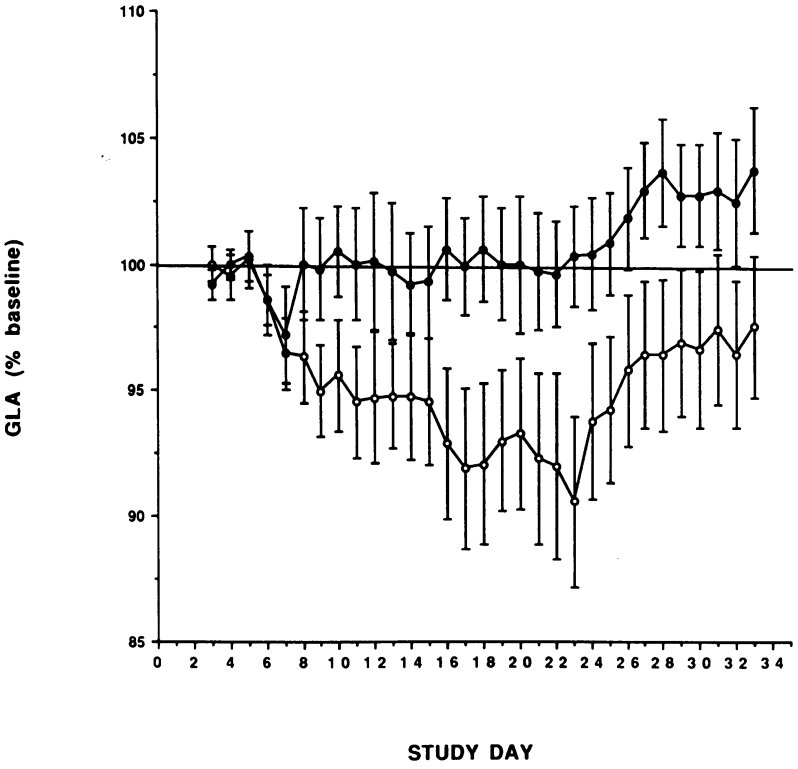

Figure 3. Effect of dietary vitamin K_1 restriction and repletion on urinary gla excretion expressed as percentage of baseline values in elderly (●) and young (○) subjects. Mean±SEM, $n = 32$. Urinary gla excretion was significantly affected by age ($P < 0.03$) and vitamin K_1 intake ($P < 0.01$) in young subjects.

elderly (0.22 nM) when compared to the young (0.14 nM). Furthermore, during the dietary vitamin K_1 restriction (days 5–17) plasma vitamin K_1 did not decrease as rapidly (when compared to day 1) in the elderly as it did in the younger subjects. On the other hand, the increase in plasma vitamin K_1 concentrations observed during the dietary vitamin K_1 repletion period (days 18–33) was more pronounced in the older subjects (from 0.22 to 0.38 nM) than in the young (from 0.14 to 0.22 nM).

Urinary gla excretion expressed as percent of baseline values obtained during the first 4 d of the study are presented in Fig. 3. Interestingly, dietary vitamin K_1 intake during the depletion period of the study produced significantly ($P < 0.03$) different responses according to age. Urinary gla excretion decreased by 10% in the young ($P < 0.01$) while in the elderly subjects urinary excretion remained unaffected by the depletion. Repletion with 5 μg/d of vitamin K_1 (days 18–21) had little effect on gla excretion, while repletion with 15, 25, and 45 μg/d of vitamin K_1 (days 22–25, 26–29, and 30–33) resulted in a gradual increase in urinary gla excretion. It is noteworthy that supplementing subjects with 45 μg of vitamin K_1 per day for 4 d did not restore urinary gla excretion to baseline levels in the young, with values at the end of the study being 96% of the values observed during the baseline period. Response in urinary gla to dietary vitamin K intakes was similar whether values were expressed as a percentage of baseline or as absolute values. Urinary concentrations (μM, mean±SEM) at baseline, end of depletion, and end of repletion were 38.5±1.5, 35.2±1.4, and 36.7±1.1, respectively, for the young and 38.2±2.6, 38.0±2.4, and 39.4±2.7 for the elderly.

PT and APTT were not significantly affected by dietary vitamin K_1 intake and the results are shown in Fig. 4. Similarly, no statistically significant changes were observed for Factor VII and protein C when evaluated by both antigenic and functional assays (Table I) and all values remained in the normal range throughout the study. Observed changes in PIVKA-II assayed using the P1-2B9 monoclonal antibody are presented in Fig. 5. PIVKA-II antigen measured on days 1 and 17 increased significantly ($P < 0.05$) as a result of the decreased intake of dietary vitamin K_1. On the other hand, the H-11 monoclonal antibody failed to detect any abnormal prothrombin or protein C (data not shown).

Discussion

Recent advances in the metabolism and function of vitamin K and vitamin K–dependent proteins clearly establish the role for vitamin K as a cofactor in the post-translational synthesis of gla. Before the discovery of gla in prothrombin, subclinical vitamin K deficiency has been diagnosed by monitoring blood coagulation times such as the PT or APTT. Clinical deficiencies have been described when bleeding disorders respond to the administration of vitamin K.

Since the discovery of gla in prothrombin and the other known vitamin K–dependent clotting factors (Factors VII, IX, and X), gla has been identified in several other proteins involved in hemostasis: proteins C, S, and Z. In addition, gla-containing proteins osteocalcin (bone gla protein or BGP) and matrix gla protein (MGP) have been identified, isolated, and characterized from bone (20). In addition to bone, messenger RNA levels for MGP have been shown to be present in such diverse tissues as heart, lung, brain, kidney, liver, and spleen (21). These results suggest a much broader role for gla-containing proteins and vitamin K outside of the hemostatic systems.

Because it is now known that the primary function for vitamin K is in the synthesis of gla residues in vitamin K–dependent proteins, assays designed to show changes in the synthesis of this amino acid should be more sensitive indicators of vita-

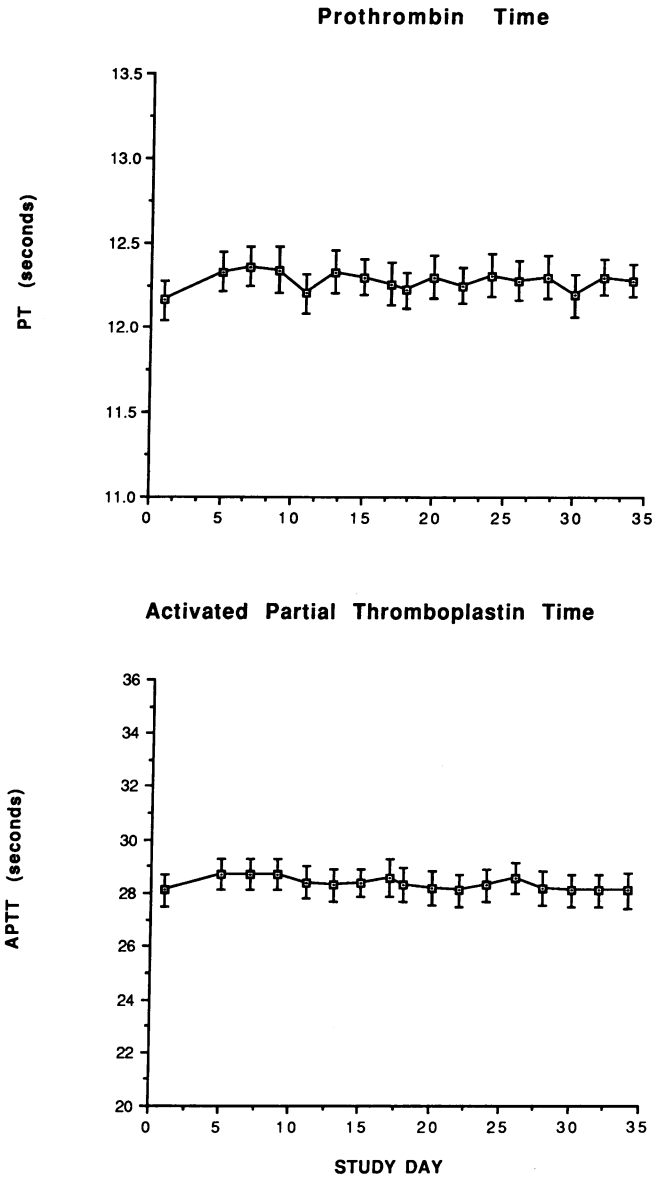

Figure 4. Effect of dietary vitamin K_1 restriction and repletion on PT and APTT. Mean±SEM, $n = 32$. Both PT and APTT were not significantly affected by dietary vitamin K_1 intake.

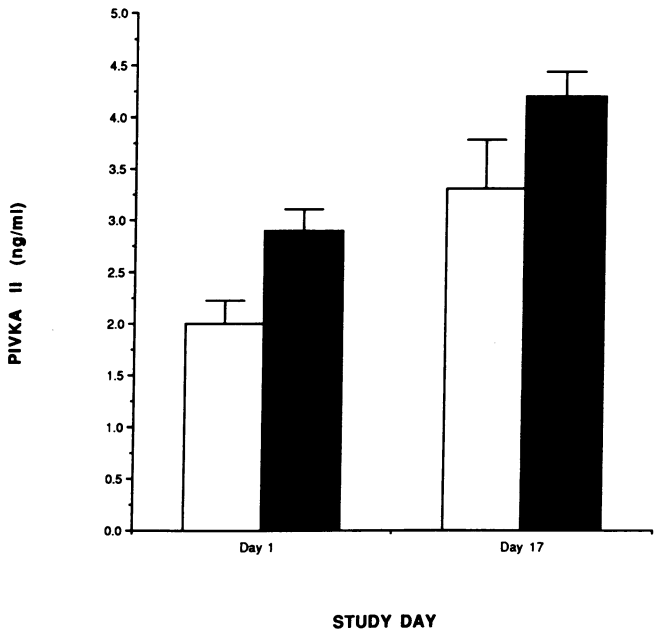

Figure 5. Effect of dietary vitamin K_1 restriction on PIVKA II (ng/ml) levels in young (*open bars*) and elderly (*filled bars*) subjects. Mean±SEM, $n = 32$. Values at the end of depletion (day 17) were significantly higher ($P < 0.05$) than at day 1.

min K status than the traditional coagulation assays. Two approaches for determining these changes are available. The first method relies on determining the daily excretion of gla in urine by collecting 24-h urine samples. When vitamin K–dependent proteins turn over, the gla is quantitatively excreted into the urine, and therefore is a good marker for the synthesis of gla residues (22). If vitamin K–dependent proteins are synthesized with deficient numbers of gla residues because of vitamin K deficiency or antagonism by a vitamin K antagonist, normal turnover of these proteins will result in decreased excretion of gla into the urine over time (11, 23–26). The other approach for determining changes in gla synthesis is to examine the vitamin K–dependent proteins themselves for their total gla content. Direct methods for the determination of total gla in vitamin K–dependent proteins has not met with success, however, indirect determination using conformation specific antibodies and altered divalent cation binding characteristics in the solid phase have recently shown some promising results (17, 27).

The aim of the present study was to establish whether dietary restriction of vitamin K intake could result in the development of a subclinical vitamin K deficiency in healthy human volunteers and to determine whether or not such factors as gender and age affected the progression of the deficiency. To achieve this goal, a weight-maintaining diet containing ~ 10

Table I. Effect of Dietary Vitamin K_1 Restriction and Repletion on Factor VII and Protein C Antigen Level and Functional Activity

Parameter	Day 1	Day 5	Day 18	Day 34	Normal range
			%		
Factor VII antigen level	88±3	87±3	87±3	89±3	70–130
Factor VII functional activity	105±5	103±4	103±5	104±5	70–130
Protein C antigen level	80±1	78±1	79±1	80±1	70–140
Protein C functional activity	103±4	101±4	105±4	104±3	70–140

Values are expressed as percentage of normal with 100% corresponding to values observed in normal pooled plasma (Factor VII 0.011 μM; protein C 0.048 μM). Mean±SEM, $n = 32$. None of the four parameters was significantly affected by dietary vitamin K intake.

μg of vitamin K_1 per day was designed and administered to 32 healthy male and female subjects aged 20–40 and 60–80 yrs and residing in the Metabolic Research Unit of the HNRCA. This study clearly demonstrates that dietary vitamin K_1 restrictions of $\sim$ 10 μg of vitamin K_1 per day bring about a rapid decrease in the concentration of vitamin K_1 in plasma below the normal range causing a decreased synthesis of gla and resulting in the development of subclinical vitamin K deficiency. This deficiency was also shown to increase circulating PIVKA-II levels in the plasma without affecting either PT or APTT or the antigenic levels or functional activities of factor VII and protein C. In addition, it appears that as a group the elderly subjects are more resistant to the development of subclinical vitamin K deficiency by dietary restriction since the excretion of gla in urine was not affected by vitamin K_1 depletion in the elderly subjects.

Plasma vitamin K_1 concentrations determined at day 1 (pre study) and shown in Fig. 2 are in agreement with the values reported in other studies (12, 28–30). In a large survey composed of more than 300 subjects aged between 20 and 90 yr, Sadowski et al. (29) reported plasma vitamin K_1 levels of 0.94 and 1.05 nM for young and elderly subjects respectively compared to 1.1 and 1.2 nM, respectively, in the present study. Consumption of the diet containing 100 μg of vitamin K_1 per day for 4 d resulted in a > 50% decrease in plasma vitamin K_1 concentrations from the baseline mean reported by Sadowski et al. (29). It can be inferred from these observations that the mean dietary intake of vitamin K_1 in the United States is probably much greater than the RDA of 80 μg/d. Furthermore, restricting dietary vitamin K to 10 μg/d for 13 d resulted in a dramatic decrease in plasma vitamin K_1 levels in the first few days resulting in levels below the normal range of 0.29–2.64 nM established by Sadowski et al. (29). These results confirm similar trends reported by Suttie et al. (11) for free-living subjects and establish the idea that plasma vitamin K_1 concentrations reflect recent dietary intake. Because in the young subjects decreased synthesis of gla was observed within a few days of initiation of the depletion phase, then recent intake of dietary vitamin K_1 is an important factor in maintaining vitamin K nutritional status on a daily basis.

Repleting the subjects for 16 d with 5, 15, 25, and 45 μg of vitamin K_1 per day (4 d per repletion level) failed to raise the plasma vitamin K_1 concentrations back into the normal range in young subjects. In contrast, the elderly subjects maintained $\sim$ 40% higher plasma vitamin K_1 levels compared to the young throughout the study. Despite similar intakes of 10 μg of vitamin K_1 per day during the depletion period, the plasma vitamin K_1 levels in the elderly subjects never dropped < 0.22 nM whereas the young subjects fell to 0.14 nM. Likewise, despite intakes of vitamin K_1 at or near the RDA for vitamin K (stage 4 of repletion), plasma levels for both groups of subjects were only near or below the lower end of the normal range established for plasma vitamin K_1 concentration, suggesting once again that the dietary intake of vitamin K_1 probably exceeds the RDA for most people.

In order to confirm that the changes observed in the vitamin K_1 levels in this study were not simply due to decreased absorption of fat-soluble compounds in general because of the very low fat content of the diet (15–17%), plasma concentrations of vitamins A (retinol), E (α-tocopherol), D (25-OH-vitamin D), and total carotenoids were determined on days 1, 5, 18, and 34 of the study. Plasma levels of vitamins A and E and 25-OH-D_3 remained normal throughout the experiment (days 1, 5, 18, and 34, respectively: vitamin A (μM), 1.8, 1.7, 1.7, 1.7; vitamin E (μM), 22.6, 21.4, 24.5, 24.9; 25-OH-vitamin D (nM), 100.5, 98.2, 100.3, 99.6), thus suggesting that the absorption of fat-soluble vitamins was not depressed by the low fat content of the diet and that the changes observed for vitamin K_1 were truly a result of the decreased dietary intake of the vitamin. Plasma carotenoids decreased with time throughout the study (days 1, 5, 18, and 34, respectively: 2.6, 2.5, 2.3, 2.2 μM) but this was to be expected since the subjects were ingesting a diet low in carotenoids. These results further confirm that the diet was adequate for all the other fat-soluble vitamins. This is an important point, since vitamins A and D are known to influence the synthesis of several vitamin K–dependent proteins such as BGP and MGP in extrahepatic tissues (31–33).

From studies performed in rats (22) it has been established that gla, the amino acid found in all vitamin K–dependent proteins, is metabolically inert and excreted quantitatively in the urine. Recently, urinary gla excretion has been used to assess vitamin K status and turnover of gla-containing proteins (11, 23–26). Decreases in gla synthesis as a result of warfarin therapy (23) or dietary vitamin K_1 restriction (11, 25, 26) have previously been demonstrated to decrease urinary gla excretion. In the present study, restriction of dietary intake of vitamin K_1 to 10 μg of vitamin K_1 per day produced a decrease in urinary gla excretion in the young subjects as a group but remained unchanged in the elderly as a group. Dietary restriction resulted in a decreased synthesis of gla in the young subjects alone as a group as determined by the decreased excretion of urinary gla in this group during the depletion period (days 5–17). The elderly subjects, as a group, failed to display any decreases in the urinary excretion of gla during the depletion period. The data, when looked at by groups, implies that all of the young subjects depleted and that the elderly were all resistant to the depletion. This, however, was not the case. During the study period, 3 out of 16 elderly subjects did show signs of depletion whereas 3 out of 16 young subjects failed to show signs of depletion. The observed discrepancy between the young and old subjects is surprising but could be due to any of a number of factors such as changes in the metabolism of the vitamin with aging, decreased turnover of vitamin K–dependent proteins in the elderly, altered gut flora in the elderly making more vitamin K in the form of menaquinones available, increased tissue storage of vitamin K in the elderly, increased utilization of vitamin K in the vitamin K cycle, or increased absorption of the vitamin by the gastrointestinal tract. Preliminary evidence from our laboratory (34) indicates that the most likely possibility for the resistance to develop vitamin K deficiency in the elderly as a group is the observation that hepatic levels of vitamin K significantly increase with aging in laboratory rats and that the development of vitamin K deficiency in laboratory rats is dependent upon the initial concentrations of vitamin K in the liver. If, indeed, hepatic concentrations of vitamin K are higher in the elderly than in the young, then, under conditions of dietary restriction, it will take longer for the elderly subjects to demonstrate symptoms of subclinical vitamin K deficiency as assessed by decreased excretion of urinary gla. In light of this information, the possibility exists that an extended length of depletion in the elderly may produce similar results to those obtained in the young population.

Table II. Relative Contribution of the Vitamin K-dependent Proteins Involved in Hemostasis to Urinary Gla Excretion

Proteins	Molecular weight	Plasma concentration	Gla residues per molecule	Gla concentration	Half-life	Gla excretion
		μM		*μM*	*h*	*μmol/d*
Factor II	72,000	1.390	10	13.90	60	9.26
Factor VII	46,000	0.011	10	0.11	6	0.61
Factor IX	55,000	0.073	12	0.87	24	1.20
Factor X	59,000	0.169	11	1.86	30	2.18
Protein C	62,000	0.048	9	0.43	6	2.37
Protein S	69,000	0.217	10	2.17	42	1.95
Protein Z	55,000	0.047	13	0.61	3	6.71
					Total	24.28

Theoretical value = 24.3 μmol/d
Observed value = 38.1 μmol/d
Percent contribution = 63.8

The biochemical data regarding half-life, molecular weight, and gla composition for each protein were taken from Sadowski et al. (35). For these calculations the average weight (70 kg) of the subjects in the study was used to calculate the plasma volume of 2.75 liters assuming a blood volume of 0.065 liter/kg body weight and a hematocrit of 40%. The observed value for the gla excretion is the average value obtained for all 32 subjects participating in the study.

The results obtained in this study clearly indicate that dietary restriction of vitamin K_1 can rapidly induce a deficiency of vitamin K in a matter of days but that this deficiency cannot be detected by using conventional blood clotting assays. The PT and APTT remained constant throughout the study period and did not increase in response to the vitamin K depletion even though plasma levels of vitamin K_1 fell below the normal range and remained below or near the lower end of the normal range throughout the study and even though there was a decrease of urinary gla excretion of in the young group. In addition, both the antigenic and functional levels of protein C and Factor VII remained unaffected by the dietary depletion of vitamin K_1 in this protocol. Factor VII and protein C were studied on the basis of their short half-lives compared to the other clotting factors (Table II). PIVKA-II antigenic levels were observed to increase on the order of 1.0 ng/ml during the depletion period in both the young and elderly subjects. If the extreme assumption is made that none of the 10 residues in prothrombin are carboxylated in PIVKA-II, then total moles of gla per day not being supplied by prothrombin can be calculated to be < 0.4 nmol/d using the data in Table II. This decrease in gla synthesis observed in prothrombin accounts for ~ 0.001% of the total urinary gla predicted to be excreted each day. Thus, the amount of gla not being synthesized in prothrombin as a result of the dietary restriction is insignificant and is not responsible for the 10% decrease in urinary gla seen in those subjects that responded to the depletion diet. Table II further demonstrates that the actual amount of gla appearing in the urine and originating in the known vitamin K–dependent clotting factors accounts for a little over 60% of the total urinary gla excreted each day. Greater than 30% of the urinary gla, therefore, cannot be accounted for as originating in any of the known vitamin K–dependent proteins involved in hemostasis. The only other known vitamin K–dependent proteins known to contain gla are BGP and MGP. It is unlikely that these two proteins alone can supply the rest of the gla to the urine. Our results suggest that there are more vitamin K–dependent proteins yet to be isolated and characterized. Our results suggest that the complete expression of the gla domain in these proteins may require adequate intakes of vitamin K on a daily basis. The consequences of underexpression of gla in the gla domains of these proteins remain to be determined, however, our data strongly suggest that these proteins can be undercarboxylated under times of mild dietary restriction such as that associated with decreased food consumption associated with disease and illness. The long-term consequences of decreased dietary intake of vitamin K_1 on the function of extrahepatic proteins needs to be explored.

In conclusion, we have demonstrated that dietary restriction of vitamin K_1 to 10 μg/d (< 12.5% of the RDA) can precipitate a subclinical deficiency of vitamin K in normal human volunteers and that younger subjects are more susceptible to this acute deficiency than older subjects. In addition, this deficiency is not associated with changes in normal blood coagulation nor with significant changes in the gla domain of prothrombin. In that vitamin K is now known to be required for proteins involved in functions outside of hemostasis, these results indicate that further research needs to be conducted in order to determine whether or not there are any pathological consequences associated with this vitamin K deficiency.

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