Vitamin K deficiency from dietary vitamin K restriction in humans

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ABSTRACT Vitamin K is required for the maintenance of normal hemostatic function. Ten college-aged male subjects chose diets restricted in vitamin K content for 40 d. Median phylloquinone intakes based on analysis of food composites dropped from 82 μg/d during the prestudy period to 40 and 32 μg/d at d 9 and 27 of dietary restriction, respectively. Serum phylloquinone concentrations fell from a mean of 0.87 to 0.46 ng/mL during a 21-d period of vitamin K restriction. Supplementation with 50 μg phylloquinone/d for 12 d increased serum phylloquinone to 0.56 ng/mL, and supplementation with 500 μg phylloquinone/d increased serum phylloquinone to 1.66 ng/mL. Vitamin K restriction resulted in alterations in a functional clotting assay that detects undercarboxylated prothrombin species in plasma and in a decrease in urinary γ-carboxyglutamic acid. Supplementation with either 50 or 500 μg of phylloquinone restored both these indices to near normal values. These data are consistent with a human dietary vitamin K requirement of ~1 μg/kg body wt/d. Am J Clin Nutr 1988;47:475–80.

KEY WORDS Vitamin K, phylloquinone, coagulation

Introduction

Vitamin K is required for the synthesis of γ-carboxyglutamyl residues in the vitamin K-dependent clotting factors (prothrombin and factors VII, IX, and X) and a number of other proteins (1). Other than rare cases of hemorrhagic disease of the newborn, uncomplicated deficiencies of vitamin K in the adult have seldom been reported (2, 3). The dietary requirement of vitamin K for the adult human is usually stated to be ~1 μg/kg body wt (2), but there are few data to support this value. Assessments of the human requirement for this fat-soluble vitamin have been hampered by two technical problems: the lack of sensitive methods to detect a functional deficiency of the vitamin and an inability to quantitate the low levels of vitamin K in tissues or in circulation.

Vitamin K sufficiency has commonly been assessed by the use of relatively insensitive prothrombin-time measurements. Impairment of vitamin K action results in the secretion of des(γ-carboxy) and partially carboxylated forms of vitamin K-dependent proteins into plasma (4). The recent development of immunochemical methods (5) and modified chromogenic assays (6) for the detection of des(γ-carboxy) prothrombin has greatly increased the sensitivity of assays designed to monitor this index of vitamin K sufficiency.

The predominant dietary source of the vitamin is phylloquinone in plant material. The development of high-performance liquid chromatography (HPLC) separation methods and a variety of detection systems has now opened the possibility of rapid quantitative assessment of phylloquinone status (7). Values reported for plasma phylloquinone (8–11) have been somewhat variable, but recent data (12) suggest that the plasma phylloquinone concentration in the normal US population is ~1 ng/mL. The relationship between phylloquinone intake in free-living subjects consuming typical or slightly modified diets and tissue or serum phylloquinone levels has not yet been established. This report describes the results of a study designed to determine this relationship. The ability of simple dietary restriction of vitamin K to influence a functional measurement of vitamin K-dependent clotting factor levels and to decrease urinary γ-carboxyglutamic acid (Gla) excretion is also described.

Methods

Ten college-aged nonsmoking males were recruited for the study following interviews with two of the investigators who

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explained procedures and obtained consent agreements approved by the University of Wisconsin-Madison Human Subjects Committee. The subjects, aged 28.3 ± 3.2 y (± SD) were 181 ± 7 cm tall and weighed 72 ± 9 kg. During days 1–7 of the study the subjects were instructed to continue normal dietary habits. On days 8–47 they were instructed to consume a diet self-selected to eliminate foods known (1) to be high in vitamin K. They were instructed to omit green vegetables and salad greens from their diet, including but not limited to peas, green beans, asparagus, broccoli, cauliflower, cabbage, lettuce, Brussels sprouts, and spinach. They were instructed not to eat liver, to omit tea, and to limit coffee consumption to five 8-oz cups/day.

During the restricted portion of the study, the subjects were given a commercial Walgreen (Chicago, IL) multivitamin plus iron supplement.

Compliance with the dietary instructions was assessed by dietary records and dietary recalls at the times indicated in the study protocol shown in Figure 1. Subjects collected duplicate portions of all foods and beverages consumed on three different days. Intake of foods on the collection days did not vary from intake on other days as judged by the number of servings from various food groups. Composites of all the solid foods and beverages consumed by each subject on each day were prepared as described previously (14) and frozen for later analysis. After consuming the vitamin K-restricted diet for 21 d, the subjects were divided into two groups and given a supplement of either 50 or 500 μg of phylloquinone for d 29–40. All subjects were given 1 mg vitamin K/d on d 41–47 (Fig 1). Five of the 10 subjects were given 15 mg Mn as chelated manganese (Nutritional Factors, Concord, CA). The vitamin K supplement was prepared by dissolving phylloquinone (Sigma Chemical Co, St Louis, MO) in corn oil and adding the appropriate amount to a 0.01% gelatin capsule containing cornstarch. Each daily supplement was divided into two equal amounts and consumed with the morning and evening meals.

Plasma and serum samples were obtained before the morning meal on the days indicated (Fig 1), and four 3-d composite urine samples were collected in acid-rinsed plastic bottles, prepared with deionized water, and frozen for storage in acid-rinsed plastic tubes or bottles. Urinary creatinine (15) and γ-carboxyglutamic acid (16) were analyzed by standard methods.

Serum phylloquinone was determined as described by Mummah-Schendel and Suttie (12). Phylloquinone content of aliquots of the homogenized food composites was determined following addition of 3H-phylloquinone as a recovery standard. The samples were extracted with acetone and the vitamin transferred from the acetone extract to hexane as described by Satter et al (17). The hexane extracts were dried under N2 and redissolved in hexane (2 mL/100 mg of lipid), and 2 mL was loaded onto a hexane washed Sep-pak® (Waters Associates, Medford, MA). The Sep-Pak® was washed with 8–10 mL of hexane before vitamin K was eluted with 8–10 mL of 3% ether in hexane. The eluate was dried under N2, redissolved in 250 μL of hexane, and 200 μL injected onto a μPorasil® column (Waters Associates, Medford, MA) and eluted with 0.2% CH3CN in hexane at a flow rate of 1.5 mL/min with a Waters Associates model 6000A pump. The elute from the column was monitored at 254 nm, and the fractions corresponding to the elution time of a trans-phylloquinone standard were collected. The pooled fractions were dried under N2, were redissolved in 250 μL of methanol, and 200 μL was injected onto a du Pont Zorbax® ODS column (Fisher Scientific, Itasca, IL). The column was eluted with 3.5% H2O in ethanol containing 3.0 mM Na acetate at pH 6.0 and the eluate was passed through an ESA® Guard Cell (ESA, Bedford, MA) mainitained at −0.65 V to reduce vitamin K in the effluent. Reduced phylloquinone was detected with a model 650-10 LC fluorimeter (Perkin Elmer, Norwalk, CT) with excitation at 330 nm and emission at 530 nm. The remainder of the methanol solution was mixed with 4 mL of Aqualos® (NEN Research Products, Boston, MA) and recovery of 3H-vitamin K was determined in a liquid scintillation spectrometer. The amount of vitamin K in the food composites was calculated from the recovery of the added phylloquinone and chromatography of standard solution of the vitamin on the Zorbax® column.

One-stage prothrombin times were performed using thromboplastin C reagent (Dade Diagnostics, Miami, FL) and an automated clot timer. An increase in the plasma concentration of circulating abnormal (des-γ-carboxy) prothrombin was assessed by a modification of the method described by Allison et al (18). The assay compares the thrombin generated by action of a commercial thromboplastin preparation, Simplastin® (Warner-Lambert, Morris Plains, NJ), with that generated with a snake venom protease from E carinatus. Citrated plasma was diluted 1:100 with 0.1 M tris buffer pH 8.3. Samples activated by Simplastin® contained 40 μL diluted plasma, 460 μL tris buffer, 100 μL (1000 Kalikrein inactivation units) Trasylol® (Sigma Chemical Co, St Louis, MO), and 240 μL Simplastin®. Samples activated with snake venom contained 40 μL diluted plasma, 640 μL tris buffer, 80 μL Trasylol®, and 80 μL (0.4 μU) Ecarin® (Sigma Chemical Co, St Louis, MO). Both samples were incubated at 37°C for 10 min, 200 μL of 1 mM chromogenic substrate S-2238 (Helena Laboratories, Beaumont, TX).
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FIG 2. Effect of dietary vitamin K restriction on serum phylloquinone concentration. Values plotted are serum phylloquinone concentrations for individual subjects for that day. Data from one subject with consistent fasting serum lipemia and elevated phylloquinone concentrations have not been plotted.

was added, and incubation was continued for an additional 30 min at 37 °C. The reaction was stopped by the addition of 60 μL of 20% sodium dodecyl sulfate, and absorbance at 405 nm was determined. A ratio of the absorbance of the Simplastin®-activated incubation to that of the Ecarin®-activated incubation was expressed as a S:E ratio. Data were analyzed for statistical differences by ANOVA and Tukey’s omega test following log transformation to control the variance (19).

Results

Serum phylloquinone values obtained during the first 29 d of the study are plotted in Figure 2. As previously observed (12), there was a wide range of values during the 3 d the subjects were consuming their normal diet and a general decrease in serum phylloquinone concentrations when the subjects were consuming the vitamin K-restricted diet. During this period <10% of the values were >1 ng/mL (2.2 nmol/L), and 70% of the serum phylloquinone values were ≤0.5 ng/mL (1.1 nmol/L). Only 30% of the values obtained when the subjects were consuming a normal diet were in this range. One subject consistently demonstrated a fasting serum lipemia and also had elevated serum phylloquinone concentrations during all phases of the study. This phenomenon was previously observed (8), and these values were not included in Figure 2.

The influence of supplementation with either 50 or 500 μg of phylloquinone/d for 12 d followed by supplementation with 1 mg phylloquinone/d for 7 d on serum phylloquinone concentrations is shown in Table 1. These data indicate that simple restriction of foods known to be high in phylloquinone resulted in a significant decrease in serum phylloquinone concentrations which could be restored to approximately double the normal values by supplementation of 500 μg of phylloquinone per day. Supplementation with 50 μg phylloquinone/d did not significantly alter serum phylloquinone concentrations. Supplementation with 1 mg phylloquinone/d raised serum phylloquinone to about three times that observed when the subjects were consuming a normal diet.

Prothrombin time measurements for all subjects were obtained once during the normal diet phase and twice during the vitamin K-restricted period. The values for all subjects at all time periods ranged from 11.0 to 12.9 s, and no values were considered to be outside the normal range. However, a more sensitive measure of functionally active prothrombin, the S:E ratio, did respond to dietary restriction of vitamin K (Table 2). There was a drop in the S:E ratio at the end of the dietary vitamin K restriction period which was restored to normal by supplementation with either 50 or 500 μg phylloquinone/d for 12 d. Supplementation with 1 mg phylloquinone/d for an additional week did not influence these values.

The effect of dietary vitamin K restriction on a functional clotting-factor assay was confirmed by measurement of Gla. Urinary Gla values were quite variable between subjects consuming a normal diet, and apparent decrease in Gla excretion when the subjects were consuming the vitamin K-restricted diet was not of statistical significance (Table 3). However, when each subject’s Gla excretion for subsequent periods was expressed as a percentage of the excretion of the normal diet period, a statistically significant drop from the normal value was observed. Supplementation with phylloquinone resulted in a significant increase from the excretion level observed during the restricted intake period. The apparent increase in Gla excretion during the period of supplementation with 1 mg phylloquinone compared with the normal diet period was of no statistical significance.

Direct analysis of the duplicate-portion food composites provided information on the dietary vitamin K intake of the study subjects. The liquid component of the diet contained less than 2 μg vitamin K/d for any of the subjects. The content of vitamin K in the solid food portion of the daily diet is presented in Table 4. The median intake of vitamin K during the pre-study period was 82 μg/d (1.02 μg/kg). Data obtained on two different days of dietary vitamin K restriction indicated that the phylloquinone intake was halved by the dietary restrictions imposed. One subject consumed 161 μg vitamin K (2.15 μg/kg) on day 16. Mean intake of other subjects on day 16 was 48 ± 31 μg and 0.64 ± 0.40 μg/kg body wt.

Five of the 10 subjects, 3 in the 500 μg supplementation group and 2 in the 50 μg supplementation group, also received a 15 mg daily supplement of Mn. This supplement had no effect on any of the indices measured.

Discussion

Although a rapid drop in serum vitamin K was observed in subjects consuming an essentially vitamin K-free diet (18), this study represents the first demonstration that serum vitamin K concentrations can be altered...
TABLE 1
Effect of dietary vitamin K restriction and phylloquinone supplementation on serum phylloquinone

<table>
<thead>
<tr>
<th>Diet</th>
<th>Study days</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ng/mL (nmol/L)</td>
<td>ng/mL (nmol/L)</td>
</tr>
<tr>
<td>Normal</td>
<td>3, 5, 8</td>
<td>0.83 ± 0.52 (1.83 ± 1.14)</td>
<td>0.92 ± 0.60 (2.02 ± 1.32)</td>
</tr>
<tr>
<td>Restricted</td>
<td>12, 17, 22, 26, 29</td>
<td>0.49 ± 0.27 (1.08 ± 0.59)†</td>
<td>0.43 ± 0.38 (0.95 ± 0.84)†</td>
</tr>
<tr>
<td>Supplemented I</td>
<td>32, 36, 40</td>
<td>1.66 ± 0.84 (3.65 ± 1.85)‡</td>
<td>0.57 ± 0.40 (1.25 ± 0.88)‡</td>
</tr>
<tr>
<td>Supplemented II</td>
<td>47</td>
<td>2.68 ± 0.99 (5.90 ± 2.18)‡</td>
<td>3.10 ± 1.13 (6.82 ± 2.49)‖</td>
</tr>
</tbody>
</table>

* Values are mean ± SD. Group A contained five subjects on each day and group B contained four subjects on each day. During the supplemented I period group A received 500 μg phylloquinone/d and group B received 50 μg/d; during the supplemented II period both groups received 1 mg phylloquinone/d.
† Significantly lower (p < 0.05) than normal diet period for same group.
‡ Significantly greater (p < 0.01) than normal or restricted period for this group.
§ Significantly greater (p < 0.01) than group B for this period.
‖ Significantly greater (p < 0.01) than all other periods for this group.

by rather simple dietary modifications. A decrease in daily vitamin K intake to about 50% of that normally consumed clearly resulted in a drop in serum phylloquinone concentration. Administration of 50 μg/d of phylloquinone, which raised the total intake above the pre-restriction values, did not significantly increase these values, whereas 500 μg/d doubled the pretreatment concentrations. Methods for quantitating endogenous phylloquinone levels have only recently been available, and there are rather substantial variations in the few values reported. Reported mean values from a number of small populations ranged from 0.3 ng/mL (0.66 nmol/L) (8) to 2.6 ng/mL (5.72 nmol/L) (9) from two groups using a UV detector and from 0.4 ng/mL (0.88 nmol/L) (11) to 1.1 ng/mL (2.42 nmol/L) (10) for two groups utilizing an electrochemical detector in a reductive mode. From the available data it is not possible to determine whether there were real differences in the vitamin K intakes of the population that were studied or if analytical problems still exist. Utilizing the methodology employed in this study, we observed a mean value of 1.3 ng/mL (2.86 nmol/L) in 95 adult Red Cross donors (12) and 1.4 ng/mL (3.08 nmol/L) in 33 metabolic ward subjects

18. The available data suggest that normal serum phylloquinone concentrations in the adult US population are ~1 ng/mL (2.2 nmol/L), and the data presented here indicate that a vitamin K intake > 100 μg/d but < 550 μg/d might be required to maintain this concentration.

Historically, changes in vitamin K-dependent clotting factor activity in response to changes in dietary intake of vitamin K have been difficult to document. Frick et al (20) administered a parenteral vitamin K-free nutrient solution for 30 d to patients also receiving antibiotics and observed prothrombin concentrations (Quick test) of 20%. Doisy (21) fed a chemically defined diet containing < 10 μg vitamin K/d and was able to reduce prothrombin concentration ~50% in ~20 wk. Udall (22) fed a diet reported to be free of vitamin K and observed a 2-3 s increase in prothrombin times in 10 subjects after 3 wk. O'Reilly (23) did not observe prothrombin times out of the normal range in four subjects ingesting ~25 μg of

TABLE 2
Effect of dietary vitamin K restriction and phylloquinone supplementation on functionally active prothrombin

<table>
<thead>
<tr>
<th>Diet</th>
<th>S:E ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal, study day 8</td>
<td>1.024 ± 0.030</td>
</tr>
<tr>
<td>Restricted, study day 29</td>
<td>0.911 ± 0.095†</td>
</tr>
<tr>
<td>Supplemented (50 or 500 μg phylloquinone), study day 40‡</td>
<td>0.980 ± 0.020</td>
</tr>
<tr>
<td>Supplemented (1 mg phylloquinone), study day 47</td>
<td>0.995 ± 0.020</td>
</tr>
</tbody>
</table>

* Values are mean ± SD for 10 subjects on each day.
† Significantly (p < 0.01) lower than all other groups.
‡ There was no difference in the response of the subgroups supplemented with 50 or 500 μg/d and data from the two subgroups were combined.

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TABLE 3
Effect of dietary vitamin K restriction and phylloquinone supplementation on urinary γ-carboxyglutamic acid excretion

<table>
<thead>
<tr>
<th>Diet</th>
<th>Urine pool</th>
<th>Creatinine (nmol/mg)</th>
<th>Percent of normal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Days 3–5</td>
<td>63.1 ± 21.9</td>
<td>100</td>
</tr>
<tr>
<td>Restricted</td>
<td>Days 23–25</td>
<td>47.9 ± 13.5</td>
<td>77.9 ± 10.0†</td>
</tr>
<tr>
<td>Supplemented (50–500 μg phylloquinone K1)</td>
<td>Days 37–39‡</td>
<td>56.0 ± 15.1</td>
<td>97.2 ± 41.9§</td>
</tr>
<tr>
<td>Supplemented (1 mg phylloquinone K1)</td>
<td>Days 43–47§</td>
<td>68.4 ± 31.7</td>
<td>122.5 ± 64.8§</td>
</tr>
</tbody>
</table>

* Values are mean ± SD for 10 subjects in each period.
† Significant drop from normal (p < 0.01).
‡ There was no difference in response of subgroups supplemented with 50 or 500 μg/d. Data from the two subgroups have been combined.
§ Significant increase from restricted (p < 0.01).
VITAMIN K DEFICIENCY

TABLE 4
Vitamin K content of solid foods consumed by study subjects

<table>
<thead>
<tr>
<th></th>
<th>Median intake µg/d</th>
<th>µg/kg body wt</th>
<th>Mean intake* µg/d</th>
<th>µg/kg body wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal self-selected, day 4</td>
<td>82</td>
<td>1.02</td>
<td>77 ± 29</td>
<td>1.04 ± 0.35</td>
</tr>
<tr>
<td>Vitamin K-restricted, day 16</td>
<td>40</td>
<td>0.61</td>
<td>60 ± 48</td>
<td>0.84 ± 0.63</td>
</tr>
<tr>
<td>Vitamin K-restricted, day 31</td>
<td>32</td>
<td>0.51</td>
<td>37 ± 24</td>
<td>0.50 ± 0.31</td>
</tr>
</tbody>
</table>

* Data are mean ± SD for 10 subjects in each period.

vitamin K/d for 28 d nor did Olson et al (24) in six subjects fed a diet containing 10 µg of vitamin K/d for 3–8 wk. These results point to the lack of sensitivity of the standard one-stage prothrombin times in monitoring vitamin K status.

Vitamin K deficiency results in the appearance of (abnormal) des-γ-carboxyprothrombin and partially carboxylated forms of prothrombin in the plasma (1, 4). These species can be activated to thrombin by E carinatus venom but not to the physiological (factor Va, factor Xa, phospholipid, Ca++) pathway activated by commercial thromboplastin preparations such as Simplastin®. The S:E ratio used here is, therefore, an indirect measurement of the relative amount of abnormal prothrombin in circulation. A simple restriction of vitamin K intake to about 50 µg/d by rather simple dietary modifications.

TABLE 4
Vitamin K content of solid foods consumed by study subjects

On the basis of data from Frick et al (20), the human requirement for dietary vitamin K is usually considered ~1 µg/kg body wt per day. The data presented here are consistent with a requirement in that range. A dietary phylloquinone intake of <1 µg/kg body wt resulted in decreases in circulating serum phylloquinone, an alteration in a functional clotting assay, and a decrease in urinary Gla excretion. Routine one-stage prothrombin times were not influenced by this dietary modification, and the measurements used to detect a deficiency are much more sensitive than those used historically (1). Additional studies will be needed to establish more accurately the amount of vitamin needed to sustain various indices of vitamin K sufficiency in a normal range. Menaquinones are found in human liver (27, 28), and they are usually assumed (2, 3) to contribute to maintaining adequate vitamin K status. However, evidence that they comprise a significant source of the vitamin for the human is lacking. Data from this study clearly demonstrate that menaquinone utilization was not sufficient to prevent a number of signs of a vitamin K deficiency in subjects consuming ~0.7 µg phylloquinone/d.

References