Interaction between vitamin K nutrure and bacterial overgrowth in hypochlorhydria induced by omeprazole\textsuperscript{1–4}

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ABSTRACT Subjects taking a hydrogen pump blocking agent (omeprazole) develop bacterial overgrowth of the small intestine. We tested the hypothesis that this bacterial overgrowth produces menaquinones, which would meet the vitamin requirement in situations of vitamin K deficiency. In a crossover-type design, 13 healthy volunteers eating a phylloquinone-restricted diet for 35 d were randomly assigned to take omeprazole during the first period of study or starting on day 15 until the end of the study. Coagulation times, serum osteocalcin [total osteocalcin (ucOC), plasma phylloquinone, urinary γ-carboxyglutamic acid, and plasma undercarboxylated prothrombin (PIVKA-II)] were measured. Plasma phylloquinone concentrations declined 82% with dietary phylloquinone restriction ($P < 0.05$) and were not significantly different in the period when the diet was combined with omeprazole treatment ($P > 0.05$). The mean value for PIVKA-II during the phylloquinone-restricted diet significantly increased 5.7-fold from baseline ($P < 0.05$); however, the combination of omeprazole treatment and the phylloquinone-restricted diet significantly reduced PIVKA-II values by 21% ($P < 0.05$) compared with the diet period alone. There were no alterations in total or percentage ucOC concentrations during the phylloquinone-restricted diet or during the period of diet plus omeprazole treatment. Our data support the hypothesis that bacterial overgrowth results in the synthesis and absorption of menaquinones. These menaquinones contribute to vitamin K nutriture during dietary phylloquinone restriction, but not enough to restore normal vitamin K status. \textit{Am J Clin Nutr} 1998;68:699–704.

KEY WORDS Phylloquinone, menaquinone, vitamin K status, osteocalcin, prothrombin, PIVKA-II, γ-carboxyglutamic acid, urinary Gla, bacterial overgrowth, omeprazole

INTRODUCTION Vitamin K is the generic name for a related group of compounds that serve as essential cofactors for the posttranslational biosynthesis of γ-carboxyglutamic acid (Gla) residues from glutamic acid in several proteins. Gla-containing proteins are found in body fluids (eg, plasma and urine) and in calcified tissues (eg, bone and hardened atherosclerotic plaques). In all of these proteins the only known function of Gla is to bind calcium ions (1). In the absence of dietary vitamin K or in the presence of vitamin K antagonists such as warfarin, these proteins are still synthesized but are less functional or nonfunctional because they lack some or all of the γ-carboxyglutamyl residues (2). Proteins missing one or more Gla residues are called undercarboxylated or descarboxy proteins.

During periods of suboptimal vitamin K status, various Gla proteins enter the bloodstream in an undercarboxylated form (eg, undercarboxylated prothrombin (PIVKA-II), proteins induced by vitamin K absence or antagonist-II) or undercarboxylated osteocalcin (ucOC)). When vitamin K proteins turn over, Gla is excreted in the urine. If vitamin K–dependent proteins are synthesized with deficient numbers of Gla residues, such as happens in vitamin K deficiency or antagonism, turnover of these proteins results in a decreased excretion of Gla into the urine over time (3).

Two forms of vitamin K are available to humans: 1) vitamin $K_1$, or phylloquinone, which is mostly found in green leafy vegetables, and 2) vitamin $K_2$, or menaquinone, which is synthesized by intestinal bacterial flora and is present in fermented foods. The term \textit{menaquinone} refers to a group of vitamin K compounds with polyunsaturated aliphatic side chains of various lengths. These compounds are denoted as menaquinone-$n$, where $n$ represents the number of isoprenoid residues in the side chain. Natural menaquinones ranging from menaquinone-4 to menaquinone-13 are produced by bacteria (4).

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Gastric acid plays an important role in the prevention of bacterial colonization of the stomach and small intestine (5). A reduction in gastric acid secretion, a situation seen with use of a hydrogen pump blocking agent (eg, omeprazole) and atrophic gastritis, allows bacteria to survive in the stomach and proximal small intestine (6). The bacteria that overgrow in the proximal gastrointestinal tract in patients with low gastric acid do not generally deconjugate bile acids or cause fat (6) and fat-soluble vitamin malabsorption. Such bacteria could produce more menaquinones, which might be bioavailable to the host. Although the colonic absorption of menaquinone-7 is poor (7), menaquinones can be absorbed from the small intestine (8, 9).

It is not known how important the bacterial menaquinone contribution is to the body store of vitamin K. Olson (10), in an article on recommendations for the dietary intake of vitamin K in humans, stated that 50% of vitamin K was supplied by intestinal bacteria. Sutty (11) stated that menaquinones are important in human nutrition but the degree to which they are is not yet determined and suggested that menaquinones are less important than previously thought.

We tested the hypothesis that the bacterial overgrowth that arises in subjects taking omeprazole produces additional menaquinones, which may be bioavailable to the host. Such menaquinone-7 production could help meet the vitamin K requirement in situations of vitamin K deficiency induced by a vitamin K–deficient diet. A vitamin K–deficient diet was given to subjects to maximize the response of the indexes analyzed in our study.

SUBJECTS AND METHODS

The research protocol was approved by the Tufts University–New England Medical Center Human Investigation Review Committee, and written, informed consent was obtained from each subject. All subjects accepted into the study underwent physical examinations and standard clinical laboratory testing [clinical chemistry and hematologic profiles, urinalysis, and measurement of prothrombin time (PT) and activated partial thromboplastin time (APTT)]. No subject took oral anticoagulants, used antiinflammatory medications including aspirin on a regular basis, or used antibiotics, anticonvulsants or barbiturates throughout the study for measurement of urinary Gla, which was passed through the mouth into the stomach. By fluoroscopy, the tip of the tube was positioned at the duodenal jejunal junction. Once in place, a small-intestinal fluid sample was aspirated. The number of CFU/L in the intestinal aspirates was obtained by serial dilution (1:10) in sterile 0.1 mol phosphate buffered saline/L through the 10th dilution. After being mixed, a 0.1-mL aliquot of each dilution was plated on 2 sets of blood agar plates. One set was incubated in an anaerobic chamber (5% carbon dioxide, 10% hydrogen and carbon monoxide, and 85% nitrogen) for measurement of facultative anaerobes and another set was incubated in a carbon dioxide jar for measurement of aerobes. Each chamber was incubated at 37°C for 48 h. Colonies were counted after the incubation and adjusted for dilution (6, 13–17). Cultures showing no growth at the lowest dilution (10/10/L) were recorded as 1 for purposes of statistical analysis.

Twenty-four–hour urine specimens were collected daily throughout the study for measurement of urinary Gla, which was determined by ortho-phthalaldehyde derivatives by reversed-phase HPLC with fluorometric detection (18). Fasting blood specimens were obtained on every third day, beginning with day 1. Serum and plasma were frozen at −7°C until analyzed for plasma phylloquinone, serum total and ucOC, and PIVKA-II. Specimens from each individual were analyzed in the same run.

Phylloquinone was measured in plasma with EDTA by reversed-phase HPLC with postcolumn, solid-phase chemical reduction of phylloquinone to its hydroquinone, followed by fluorometric detection (19). Serum osteocalcin and ucOC were quantitated before and after treatment with BaSO₄ by radioimmunoassay with a rabbit polyclonal antibody raised against purified bovine bone osteocalcin, which recognized both native and ucOC (20). PIVKA-II was measured in citrated plasma with an enzyme-linked immunosorbent assay from American Bioproducts Company ( Parsippany, NJ).

Statistical analysis of effects from the period of administration of omeprazole

To test if the responses of clotting time (PT and APTT), plasma phylloquinone, plasma PIVKA-II, serum osteocalcin, serum percentage ucOC, and urinary Gla were altered by whether
omeprazole was given for the first half of the study (group 1) or for the second half of the study (group 2), the data were analyzed in the manner of a crossover study by using repeated-measures analysis of variance (ANOVA). Because none of the responses were affected by period of administration, this variable was ignored in subsequent analyses. Responses were then analyzed along with baseline values.

**Statistical analysis of differences between treatments**

Repeated-measures ANOVA or Friedman’s nonparametric ANOVA was used to compare all data together from baseline (day 1 in group 1 plus day 1 in group 2), dietary vitamin K restriction (day 34 in group 1 plus day 13 in group 2), and dietary vitamin K restriction plus omeprazole treatment (day 13 in group 1 plus day 34 in group 2). If differences were found among the treatments, we performed multiple comparisons between treatment pairs, using the Student-Neuman-Keuls test.

Statistical significance was set at $P < 0.05$. Values are presented as means ± SDs or as medians with 25th and 75th percentiles. To represent the statistical values, 2 kinds of graphics are used: in the normal distribution, a scatter plot with SD bars, and for a nonnormal distribution, a box plot graph with 10th, 25th, 50th, 75th, and 90th percentiles indicated.

**RESULTS**

The 18 subjects recruited for the study tolerated the phylloquinone-restricted diet without any adverse events. Because the purpose of this study was to determine bacterial contribution to vitamin K nutriture, 3 subjects in group 1 and 2 subjects in group 2 were excluded from the statistical analysis because $J$) in 3 subjects the bacterial content of their duodenal juice was $< 1 \times 10^8$ CFU/L at the end of omeprazole treatment, 2) in 1 subject bacterial counts were $\geq 1 \times 10^8$ before omeprazole treatment, and 3) in 1 subject phylloquinone concentrations during the phylloquinone-restricted diet plus omeprazole period were higher than baseline concentrations (5.04 and 2.25 nmol/L, respectively), indicating dietary noncompliance. As a result, the data analyzed were from 13 subjects, 6 in group 1 and 7 in group 2.

The logarithmically transformed values for the number of CFU/L in the intestinal aspirates are presented in Figure 1. As expected, intestinal counts of bacteria were significantly higher at the end of the omeprazole treatment periods. In both groups at the end of the treatment periods, intestinal aerobes were the major fraction of bacteria in the duodenal fluid (mean: 70%). Only 1 subject, in group 1, had a predominance of anaerobes.

Clotting times (baseline: PT, 12.5 ± 0.4 s; APTT, 29.1 ± 2.1 s) did not change with dietary restriction of phylloquinone (PT, 12.5 ± 0.5 s; APTT, 30.1 ± 2.4 s), nor did it change with omeprazole treatment (PT, 12.7 ± 0.2 s; APTT, 29.8 ± 2.2 s) ($P = 0.17$ and 0.18, respectively).

The reference range for plasma phylloquinone in our laboratory is 0.29–2.64 nmol/L. None of the subjects at baseline had plasma phylloquinone concentrations < 0.29 nmol/L and only 1 subject had concentrations above the normal range. Median plasma phylloquinone concentrations declined 82% with the phylloquinone-restricted diet ($P < 0.05$) and 11 subjects had values < 0.29 nmol/L. During the period of treatment with omeprazole plus the phylloquinone-restricted diet, median plasma phylloquinone concentrations were significantly lower than at baseline ($P < 0.05$), but were not significantly different from concentrations during the period of phylloquinone dietary restriction alone ($P > 0.05$).

With the phylloquinone-restricted diet, Gla excretion tended to decline 8.9% (to 91.1 ± 11.8% of baseline values, NS). With the phylloquinone-restricted diet plus omeprazole treatment, mean urinary Gla excretion tended to be higher (95.8 ± 17.8% of baseline) than during the phylloquinone-restricted diet period alone (NS). The mean differences between the values at baseline or during the 2 treatment periods were not significant ($P = 0.068$). However, urinary Gla excretion decreased during the phylloquinone-restricted diet period in 10 of 13 subjects and was higher (compared with the phylloquinone-restricted diet period) in 10 of 13 subjects during the phylloquinone-restricted diet plus omeprazole treatment period.

Influences of the phylloquinone-restricted diet on plasma PIVKA-II concentrations are shown in Figure 2. All PIVKA-II baseline concentrations were within the normal range (0–2 µg/L). The median value for PIVKA-II increased 5.7-fold from baseline during the phylloquinone-restricted diet period ($P < 0.05$). PIVKA-II concentrations were significantly lower (21%, $P < 0.05$) during the phylloquinone-restricted diet and omeprazole treatment period than during the restricted diet period alone. However, the median values remained significantly greater (threelfold) than at baseline ($P < 0.05$).

Serum total osteocalcin concentrations (baseline: 10.6 ± 3.3 µg/L) did not change with the phylloquinone-restricted diet (9.8 ± 3.1 µg/L) or with the phylloquinone-restricted diet plus omeprazole treatment (10.6 ± 3.0 µg/L) ($P = 0.28$). The influence of the phylloquinone-restricted diet alone and the administration of omeprazole combined with the phylloquinone-restricted diet on percentage ucOC is presented in Figure 3. These values were not significantly different ($P = 0.957$).
The dotted line represents the plasma PIVKA-II concentration of 2 μg/L, which is the cutoff for the normal range. In the box plot, the bottom boundary of the box indicates the 25th percentile, the line within the box indicates the median, and the top boundary of the box indicates the 75th percentile. Whiskers above and below the box indicate the 90th and 10th percentiles, respectively. The P value for the overall comparison of PIVKA-II during the baseline, diet, and diet + omeprazole periods is by Friedman’s nonparametric ANOVA (P < 0.001). Means with different letters are significantly different, P < 0.05.

FIGURE 2. Individual plots of data and median and box plot of plasma undercarboxylated prothrombin (PIVKA-II) concentrations at baseline, during the phylloquinone-restricted diet (diet), and during the phylloquinone-restricted diet plus administration of omeprazole (diet + omeprazole). The values were not significantly different (P = 0.957, repeated-measures ANOVA).

DISCUSSION

Before the discovery of Gla in prothrombin, subclinical vitamin K deficiency was diagnosed with blood coagulation tests, such as tests of PT and APTT. Recent approaches for assessing vitamin K nutritional status have included static measurement of plasma phylloquinone concentrations and functional measurements based on the Gla domain and its carboxylation state (urinary Gla excretion, PIVKA-II, and ucOC). Knapen et al (21) proposed recently that a person should be considered vitamin K sufficient if all known Gla proteins occur in a fully carboxylated form. According to this definition, vitamin K deficiency might be regarded as a state in which at least one of the Gla proteins occurs in an undercarboxylated form (4).

The significant decrease in plasma phylloquinone concentrations, the increase in circulating PIVKA-II concentrations (Figure 2), and the trend toward a decrease in urinary Gla excretion suggest that subclinical vitamin K deficiency was produced in normal subjects by the administration of a diet deficient in phylloquinone. Ferland et al (3) also found that dietary restriction of phylloquinone to 10 mg/d (<12.5% of the RDA) could precipitate a subclinical deficiency of vitamin K in normal human volunteers, as measured by these same indexes (3). However, as found in our study, the vitamin K deficiency was not associated with changes in blood coagulation (3).

Although significant decreases in urinary Gla excretion during dietary phylloquinone restriction have been reported previously (3, 22), nonsignificant decreases such as observed in our study have also been reported in response to mild vitamin K antagonism (23). Crăciun et al (2), using a rat model, observed a more pronounced reduction in Gla excretion with the use of a vitamin K antagonist than in the case of dietary vitamin K defi-

FIGURE 3. Individual plots of data and mean (±SD) serum percentage undercarboxylated osteocalcin at baseline, during the phylloquinone-restricted diet (diet), and during the phylloquinone-restricted diet plus administration of omeprazole (diet + omeprazole). The values were not significantly different (P = 0.957, repeated-measures ANOVA).

ciency. These authors proposed that during vitamin K deficiency, some residual vitamin K is still available and might be sufficient to produce coagulation factors (or precursors) with a reduced amount of Gla residues. These undercarboxylated coagulation factors have little procoagulant activity but still might contribute to urinary Gla excretion. This could be the explanation for the nonsignificant trend toward reduced urinary Gla excretion during conditions of dietary vitamin K deficiency in our study.

It has been reported that the circulating osteocalcin concentration is the most sensitive marker of vitamin K status (4) and that an increase in the circulating percentage of ucOC is the most sensitive indicator of a vitamin K deficiency acquired as a result of warfarin treatment (23). We expected, therefore, that the phylloquinone-restricted diet in our study would result in an increase in ucOC concentrations; however, the subjects showed no alterations in total osteocalcin or ucOC concentrations while consuming the phylloquinone-restricted diet (Figure 3). Garber et al (24) also showed in rats that percentage ucOC did not respond to a 20-d vitamin K dietary deficiency period. The reason for the lack of response of osteocalcin to the vitamin K–restricted diet is unknown.

Our baseline values for ucOC concentrations were similar to those found in a study that assessed biochemical indexes for vitamin K nutritional status in a healthy adult population (25) from the New England region and were consistent with the ucOC concentrations of healthy subjects after they consumed a mixed-diet containing 100 mg phylloquinone/d for 15 d, during which ucOC concentrations increased significantly (26). In the latter study, the authors suggested that a diet sufficient for maintaining normal blood clotting may not be able to maximize carboxylation of osteocalcin. Also, Vermeer et al (27) stated that, “based on the Gla-content of the bone protein osteocalcin, a substantial part of the population must be characterized as biochemically vitamin K deficient.”

Although biochemical markers of vitamin K status were within normal ranges in our study, mean plasma phylloquinone concentrations were below average (0.94 compared with 1.1 nmol/L) for our laboratory and mean percentage ucOC was above average (27% compared with 18–26%). Average dietary
intakes in the United States are below the current RDA of 65–80 μg/d (28) and some data suggest that the current RDA does not support optimal vitamin K status (23, 26). Collectively, these observations suggest that these participants as a group did not have an adequate intake of vitamin K.

Liver is one of the main target tissues of vitamin K action, and in rats it has been shown that only when the liver contains sufficient vitamin K for optimal activity of the hepatic vitamin K–dependent carboxylase do circulating phylloquinone concentrations increase in response to phylloquinone supplementation (29). Therefore, the healthy subjects in our study were probably not consuming a vitamin K–adequate diet before the study; thus, there was a preferential uptake of phylloquinone for hepatic vitamin K–dependent proteins. During the phylloquinone dietary restriction period, as confirmed by significantly lower plasma phylloquinone concentrations, the amount of vitamin K available to the liver decreased, resulting in increased PIVKA-II concentrations (Figure 2) but no change in ucOC concentrations (Figure 3). We speculate that a mechanism exists that protects further undercarboxylation of osteocalcin and, possibly, of other extrahepatic vitamin K–dependent proteins.

The intestinal absorption of vitamin K (of phylloquinone and probably menaquinones) is thought to be governed by the same principles established for other fat-soluble vitamins. Most of the bacteria identified in subjects taking omeprazole belong to species colonizing the oral cavity and pharynx, suggesting a descending route of colonization (30). Bacteria that normally inhabit the terminal ileum can synthesize menaquinones, which may be absorbed from this region through pathways mediated by bile salts (31). Given orally, menaquinones are absorbed and can counteract the effect of vitamin K antagonists in rats (32) and humans (8). If bacterial synthesis of menaquinones were to contribute to vitamin K status, subjects with small-intestinal bacterial overgrowth (ie, those treated with omeprazole) would show a resistance to the subclinical effects of dietary phylloquinone restriction. Although menaquinones were not directly measured in this study, the significant decrease in PIVKA-II concentrations (Figure 2) and the trend toward increased excretion of urinary Gla during the period of omeprazole treatment plus dietary phylloquinone restriction provides some evidence for a contribution of menaquinones synthesized by intestinal bacteria in vivo to vitamin K status in humans.

During treatment with omeprazole, the subjects in our study showed no changes in ucOC concentrations. Given the significant positive relation between percentage ucOC and PIVKA-II concentrations (25), we expected that ucOC concentrations would decrease in parallel to PIVKA-II. Our results were surprising because significant decreases in percentage ucOC had been shown in healthy subjects after vitamin K administration (23) and dietary phylloquinone supplementation (26). In our study, PIVKA-II concentrations decreased during omeprazole treatment but were still 3 times greater than the concentrations measured at baseline. This suggests that the amount of vitamin K produced by the bacteria was not enough to ensure the optimal activity of the vitamin K–dependent carboxylase in the liver, and it is likely that there was still not a sufficient amount of vitamin K available for extrahepatic tissues. PIVKA-II has also been shown to respond significantly to dietary phylloquinone deficiency (3) and vitamin K antagonism (23). Collectively, these data suggest that the PIVKA-II concentration is a more sensitive marker than percentage ucOC for diagnosing subclinical vitamin K deficiency produced by dietary phylloquinone restriction and the marginal reversal of this deficiency under conditions of bacterial overgrowth.

There are 2 possible explanations for this small contribution. First, the predominant bacteria that proliferate in the small intestine in subjects taking omeprazole are not producers of large amounts of menaquinones. In fact, relatively few of the bacteria that make up the normal small-intestinal flora are major producers of menaquinones. Obligate anaerobes such as Bacteroides fragilis and facultative anaerobic organisms such as Escherichia coli, which are predominantly in the colon, are menaquinone producers. However, major small-intestinal organisms such as bifidobacterium and lactobacillus species do not produce the vitamin (33). In the bacterial overgrowth in hypochlorhydria induced by omeprazole, the primary sources of bacteria are saliva and food (34). These bacteria consist predominantly of aerobic Gram-positive organisms (35), which may not be large producers of menaquinones. Second, most bacterial menaquinones are not available for absorption because they are tightly bound to insoluble material (such as membrane remnants) (11, 31).

In summary, our data confirm earlier findings of Suttie et al (22) and Ferland et al (3) that a vitamin K deficiency can be induced by a phylloquinone-restricted diet, as seen by the significant decrease in plasma phylloquinone concentrations and increase in plasma PIVKA-II concentrations. Subjects in this study did not show any alterations in total osteocalcin concentrations or percentage ucOC when consuming the phylloquinone-restricted diet or during the period of combined omeprazole treatment and dietary phylloquinone restriction. Because vitamin K status improved during bacterial overgrowth while phylloquinone intake was restricted, our data support the hypothesis that bacterial overgrowth can produce bioavailable menaquinones. These menaquinones contribute to vitamin K nutriture during dietary phylloquinone restriction, but not enough to restore normal vitamin K status.

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