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Deuterium-labeled Phylloquinone Fed to α -Tocopherol Injected Rats Demonstrates Sensitivity of Low Phylloquinone-Containing Tissues to Menaquinone-4 Depletion

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Abstract

The influence of excess α -tocopherol (α -T) on tissue depletion of phylloquinone (PK) and menaquinone-4 (MK-4) was evaluated by feeding rats (n=5/group) deuterium-labeled-PK (d₄-PK, 2 μ mol/kg) for 17 d, thereby labeling the conversion from d₄-PK to d₄-MK-4. Then they were injected subcutaneously daily for the last seven days with saline, vehicle, or α -T (100 mg/kg BW). α -T injections 1) increased α -T concentrations by 10-fold in liver, doubled them in plasma and most tissues, but they were unchanged in brain; 2) increased the α -T metabolite, carboxyethyl hydroxychromanol (α -CEHC) concentrations: >25-fold in liver and kidney, 10-fold in plasma and lung, and 50-fold in heart; brain contained detectable α -CEHC (0.26 ± 0.03 nmol/g) only in α -T injected animals and 3) depleted most tissues' vitamin K. Compared with vehicle-injected rats, brains from α -T rats contained half the total vitamin K (10.3 ± 0.5 vs. 21 ± 2 pmol/g, $P=0.0002$) and one third the d₄-MK-4 (5.8 ± 0.5 vs. 14.6 ± 1.7 pmol/g, $P=0.0002$). Tissues with high PK concentrations (liver, 21–30 pmol/g and heart, 28–50 pmol/g) were resistant to K depletion. We propose that α -T dependent vitamin K depletion is likely mediated at an intermediate step in MK-4 production; thus, tissues with high PK are unaffected.

Keywords

5C-and 7C-aglycones; CEHC; CYP4F2; urine; bile; brain; heart

Introduction

More than 90% of Americans do not consume sufficient dietary vitamin E (α -tocopherol, α -T) to meet estimated average requirements (EAR) [1]. Although supplement intake in

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Statement of authors' contributions to manuscript

SMF, JFS and MGT designed research; SMF, DJC, DJH, and SWL conducted research; JFS provided essential materials; SWF and MGT analyzed data; SMF, SWL and MGT wrote the paper. MGT had primary responsibility for final content. All authors read, edited and approved the final manuscript.

associated with adequate micronutrient intakes [2], the public is concerned that vitamin E supplements are dangerous [3] because meta-analyses of randomized clinical trials of antioxidants claim that vitamin E increases risk of death [4–6]. However, the documented adverse consequences of vitamin E in humans do not include death, but rather include increased bleeding [7], heart failure [8], and hemorrhagic stroke [9, 10]. Hypothetically, these adverse effects in humans are a result of vitamin E and K interactions, which lead to decreased vitamin K status.

Studies in rats suggest that vitamin E antagonizes vitamin K because the increased bleeding that is associated with excess dietary vitamin E [11–13] can be prevented by dietary vitamin K supplementation [11, 14, 15]. Since the 1940s animal studies have shown that high dietary vitamin E intakes lead to impaired coagulation [11, 14–17]. The Institute of Medicine used this information to set the upper tolerable limit of daily α -T supplements in humans to 1000 mg (1100 *all racemic* or 1500 IU *RRR*- α -T) [18]. In humans, vitamin E supplements (1000 IU per day) for 12 weeks also increased a biomarker of low vitamin K status, PIVKA-II (protein induced by vitamin K antagonism-factor II) [19]. The Women's Health Study demonstrated decreased risk of venous thromboembolism in women supplemented every other day for 10 years with vitamin E (600 IU) and attributed this benefit to vitamin K antagonism [20]. Thus, the claim that high dietary vitamin E adversely impacts vitamin K status is well supported, but the mechanism by which vitamin E supplementation adversely impacts vitamin K status is not known.

The various forms of vitamin K, the interconversion between forms and vitamin K metabolism have recently been extensively reviewed [21]. In brief, phyloquinone (PK), the plant-derived, dietary vitamin K form, is converted endogenously by UbiA prenyltransferase containing 1 (UBIAD1) in tissues to menaquinone-4 (MK-4) [22]. Menadione is an intermediate in this process [23]. Menadione has been suggested to be generated following intestinal PK uptake; the menadione then circulates and is taken up by tissues with subsequent conversion to MK-4 [24]. However, chylomicron transport PK from the intestine to the circulation has also reported [25, 26]. Thus, the extent of intestinal PK conversion to menadione is not known. Okano et al [27] have suggested that the cerebrum takes up both PK and menadione, and then converts them to MK-4.

UBIAD1, a prenyl transferase, likely controls tissue MK-4 concentrations. MK-4 is a potent ligand for the nuclear steroid X receptor (SXR) [28, 29]. In addition to converting PK to MK-4 or menadione to MK-4 [22], UBIAD1 is also known as the product of the transitional epithelial response (TERE1), a tumor suppressor gene [30]. UBIAD1 is also involved in ubiquinol synthesis [31] and regulation of cholesterol metabolism [32]. These findings suggest a link between UBIAD1, regulation of MK-4 concentrations and SXR target gene responses [33], especially with regard to tumor suppression [31].

Vitamin K is better known for its co-factor role in the post-translational processing of vitamin K-dependent proteins, which regulate calcium levels in the blood-clotting cascade, artery walls, bone matrix and brain [34]. Both PK and MK-4 (but not menadione) function as co-factors [35]. Vitamin K (either PK or MK-4) first must be reduced to the hydroquinone (KH₂), which is used as a cofactor by vitamin K carboxylase (GGCX) to

carboxylate glutamic acids (Glu) to γ -carboxyglutamic acids (Gla) in vitamin K-dependent proteins [36]. Gla are necessary for the calcium-chelating function of vitamin K-dependent proteins [37]. Gla production generates vitamin K epoxide, which then requires a 4-electron reduction to form KH_2 . The vitamin K oxidoreductase complex 1 (VKORC1) carries out the reduction steps and drives the reaction [38]. GGCX and VKORC1 form a hetero-dimeric complex to promote efficient vitamin K function and recycling [39]. This recycling process is critical for vitamin K function [40], allowing each vitamin K to participate in more than 500 times in carboxylation reactions [41], thereby allowing tissue vitamin K concentrations to be relatively low, e.g. in the nmol/g range.

Vitamin E, α -T, functions as a lipid soluble antioxidant, primarily as a peroxyl radical scavenger [42]. Both vitamins E and K are fat-soluble, share similar side-chains and are metabolized to tail-shortened, carboxy-compounds [21, 43]. PK and MK-4 are catabolized to 5-carbon side chain- (5C-) and seven carbon side chain (7C-) aglycone catabolites, a process that is initiated with an ω -hydroxylation by CYP4F2 [44, 45], followed by multiple β -oxidation rounds to yield the 5C- and 7C-side chain catabolites [46]. Similarly, α -T is ω -hydroxylated by CYP4F2 [47, 48] and metabolized to tail shorted metabolite, α -carboxyethyl hydroxychromanol (α -CEHC), as reviewed [49]. It is plausible that α -T excess up-regulates this shared metabolic pathway and thus might increase vitamin K metabolism and excretion. However, we previously used a model of excess vitamin E: subcutaneous α -T injections administered to rats to study vitamin E and K interactions [50]. We found that irrespective of whether rats were fed low dietary concentrations of menadione or PK (2 $\mu\text{mol/ kg}$ diet), α -T injections (100 mg α -T/kg body weight) similarly depleted extra-hepatic tissue MK-4 concentrations, when measured in fasted rats. Urinary excretion of vitamin K metabolites was not increased and hepatic expression of CYP4F2 mRNA and protein were decreased, rather than increased [50]. Moreover, our in vitro studies demonstrated that α -T did not up-regulate or activate vitamin K hydroxylation by CYP4F2 [51].

We hypothesized that the influence of excess α -tocopherol (α -T) on tissue depletion of phylloquinone (PK) and menaquinone-4 (MK-4) could be evaluated by feeding deuterium-labeled-PK (d_4 -PK), thereby labeling the conversion from d_4 -PK to d_4 -MK-4. The use of d_4 -PK is a novel approach to define the impact of vitamin E excess on vitamin K status in various tissues.

Methods

Animal studies

The Oregon State University (OSU) Institutional Animal Care and Use Committee approved all procedures (ACUP 4241).

Male Sprague-Dawley rats (Charles River, 250–300 g) were housed in plastic cages with hardwood chips and were kept on a 12-h light/dark schedule. Upon arrival, rats were fed a defined diet (TD.120038, Harlan Teklad, Madison, WI) that contained per kg diet both vitamin E (60 IU *all-rac*- α -tocopheryl acetate/ kg) and d_4 -PK (2 $\mu\text{mol/ kg}$ diet) as Vitamin K1-ring-D4 (Buchem BV, Apeldoorn, The Netherlands). Vitamins E and K were dissolved in tocopherol-stripped corn oil (USB Corporation, Cleveland, OH); the oil was then used in

the manufacture of the otherwise vitamin K-deficient semi-purified diet (Table 1). Diets were pelleted, vacuum-sealed in 1 kg amounts in plastic bags and kept frozen (-20°C) until use. Food and water were allowed ad libitum.

After 10 days of consuming the d_4 -PK-diet, rats received daily subcutaneous (SC) injections of saline (control), α -T (100 mg *RRR*- α -tocopherol/kg body weight, Emcelle, Stuart Products, Bedford, TX) or of vehicle, which was the same emulsion without α -T addition (Stuart Products, Bedford, TX). Injections were carried out daily at 9 AM for 7 days. Twenty-four h after the last injection (including a 12-h fast), rats were anesthetized using isofluorane, exsanguinated, then blood and tissues were collected as previously described [50] and stored at -80°C until analysis. Rats' weights (g, mean $\pm$ SEM) before starting injections (d 0) were similar for control (saline-injected, 331 ± 12 g), vehicle-injected (346 ± 5) and α -T-injected (343 ± 8) rats. However, at d 8 α -T-injected rats had significantly lower body weights (326 ± 7) compared with vehicle-injected animals (371 ± 4), but not saline injected rats (350 ± 15 g; two-way ANOVA (interaction, $P < 0.0001$; Tukey multiple comparisons, $p < 0.05$).

Vitamin E, α -carboxy ethyl hydroxy chomanol and vitamin K analyses

Plasma and tissue α -T concentrations were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ECD), as described previously [52]. α -CEHC concentrations were determined by high-performance liquid chromatography with mass spectrometry (HPLC-MS), as described previously [53] with the exception that plasma was pretreated with 0.5 mL 6N HCL instead of glucuronidase prior to extraction, as described [54]. Following incubation (60 min at 37°C), the samples were extracted with 4 mL diethyl ether and an aliquot of the ether fraction was collected and dried under nitrogen. The extracts were resuspended in 1:1 (v/v) water:methanol with trolox (500 ng/mL, 10 μL added, as an internal standard), and analyzed using by HPLC-MS (Waters (Milford, MA) 2695 Separations Module and a Micromass ZQ2000 (Milford, MA)). Instrument control and acquisition was performed using Waters Masslynx version 4.0 software. The column used was a SymmetryShield^{TM} RP-18 column (3.0 $\times$ 150 mm, 3.5 μm particle; Waters) with a Symmetry-Shield^{TM} Sentry^{TM} RP-18 pre-column (3.9 $\times$ 20 mm, 3.5 μm particle; Waters). Single-ion recording mass-to-charge ratio (m/z) data were obtained for α -CEHC (m/z 277), γ -CEHC (m/z 263) and the internal standard trolox (m/z 249). Typical retention times were 14.2, 14.6 and 15.4 minutes for trolox, γ -CEHC, and α -CEHC respectively. Sample CEHC concentrations were calculated from the peak area of the corresponding ion to that of the internal standard peak.

Plasma and tissue d_4 -PK, PK, d_4 -MK-4, and MK-4 were extracted and measured with the HPLC-MS described above with an atmospheric pressure chemical ionization (APCI) source operating in negative mode as previously described [50], using MK-7 (Caymen Chemical, Ann Arbor, MI), as the internal standard. Single-ion recording (SIR) data were obtained for d_4 -PK (m/z 454), PK (m/z 450), d_4 -MK-4 (m/z 448), MK-4 (m/z 444), and MK-7 (m/z 648). Typical retention times for MK-4, PK and d_4 -PK, and MK-7 were 8.0, 13.3 and 24.5 minutes, respectively. Peak areas were integrated using Waters Masslynx version 4.0 software. Labeled and unlabeled PK and MK-4 concentrations were quantitated using

calibration standards prepared using authentic compounds. The lower limit of quantification (LLOQ) for plasma PK and MK-4 was 0.4 nM, and the lower limit of detection (LLOD) was 0.2 nM, with signal-to-noise ratios of 10/1 and 3/1, respectively. Analyte concentrations were quantitated by comparison of peak areas relative to the internal standard.

Plasma total cholesterol was determined using Thermo Infinity cholesterol reagent (Fisher Diagnostics, Middletown, VA), at 500 nm.

Statistical Analyses

Data are reported as means $\pm$ SEM (n=5 rats per group). Statistical analyses were performed using Prism 5 statistical software (Graphpad Software, Inc.). Data were analyzed by one or two-way (for time courses) ANOVA; when interactions, or overall group effects were significant ($P < 0.05$), then Tukey multiple-comparisons (TMC) were performed; or by using Student's t-test where indicated. When variances were different between groups, data were log-transformed to normalize variances. Brain α -CEHC was only detectable in the α -T injected rats, therefore Fisher's exact test was used.

To estimate the percentage tissue conversion of d₄-PK to d₄-MK-4, the product (d₄-MK-4) concentrations were divided by the labeled vitamin K (sum of d₄-PK and d₄-MK-4 concentrations).

Results

Plasma and Tissue α -T and α -CEHC Concentrations

Rats were fed d₄-PK-containing diets for 17 days; beginning on day 10, they were SC injected daily for 7 days with saline, vehicle, or α -T. To evaluate the extent of increase in α -T concentrations by the injections, the rats were sacrificed 24 h after the last injection (including a 12-h fast). The result of the seven daily α -T injections was to increase liver α -T concentrations 10-fold; plasma and most tissues' α -T concentrations approximately doubled, while brain α -T concentrations were not significantly changed by any of the treatments (Figure 1A).

α -T injections also increased α -CEHC concentrations in plasma and all tissues examined. Liver, kidney and heart α -CEHC concentrations increased 40-, 25- and 40-fold, respectively (Figure 1B). Importantly, brain α -CEHC concentrations were only detectable in α -T injected rats (Fisher's exact test, $P = 0.0003$).

Plasma and Tissue Vitamin K Concentrations

To evaluate the changes in vitamin K concentrations resulting from the α -T injections, the plasma and tissues from the rats described above were analyzed for labeled and unlabeled PK and MK-4. Plasma contained only d₄-PK, not unlabeled PK, or d₄- or unlabeled MK-4. Plasma d₄-PK concentrations were not statistically different between treatment groups (saline 0.4 ± 0.1 , vehicle 0.3 ± 0.1 , α -T 0.2 ± 0.1 nmol/L).

Tissue vitamin K concentrations varied widely with regard to distribution of unlabeled PK, d₄-PK and d₄-MK-4 concentrations (Table 2). Of the tissues examined, only brain contained substantial amounts of unlabeled MK-4, in addition to d₄-MK-4 concentrations (Table 2).

The effect of α -T injections on vitamin K concentrations also varied with the tissue type. In the liver, α -T injections had no statistically significant effects on vitamin K concentrations (Table 2). In extra-hepatic tissues, α -T injections decreased brain, kidney and lung total vitamin K concentrations by more than half, while in heart tissue, α -T injections had no significant effect on vitamin K concentrations (Table 2). Additionally, heart d₄-PK concentrations were more than double those of other tissues measured; α -T injections had a tendency to increase d₄-PK concentrations (Table 2).

Conversion of d₄-PK to d₄-MK-4

The rats' diets only contained d₄-PK. No Mk-4 was detected in plasma, suggesting transport of d₄-MK-4 from one tissue to another is not likely to contribute to tissue MK-4 concentrations. Therefore, d₄-MK-4 is likely to be synthesized in the tissues. d₄-PK could have been converted to d₄-menadione in the intestine with subsequent plasma transport of d₄-menadione [24]; however, we were not successful in measuring plasma menadione.

The apparent conversion of d₄-PK to d₄-MK-4 was calculated by dividing the d₄-MK-4 by the sum of d₄-MK-4 and d₄-PK concentrations in each tissue. This calculation assumes that tissues are at steady state, that the influx and efflux from other tissues are balanced and that no other forms of vitamin K, e.g. menadione, vitamin K-epoxide, etc. contribute to the total deuterated-vitamin K body pool. The percentage conversion of d₄-PK to d₄-MK-4 varied dramatically between tissues, as has been reported previously [55]. The d₄-PK to d₄-MK-4 conversion in brain was approximately 90%, which was the highest d₄-PK fractional conversion of any of the tissues, while kidney and lung were lower at approximately 50%, while liver was only 20% (Table 3). α -T injections had virtually no effect on this parameter in any of the tissues examined with the exception of the heart. In the heart, α -T injections decreased the percentage d₄-PK conversion from ~20% to 8%. The heart also accumulated high concentrations of d₄-PK and did not show any significant decrease in d₄-MK-4 concentrations in response to α -T injections (Table 2). Overall, these data indicate that the process by which vitamin E effects vitamin K status does not change the fraction of d₄-PK that is converted to d₄-MK-4 with the exception of in the heart, which had exceptionally high PK concentrations.

Discussion

In the present study, rats were fed a diet containing only d₄-PK as the source vitamin K. Following 17 days of consuming this diet, most tissues, including the brain, contained only d₄-vitamin K with <5% unlabeled PK, and no unlabeled MK-4. This response is quite different from studies in rats fed labeled vitamin E, which has a much slower turnover, especially in nervous tissues e.g. brain and spinal cord [56, 57]. Since vitamin E is transported solely in lipoproteins [43], these data strongly suggest that vitamin K transport is not solely dependent upon lipoprotein delivery to tissues. This finding is consistent with the relatively low concentrations of vitamin K in lipoproteins, such as LDL and HDL [26].

However, d₄-PK absorption and transport in chylomicrons, e.g. triglyceride-rich lipoproteins [26, 58], with subsequent delivery to tissues is supported by our findings.

Recently, the intestinal release of menadione has been proposed as a transport form of vitamin K [24]. Our data neither supports nor refutes this proposal, because we were not successful in measuring menadione (data not shown). Tissue MK-4 synthesis requires cleavage of the PK side chain, producing menadione as an intermediate, which is then geranylgeranylated [22]. Hypothetically, tissue uptake of intestinally derived menadione from the circulation would provide a shortcut for tissues to synthesize MK-4.

To evaluate vitamin E and K interactions, α -T injections were used to rapidly expose rats to high α -T concentrations. It should be noted that dietary α -T has been used previously in rats to evaluate these vitamin interactions and Tovar et al [59] reported lower tissue concentrations of both PK and MK-4 in spleen, kidney and brain, but not plasma or liver. Thus, the route of vitamin E administration did not affect the outcomes of lower vitamin K status. Herein we show that following seven days of daily α -T injections, brain, kidney, and lung, were especially vulnerable to vitamin K depletion (Table 2). The heart and liver were exceptions; notably they tended to contain higher total vitamin K concentrations and a higher proportion (>75%) of d₄-PK. Thijssen et al [60, 61] previously emphasized that heart and liver are unique in that they have proportionately greater PK relative to MK-4 compared with other tissues. Apparently efficient delivery of dietary PK to the liver and heart provided resistance to vitamin K depletion by excess α -T. It has been shown that PK-carrying chylomicrons deliver PK to the liver and bone [62]. Because the heart requires a high input of fatty acids [63, 64], it is likely that both chylomicrons and triglyceride-rich lipoproteins (e.g. very low density lipoproteins) containing PK also efficiently deliver PK to the heart. This postulated high influx of dietary PK to both the heart and the liver appears protective for vitamin K status.

Despite the major effects of the α -T injections on total vitamin K concentrations in the extra-hepatic tissues examined, the relative proportions of the vitamin K forms in each of the tissues were largely unaffected (Figure 2). For example, d₄-PK represented approximately 80% of liver or heart vitamin K, while in the kidney d₄-MK-4 and d₄-PK were present in nearly equal proportions, irrespective of whether the animals were injected with saline, vehicle, or α -T. In the brain, the d₄-MK-4 plus unlabeled MK-4 represented more than 90% of vitamin K, again with no changes due to treatment. These findings suggest that overall delivery of vitamin K to tissues was decreased, but once the tissue obtained the vitamin K there was a consistent portion converted to MK-4.

The percentage d₄-PK to d₄-MK-4 conversion varied dramatically between tissues (Table 3). This percentage was approximately 90% in brain, which was the highest d₄-PK fractional conversion to d₄-MK-4 of any of the tissues, while in kidney and lung it was approximately 50%, and in liver it was approximately 20%. α -T had virtually no effect on conversion in any of the tissues examined (with the exception of the heart, discussed below). It is unclear as to why synthesis of MK-4 is necessary since both PK and MK-4 function as co-factors for Gla synthesis by GGCX [35].

This is the first study to report tissue vitamin E metabolite (α -CEHC) concentrations. The extraordinarily high α -T (100 mg/kg daily for 7 days) amounts administered to the rats lead to detectable tissue α -CEHC concentrations in all tissues examined (Figure 1B). It is likely that α -CEHC, which is water soluble, was secreted from the liver for uptake by the kidney and excretion in urine, since urine is a major excretory route for α -CEHC [43]. The 10-fold increase in circulating concentrations in the α -T injected animals suggests that this high circulating concentration led to non-specific tissue uptake of α -CEHC. Notably, most tissues displayed only a 2-fold increase in tissue α -T and in the case of brain, no change in α -T concentrations.

As for the mechanism for the observed decrease in vitamin K status by vitamin E excess, the increases in circulating and tissue α -CEHC concentrations possibly could lead to decreases in menadiol production in the intestine or tissues, or decreases in an as yet undescribed receptor mediated uptake of circulating menadiol. We reported previously that hearts from menadiol-fed rats contained higher MK-4 concentrations than did those from PK-fed animals, suggesting that menadiol compared with PK is more readily converted to MK-4 [50]. However, α -T administration led to similarly depleted MK-4 concentrations in both diet groups [50]. In the present study, hearts from rats receiving α -T injections similarly displayed a lower percentage d_4 -PK conversion to d_4 -MK-4 (8%) compared with rats receiving injections of saline (24%) or vehicle (18%) (Table 2). Taken together these data suggest that the conversion of menadiol to MK-4 is impaired in α -T injected rats (Figure 3). This study sought to discover the fate of PK by using d_4 -PK to identify recently absorbed dietary PK separate from tissue reserves. Our data clearly demonstrate that d_4 -PK replaced most of the tissue unlabeled PK, but more importantly many tissues in α -T excess-treated rats contained depleted d_4 -PK concentrations compared with rats in the saline or vehicle treatments. Thus, any mechanism to explain the decrease in tissue vitamin K must explain decreases in both PK and MK-4. Hypothetically, uptake of d_4 -PK could be affected by α -CEHC, but this seems unlikely since d_4 -PK is fat-soluble and α -CEHC is water-soluble. Given how little information is available about mechanisms for tissue uptake of the various vitamin K forms and the mechanisms for plasma transport, further research is needed.

In conclusion, this study demonstrates that in response to α -T excess, vitamin K status decreased in most of the extrahepatic tissues studied. Tissues resistant to the effects of α -T administration are those with high PK concentrations, such as the liver and the heart, which may be able to maintain adequate concentrations of vitamin K. Further research is needed to clarify the role of excess α -T interference with conversion of PK to MK-4, especially with regard to the vitamin E metabolite, α -CEHC.

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Abbreviations

CEHC	carboxyethyl hydroxychromanol
CYP4F2	cytochrome P450 4F2
d	deuterated
MK-4	menaquinone-4
PK	phyloquinone
T	tocopherol

References

1. Moshfegh A, Goldman J, Cleveland L. United States Department of Agriculture. Agricultural Research Service. 2005
2. Bailey RL, Fulgoni VL 3rd, Keast DR, Dwyer JT. Examination of vitamin intakes among US adults by dietary supplement use. *Journal of the Academy of Nutrition and Dietetics*. 2012; 112:657–663. e654. [PubMed: 22709770]
3. Miller, A. Popular but Dangerous: 3 Vitamins That Can Hurt You If you take a vitamin pill, these warnings probably apply to you. U.S. News & World Report LP; 2012. Available at: <http://health.usnews.com/health-news/diet-fitness/articles/2012/02/24/popular-but-dangerous-3-vitamins-that-can-hurt-you>. [Date accessed: November 10, 2013]
4. Miller ER 3rd, Paston-Barriuso R, Dalal D, Riemersma RA, et al. Meta-analysis: high-dosage vitamin E supplementation may increase all-cause mortality. *Ann Intern Med*. 2005; 142:37–46. [PubMed: 15537682]
5. Myung SK, Ju W, Cho B, Oh SW, et al. Efficacy of vitamin and antioxidant supplements in prevention of cardiovascular disease: systematic review and meta-analysis of randomised controlled trials. *BMJ*. 2013; 346:f10. [PubMed: 23335472]
6. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. *The Cochrane database of systematic reviews*. 2012; 3:CD007176. [PubMed: 22419320]
7. Lee IM, Cook NR, Gaziano JM, Gordon D, et al. Vitamin E in the primary prevention of cardiovascular disease and cancer: the Women's Health Study: a randomized controlled trial. *JAMA*. 2005; 294:56–65. [PubMed: 15998891]
8. Lonn E, Bosch J, Yusuf S, Sheridan P, et al. Effects of long-term vitamin E supplementation on cardiovascular events and cancer: a randomized controlled trial. *JAMA*. 2005; 293:1338–1347. [PubMed: 15769967]
9. Sesso HD, Buring JE, Christen WG, Kurth T, et al. Vitamins E and C in the prevention of cardiovascular disease in men: the Physicians' Health Study II randomized controlled trial. *JAMA*. 2008; 300:2123–2133. [PubMed: 18997197]
10. Schurks M, Glynn RJ, Rist PM, Tzourio C, Kurth T. Effects of vitamin E on stroke subtypes: meta-analysis of randomised controlled trials. *BMJ*. 2010; 341:c5702. [PubMed: 21051774]
11. Wheldon GH, Bhatt A, Keller P, Hummler H. d,l-alpha-Tocopheryl acetate (vitamin E): a long term toxicity and carcinogenicity study in rats. *Int J Vitam Nutr Res*. 1983; 53:287–296. [PubMed: 6629668]
12. Abdo KM, Rao G, Montgomery CA, Dinowitz M, Kanagalingam K. Thirteen-week toxicity study of d-alpha-tocopheryl acetate (vitamin E) in Fischer 344 rats. *Food Chem Toxicol*. 1986; 24:1043–1050. [PubMed: 3804111]
13. Takahashi O, Ichikawa H, Sasaki M. Hemorrhagic toxicity of d-alpha-tocopherol in the rat. *Toxicology*. 1990; 63:157–165. [PubMed: 2399533]
14. Frank J, Weiser H, Biesalski HK. Interaction of vitamins E and K: effect of high dietary vitamin E on phyloquinone activity in chicks. *Int J Vitam Nutr Res*. 1997; 67:242–247. [PubMed: 9285253]

15. Helson L. The effect of intravenous vitamin E and menadiol sodium diphosphate on vitamin K dependent clotting factors. *Thromb Res.* 1984; 35:11–18. [PubMed: 6474408]
16. Woolley DW. Biological antagonisms between metabolically important compounds and their structural analogs. *Harvey Lect.* 1945; 41:189–215. [PubMed: 20289739]
17. March BE, Wong E, Seier L, Sim J, Biely J. Hypervitaminosis E in the chick. *J Nutr.* 1973; 103:371–377. [PubMed: 4688149]
18. Food and Nutrition Board, Institute of Medicine. Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids. Washington: National Academy Press; 2000.
19. Booth SL, Golly I, Sackeck JM, Roubenoff R, et al. Effect of vitamin E supplementation on vitamin K status in adults with normal coagulation status. *Am J Clin Nutr.* 2004; 80:143–148. [PubMed: 15213041]
20. Glynn RJ, Ridker PM, Goldhaber SZ, Zee RY, Buring JE. Effects of random allocation to vitamin E supplementation on the occurrence of venous thromboembolism: report from the Women's Health Study. *Circulation.* 2007; 116:1497–1503. [PubMed: 17846285]
21. Shearer MJ, Newman P. Recent trends in the metabolism and cell biology of vitamin K with special reference to vitamin K cycling and MK-4 biosynthesis. *J Lipid Res.* 2014; 55:345–362. [PubMed: 24489112]
22. Nakagawa K, Hirota Y, Sawada N, Yuge N, et al. Identification of UBIAD1 as a novel human menaquinone-4 biosynthetic enzyme. *Nature.* 2010; 468:117–121. [PubMed: 20953171]
23. Thijssen HH, Vervoort LM, Schurgers LJ, Shearer MJ. Menadione is a metabolite of oral vitamin K. *Br J Nutr.* 2006; 95:260–266. [PubMed: 16469140]
24. Hirota Y, Tsugawa N, Nakagawa K, Suhara Y, et al. Menadione (vitamin K3) is a catabolic product of oral phyloquinone (vitamin K1) in the intestine and a circulating precursor of tissue menaquinone-4 (vitamin K2) in rats. *J Biol Chem.* 2013; 288:33071–33080. [PubMed: 24085302]
25. Lamou-Fava S, Sadowski JA, Davidson KW, O'Brien ME, et al. Plasma lipoproteins as carriers of phyloquinone (vitamin K1) in humans. *Am J Clin Nutr.* 1998; 67:1226–1231. [PubMed: 9625097]
26. Erkkila AT, Lichtenstein AH, Dolnikowski GG, Grusak MA, et al. Plasma transport of vitamin K in men using deuterium-labeled collard greens. *Metabolism: clinical and experimental.* 2004; 53:215–221. [PubMed: 14767874]
27. Okano T, Shimomura Y, Yamane M, Suhara Y, et al. Conversion of phyloquinone (Vitamin K1) into menaquinone-4 (Vitamin K2) in mice: two possible routes for menaquinone-4 accumulation in cerebra of mice. *J Biol Chem.* 2008; 283:11270–11279. [PubMed: 18083713]
28. Horie-Inoue K, Inoue S. Steroid and xenobiotic receptor mediates a novel vitamin K2 signaling pathway in osteoblastic cells. *Journal of bone and mineral metabolism.* 2008; 26:9–12. [PubMed: 18095058]
29. Fredericks WJ, McGarvey T, Wang H, Zheng Y, et al. The TERE1 protein interacts with mitochondrial TBL2: regulation of trans-membrane potential, ROS/RNS and SXR target genes. *Journal of cellular biochemistry.* 2013; 114:2170–2187. [PubMed: 23564352]
30. McGarvey TW, Nguyen T, Tomaszewski JE, Monson FC, Malkowicz SB. Isolation and characterization of the TERE1 gene, a gene down-regulated in transitional cell carcinoma of the bladder. *Oncogene.* 2001; 20:1042–1051. [PubMed: 11314041]
31. Mugoni V, Postel R, Catanzaro V, De Luca E, et al. Ubiad1 is an antioxidant enzyme that regulates eNOS activity by CoQ10 synthesis. *Cell.* 2013; 152:504–518. [PubMed: 23374346]
32. Nickerson ML, Bosley AD, Weiss JS, Kostih BN, et al. The UBIAD1 prenyltransferase links menaquinone-4 [corrected] synthesis to cholesterol metabolic enzymes. *Human mutation.* 2013; 34:317–329. [PubMed: 23169578]
33. Fredericks WJ, Sepulveda J, Lai P, Tomaszewski JE, et al. The tumor suppressor TERE1 (UBIAD1) prenyltransferase regulates the elevated cholesterol phenotype in castration resistant prostate cancer by controlling a program of ligand dependent SXR target genes. *Oncotarget.* 2013; 4:1075–1092. [PubMed: 23919967]
34. Ferland G. The discovery of vitamin K and its clinical applications. *Annals of nutrition & metabolism.* 2012; 61:213–218. [PubMed: 23183291]

35. Buitenhuis HC, Soute BA, Vermeer C. Comparison of the vitamins K1, K2 and K3 as cofactors for the hepatic vitamin K-dependent carboxylase. *Biochim Biophys Acta*. 1990; 1034:170–175. [PubMed: 2112953]
36. Rishavy MA, Berkner KL. Vitamin K oxygenation, glutamate carboxylation, and processivity: defining the three critical facets of catalysis by the vitamin K-dependent carboxylase. *Adv Nutr*. 2012; 3:135–148. [PubMed: 22516721]
37. Vermeer C. Vitamin K: the effect on health beyond coagulation - an overview. *Food & nutrition research*. 2012; 56
38. Rishavy MA, Hallgren KW, Wilson LA, Usabalieva A, et al. The vitamin K oxidoreductase is a multimer that efficiently reduces vitamin K epoxide to hydroquinone to allow vitamin K-dependent protein carboxylation. *J Biol Chem*. 2013
39. Wu S, Liu S, Davis CH, Stafford DW, et al. A hetero-dimer model for concerted action of vitamin K carboxylase and vitamin K reductase in vitamin K cycle. *Journal of theoretical biology*. 2011; 279:143–149. [PubMed: 21453708]
40. Suhara Y, Murakami A, Nakagawa K, Mizuguchi Y, Okano T. Comparative uptake, metabolism, and utilization of menaquinone-4 and phyloquinone in human cultured cell lines. *Bioorganic & medicinal chemistry*. 2006; 14:6601–6607. [PubMed: 16798001]
41. Chatrou ML, Reutelingsperger CP, Schurgers LJ. Role of vitamin K-dependent proteins in the arterial vessel wall. *Hamostaseologie*. 2011; 31:251–257. [PubMed: 21713318]
42. Traber MG, Atkinson J. Vitamin E, antioxidant and nothing more. *Free Radic Biol Med*. 2007; 43:4–15. [PubMed: 17561088]
43. Traber MG. Mechanisms for the prevention of vitamin E excess. *J Lipid Res*. 2013; 54:2295–2306. [PubMed: 23505319]
44. McDonald MG, Rieder MJ, Nakano M, Hsia CK, Rettie AE. CYP4F2 is a vitamin K1 oxidase: An explanation for altered warfarin dose in carriers of the V433M variant. *Mol Pharmacol*. 2009; 75:1337–1346. [PubMed: 19297519]
45. Edson KZ, Prasad B, Unadkat JD, Suhara Y, et al. Cytochrome P450-dependent catabolism of vitamin K: omega-hydroxylation catalyzed by human CYP4F2 and CYP4F11. *Biochemistry*. 2013; 52:8276–8285. [PubMed: 24138531]
46. Harrington DJ, Booth SL, Card DJ, Shearer MJ. Excretion of the urinary 5C- and 7C-aglycone metabolites of vitamin K by young adults responds to changes in dietary phyloquinone and dihydrophyloquinone intakes. *J Nutr*. 2007; 137:1763–1768. [PubMed: 17585028]
47. Sontag TJ, Parker RS. Cytochrome P450 omega-hydroxylase pathway of tocopherol catabolism. Novel mechanism of regulation of vitamin E status. *J Biol Chem*. 2002; 277:25290–25296. [PubMed: 11997390]
48. Sontag TJ, Parker RS. Influence of major structural features of tocopherols and tocotrienols on their omega-oxidation by tocopherol-omega-hydroxylase. *J Lipid Res*. 2007; 48:1090–1098. [PubMed: 17284776]
49. Jiang Q. Natural forms of vitamin E: Metabolism, antioxidant and anti-inflammatory activities and the role in disease prevention and therapy. *Free Radic Biol Med*. 2014
50. Farley SM, Leonard SW, Labut EM, Raines HF, et al. Vitamin E decreases extra-hepatic menaquinone-4 concentrations in rats fed menadione or phyloquinone. *Mol Nutr Food Res*. 2012; 56:912–922. [PubMed: 22707266]
51. Farley SM, Leonard SW, Taylor AW, Birringer M, et al. omega-Hydroxylation of phyloquinone by CYP4F2 is not increased by alpha-tocopherol. *Mol Nutr Food Res*. 2013; 57:1785–1793. [PubMed: 23650179]
52. Podda M, Weber C, Traber MG, Packer L. Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinol and ubiquinones. *J. Lipid Res*. 1996; 37:893–901. [PubMed: 8732789]
53. Leonard SW, Traber MG. Measurement of the vitamin E metabolites, carboxyethyl hydroxychromans (CEHCs), in biological samples. *Curr Protoc Toxicol*. 2006; Chapter 7(Unit 7.8.1–7.8.12)
54. Li YJ, Luo SC, Lee YJ, Lin FJ, et al. Isolation and identification of alpha-CEHC sulfate in rat urine and an improved method for the determination of conjugated alpha-CEHC. *J Agric Food Chem*. 2008; 56:11105–11113. [PubMed: 18991451]

55. Al Rajabi A, Booth SL, Peterson JW, Choi SW, et al. Deuterium-labeled phyloquinone has tissue-specific conversion to menaquinone-4 among Fischer 344 male rats. *J Nutr.* 2012; 142:841–845. [PubMed: 22437559]
56. Vatassery GT, Brin MF, Fahn S, Kayden HJ, Traber MG. Effect of high doses of dietary vitamin E upon the concentrations of vitamin E in several brain regions, plasma, liver and adipose tissue of rats. *J. Neurochem.* 1988; 51:621–623. [PubMed: 3392549]
57. Ingold KU, Burton GW, Foster DO, Hughes L, et al. Biokinetics of and discrimination between dietary RRR- and SRR-alpha-tocopherols in the male rat. *Lipids.* 1987; 22:163–172. [PubMed: 3573996]
58. Schurgers LJ, Vermeer C. Differential lipoprotein transport pathways of K-vitamins in healthy subjects. *Biochim Biophys Acta.* 2002; 1570:27–32. [PubMed: 11960685]
59. Tovar A, Ameho CK, Blumberg JB, Peterson JW, et al. Extrahepatic tissue concentrations of vitamin K are lower in rats fed a high vitamin E diet. *Nutr Metab (Lond).* 2006; 3:29. [PubMed: 16857056]
60. Thijssen HH, Drittij-Reijnders MJ. Vitamin K distribution in rat tissues: dietary phyloquinone is a source of tissue menaquinone-4. *Br J Nutr.* 1994; 72:415–425. [PubMed: 7947656]
61. Thijssen HH, Drittij-Reijnders MJ. Vitamin K status in human tissues: tissue-specific accumulation of phyloquinone and menaquinone-4. *Br J Nutr.* 1996; 75:121–127. [PubMed: 8785182]
62. Niemeier A, Niedzielska D, Secer R, Schilling A, et al. Uptake of postprandial lipoproteins into bone in vivo: impact on osteoblast function. *Bone.* 2008; 43:230–237. [PubMed: 18538644]
63. Bharadwaj KG, Hiyama Y, Hu Y, Huggins LA, et al. Chylomicron- and VLDL-derived lipids enter the heart through different pathways: in vivo evidence for receptor- and non-receptor-mediated fatty acid uptake. *J Biol Chem.* 2010; 285:37976–37986. [PubMed: 20852327]
64. Goldberg IJ, Trent CM, Schulze PC. Lipid metabolism and toxicity in the heart. *Cell metabolism.* 2012; 15:805–812. [PubMed: 22682221]
65. Reboul E, Borel P. Proteins involved in uptake, intracellular transport and basolateral secretion of fat-soluble vitamins and carotenoids by mammalian enterocytes. *Progress in lipid research.* 2011; 50:388–402. [PubMed: 21763723]
66. Podszun MC, Grebenstein N, Spruss A, Schlueter T, et al. Dietary alpha-tocopherol and atorvastatin reduce high-fat-induced lipid accumulation and down-regulate CD36 protein in the liver of guinea pigs. *J Nutr Biochem.* 2014; 25:573–579. [PubMed: 24725433]
67. Venugopal SK, Devaraj S, Jialal I. RRR-alpha-tocopherol decreases the expression of the major scavenger receptor, CD36, in human macrophages via inhibition of tyrosine kinase (Tyk2). *Atherosclerosis.* 2004; 175:213–220. [PubMed: 15262176]

Figure 1. Plasma and tissue α -T and α -CEHC

Following 1 week of daily SC injections to rats with saline (open bars), vehicle (striped bars), or α -T (solid bars), plasma and tissues taken for analysis (N=5/group). **A** α -T or **B** α -CEHC concentrations (mean $\pm$ SEM) are shown. For plasma, liver, kidney, lung and heart both α -T and α -CEHC concentrations were significantly higher in α -T injected (*) compared with saline or vehicle injected, which were not different from each other (ANOVA $P < 0.0001$; Tukey multiple comparisons (TMC) $P < 0.05$). Brain α -T concentrations were not statistically different between treatments; brain α -CEHC was only measurable

following α -T injections (ND, not detectable in saline or vehicle injected rats, Fisher's exact test $P=0.0003$).

Figure 2. Tissue Vitamin K Distribution

Labeled and unlabeled vitamin K concentrations were measured in selected tissues (liver, kidney, lung, heart and brain) following 1 week of daily injection with α -T, vehicle, or saline (labeled above each chart; see Table 2 for concentrations and statistical comparisons). Shown are the mean percentages of d₄-PK, PK, d₄-MK-4, and MK-4 in the charts for each injection condition; the total vitamin K concentration is shown below the chart and the percentages of the vitamin K forms are shown in the legend. Note that unlabeled MK-4 was only detected in brain.

Figure 3. Proposed mechanism for the interference of α -T excess on vitamin K status

Hypothetically, the decreases in MK-4 production could be caused by less menadione being available for conversion to MK-4. Decreases in menadione concentrations could be caused by decreases in production of menadione from PK in the intestine (shown as a diamond), decreased uptake of menadione by tissues (shown as a pentagon), and menadione conversion to MK-4 could be inhibited intracellularly. Since increases in α -CEHC concentrations in plasma and in all tissues were detected 24 h after the last α -T injection, high α -CEHC concentrations are a prime candidate for interference (shown as a starburst). Given that both

menadione and α -CEHC are water-soluble, either interference with or down-regulation of uptake receptors, seem the most plausible explanation for decreases in MK-4. However, no mechanisms for α -CEHC uptake have been described and circulating menadione remains a postulated vitamin K transport mechanism; thus, the mechanism is highly speculative. Importantly, we found that all tissues contained detectable d_4 -PK concentrations, which were decreased by the α -T treatment, suggesting interference with PK delivery. The known PK transport mechanisms are primarily in chylomicrons and very low-density lipoproteins (e.g. triglyceride-rich lipoproteins). It is not obvious that either α -CEHC or α -T should interfere with chylomicron secretion, but uptake/delivery of chylomicron contents to tissues is likely receptor mediated. Cluster determinant 36 (CD36) is a known mediator of cellular uptake of both fatty acids and fat-soluble vitamins [65] and is down-regulated by α -T [66, 67]. Thus, it is plausible that either α -CEHC or α -T could interfere in PK uptake by tissues. Clearly, all of these mechanisms are highly speculative and warrant further investigation.

TABLE 1 Composition of the d₄-PK diet

Formula:	<i>g/kg diet</i>
Isolated Soy Protein	192.0
DL-Methionine	1.0
Sucrose	508.0269
Maltodextrin	100.0
Corn Starch	50.0
Cellulose	50.0
Corn Oil, tocopherol-stripped	50.0
Mineral Mix, AIN-76 (170915)	35.0
Calcium Carbonate	11.25
Choline Bitartrate	2.5
Niacin	0.03
Calcium Pantothenate	0.016
Pyridoxine HCl	0.007
Thiamin HCl	0.006
Riboflavin	0.006
Vitamin B12 (0.1% in mannitol)	0.025
Folic Acid	0.002
Biotin	0.0002
Vitamin A Palmitate (500,000 IU/g)	0.008
Vitamin D3, cholecalciferol (500,000 IU/g)	0.002
Vitamin E, DL- α -tocopheryl acetate	60 IU
Vitamin K, d ₄ -PK	2 μ mol (approx. 1 mg)

Table 2

Tissue Labeled and Unlabeled Vitamin K Concentrations in Rats

Tissue	Injection	d ₄ -PK pmol/g	Unlabeled PK pmol/g	d ₄ -MK-4 pmol/g	Unlabeled MK-4 pmol/g	Total vitamin K pmol/g
Liver	α-T	25.06 ± 4.32	1.32 ± 0.21	5.35 ± 0.52		31.73 ± 4.99
	Vehicle	18.20 ± 3.80	0.85 ± 0.18	3.99 ± 0.26		23.04 ± 4.06
	Saline	17.93 ± 1.70	0.90 ± 0.11	4.46 ± 0.42		23.29 ± 2.16
	P-value	0.1856	0.1040	0.1048		0.2449
Kidney	α-T	2.31 ± 0.54 ^a	0.13 ± 0.03 ^a	2.06 ± 0.21 ^a		4.50 ± 0.53 ^a
	Vehicle	5.90 ± 1.28 ^b	0.34 ± 0.11 ^{a,b}	8.77 ± 0.88 ^b		15.01 ± 1.05 ^b
	Saline	5.69 ± 0.73 ^{a,b}	0.41 ± 0.09 ^b	6.92 ± 0.96 ^b		13.02 ± 1.35 ^b
	P-value	0.0268	0.0159	0.0001		<0.0001
Lung	α-T	1.46 ± 0.23 ^a	0.25 ± 0.01 ^a	1.93 ± 0.11 ^a		3.64 ± 0.34 ^a
	Vehicle	7.51 ± 1.09 ^b	0.48 ± 0.05 ^b	8.07 ± 0.79 ^b		16.06 ± 1.87 ^b
	Saline	8.38 ± 1.17 ^b	0.53 ± 0.06 ^b	7.43 ± 0.72 ^b		16.34 ± 1.92 ^b
	P-value	0.0004	0.0004	<0.0001		0.0001
Heart	α-T	54.23 ± 9.77	2.41 ± 0.44	4.53 ± 0.94		61.17 ± 11.07
	Vehicle	37.15 ± 7.08	1.66 ± 0.34	8.37 ± 2.00		47.18 ± 9.31
	Saline	28.21 ± 2.85	1.30 ± 0.16	9.33 ± 2.29		38.84 ± 3.82
	P-value	0.0673	0.1024	0.1924		0.2220
Brain	α-T	0.95 ± 0.10 ^a	0.18 ± 0.01 ^a	5.82 ± 0.52 ^a	3.33 ± 0.09 ^a	10.3 ± 0.5 ^a
	Vehicle	1.63 ± 0.11 ^b	0.24 ± 0.01 ^b	14.61 ± 1.68 ^b	4.82 ± 0.47 ^b	21.3 ± 2.1 ^b
	Saline	1.42 ± 0.11 ^b	0.23 ± 0.01 ^b	12.97 ± 0.60 ^b	4.15 ± 0.31 ^{a,b}	18.8 ± 0.8 ^b
	P-value	0.0021	0.0015	0.0002	0.0228	0.0002

Statistical significance of concentrations (mean ± SEM, n=5 per group) is indicated for each tissue (P-value= ANOVA). columns that are labeled with different letters indicate statistically significant differences (Tukey multiple comparisons (TMC) P<0.05). Unlabeled MK-4 was undetectable in all tissues, except brain.

Table 3

Percentage of d₄-MK-4 of total d₄-vitamin K in selected tissues

Treatment	Tissue				
	Liver	Kidney	Lung	Heart	Brain
α-T	19% ± 2% ^a	49% ± 6% ^b	58% ± 3% ^{b,c}	8% ± 1% ^{*,d}	85% ± 3% ^e
Vehicle	20% ± 2% ^a	61% ± 6% ^b	52% ± 2% ^{b,c}	18% ± 1% ^{#,ad}	90% ± 1% ^e
Saline	20% ± 1% ^a	54% ± 4% ^b	48% ± 2% ^{b,c}	24% ± 4% ^{#,ad}	90% ± 1% ^e

Shown are the mean ± SEM of the %conversion to MK-4 [%=100 × d₄-MK-4/(sum (d₄-MK-4+ d₄-PK)]. Two-factor repeated measures ANOVA P-value: Interaction P<0.0001, different symbols (^{*},[#]) in the same column, or different letters in the same row are significantly different, (P<0.05, TMC)