AN ABSTRACT OF THE DISSERTATION OF

Sherry Mae Farley for the degree of Doctor of Philosophy in Nutrition presented on November 12, 2012.

Title: Vitamin E and K Interactions: Investigating Mechanisms of Reduced Vitamin K Status in Response to Excess Vitamin E

Abstract approved:

Maret G. Traber

The primary goal of my studies was to elucidate the mechanisms for the well-recognized interaction between two nutrients, vitamins E and K. The outcomes from my studies assess mechanisms for adverse effects of vitamin E and provide novel information on mechanisms for vitamin K homeostasis. These findings will provide information relevant for assessing optimal intakes of vitamins E and K.

This dissertation presents studies aimed at evaluating three different mechanisms by which vitamin K status could be decreased by increases in whole body vitamin E concentrations in rats supplemented with vitamin E by subcutaneous injections (100 mg α-tocopherol (α-T)/ kg body weight per day), the model system developed in the Traber lab. The tested mechanisms by which vitamin E leads to reduced vitamin K status were: 1) increasing vitamin K metabolism, 2) decreasing menaquinone-4 (MK-4) synthesis from dietary phylloquinone (PK) and 3) potentiating vitamin K excretion through xenobiotic pathways.

Two approaches were undertaken to evaluate the hypothesis that vitamin E increases vitamin K metabolism. In Aim 1.1, the in vitro omega-hydroxylation of vitamin K by human cytochrome P450 CYP4F2 (expressed in insect microsomes) was tested because CYP4F2 is considered the limiting step in the catabolism of both vitamins. Chapter 2 shows that CYP4F2 more rapidly hydroxylated vitamin K compared with vitamin E. Moreover, vitamin E did not stimulate vitamin K metabolism in vitro. Thus, it is unlikely vitamin E stimulates vitamin K metabolism in vivo by direct interaction with the CYP4F2 enzyme-substrate complex. In Aim 1.2, the in vivo urinary and biliary excretion of vitamin K metabolites was investigated. Chapter 3 shows that α-T-injected rats

significantly increased urinary excretion of vitamin E catabolites, but no increases in urinary vitamin K catabolites were found. Chapter 4 shows that α-T-injected rats increased biliary excretion of 5C-aglycone, a major vitamin K catabolite shared by MK-4 and PK. However, the overall in vivo excretion of vitamin K catabolites was not changed when urinary excretion was also taken into account.

Aim 2 evaluated the hypothesis that α-T interferes with the conversion of PK to MK-4 because α-T and PK have similar side-chains. In Aim 2.1, conversion of PK or MN to MK-4 was tested in vivo. Rats were fed semi-purified diets containing equimolar concentrations of either PK or MN for 10 days, then α-T injections were undertaken. Chapter 3 shows that extra-hepatic tissues from α-T injected rats contained significantly lower MK-4 concentrations irrespective of whether the rats were fed PK or MN. These findings show that if vitamin E is interfering with the metabolic mechanism of MK-4 synthesis, then it is not specific to the cleavage of PK's side chain. In Aim 2.2, conversion of deuterium-labeled PK (d_4-PK) to d_4-MK-4 was used to evaluate the extra-hepatic tissue uptake of d_4-PK in α-T-injected rats. Rats were fed semi-purified diets containing equimolar concentrations of d_4-PK similar to my previous study for 10 days then α-T injections were undertaken for 7 days. Chapter 5 shows that total (labeled and unlabeled) vitamin K concentrations decreased in extra-hepatic tissues from α-T injected rats fed d_4-PK. Both d_4-MK-4 and d_4-PK concentrations decreased, suggesting that MK-4 concentrations were dependent upon those of d_4-PK. These findings suggest that PK, and not MN, is the primary substrate for MK-4 synthesis in extra-hepatic tissues. Moreover, both d_4-MK-4 and d_4-PK decreased in α-T-injected rats demonstrating that vitamin E's untoward effect on vitamin K status is likely a mechanism that is shared by both vitamin K forms and not specific to MK-4 synthesis. Recycling of vitamin K from the epoxide was not examined in this study and interference with the recycling mechanism for either PK or MK-4 in α-T injected rats has not been examined.

Vitamin E metabolism is greatly increased in α-T-injected rats by increasing various xenobiotic pathways. Thus, vitamin K status was hypothesized to decrease in α-T-injected rats as a result of the up-regulation of these pathways. As shown in Aim 1, urinary vitamin K metabolite excretion was not increased in α-T-injected rats. In Aim 3.1, the biliary excretion of vitamins E and K were examined to evaluate whether the

increased expression in biliary transporters, such as MDR1, led to increased vitamin K and E excretion via the bile. Chapter 4 shows that α-T increased in bile over the week of vitamin E injections and α-CEHC was the major vitamin E form excreted in bile. Although biliary PK secretion was unchanged and biliary MK-4 was undetectable, increased excretion of a major catabolite of both PK and MK-4, 5C-aglycone, was observed. In Aim, 3.2, the gene expression of enzymes and transporters in liver and extra-hepatic tissues as mechanisms involved in regulating their concentrations in these tissues was assessed. In Chapters 3 and 5, increased expression of biliary transporters were observed, one of which is known to bind the vitamin K intermediate MN as its substrate. It is possible other vitamin K catabolites, in addition to 5C-and 7C-aglycone, may have been excreted that were unaccounted for, e.g. MN or vitamin K epoxide metabolites.

In summary, my studies have shown vitamin K status is decreased in α-T-injected rats because PK and MK-4 concentrations are decreased in many extra-hepatic tissues. Although metabolism of vitamin K was not stimulated in response to α-T injections, increased excretion of a vitamin K catabolite was measured in the bile; however it may not account for all of the vitamin K loss observed in tissues. Alternatively, transport of PK and MN to extra-hepatic tissues or MK-4 recycling may have been inhibited in response to vitamin E. Further studies are needed to distinguish between these mechanisms.

Vitamin E and K interactions: Investigating Mechanisms of Reduced Vitamin K Status in Response to Excess Vitamin E

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Sherry Mae Farley

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APPROVED:

Major Professor, representing Nutrition

Co-Director of the School of Biological and Population Health Sciences

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Sherry Mae Farley, Author

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CONTRIBUTION OF AUTHORS

Scott Leonard contributed to the vitamin K and E measurements as well as the writing of that text in Chapters 2-5. Alan Taylor provided technical expertise in measurement of vitamin E and K metabolites and the writing of that text in Chapter 2. Allan Rettie provided analytical expertise in chapter 2. Ed Labut assisted in the animal handling and sample collections in chapters 3, 4, and 5. Hannah Raines contributed to the protein and qPCR data collection in Chapters 3 and 5. Dominic Harrington and David Card provided the measurement of vitamin K metabolites as well as the writing of that text in Chapters 3 and 4. Jay Kirkwood contributed the identification of sulfated tocopherol metabolite and the writing of that text in Chapter 5. Debbie Mustacich contributed to design and writing of Chapter 3. Maret G. Traber was involved in the design, analysis and writing of all experiments and chapters.

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Chapter 1:

Introduction to Vitamin E and K Interactions

Vitamin E Function and Requirements

Vitamin E is a lipid-soluble, chain-breaking antioxidant that prevents lipid peroxidation and damage to cellular membranes [1]. Vitamin E refers to eight natural compounds that include α-, β-, γ-, and δ-tocopherols and the respective tocotrienols. α-Tocopherol (α-T) is most relevant physiologically because it is the form preferentially maintained in the plasma and tissues and this is a result of the α-tocopherol transfer protein (α-TTP), which facilitates the preferential retention of α-T in the plasma [2, 3]. Thus, α-T is the form that fulfills dietary human requirements [4].

Vitamin E and Bleeding Abnormalities

The Food and Nutrition Board [4] set the vitamin E upper tolerable limit (UL) for humans based on studies conducted in animals demonstrating bleeding abnormalities resulting from vitamin E administration [4-9]. There is also evidence for potential health benefits from vitamin E supplementation as a "natural blood thinner". The Women's Health Study [10] tested the efficacy of 600 IU vitamin E or placebo taken every other day for ten years by nearly 40,000 women aged 45 years and older demonstrated that vitamin E supplements decrease the risk of mortality from thromboembolism [11]. Although there is evidence that vitamin E may also reduce platelet aggregation to antagonize blood clotting [12-14], the prevention of fatal hemorrhaging in animals was observed when vitamin K supplementation was provided with vitamin E administration [7-9], which indicates vitamin E antagonizes coagulation via a vitamin K-dependent mechanism.

Vitamin E and Vitamin K Status

In humans, high-dose vitamin E supplements increased a biomarker of inadequate vitamin K status, PIVKA-II (proteins induced by vitamin K absence-factor II, or under-γ-carboxylated prothrombin) [15], suggesting that vitamin K concentrations may be compromised in response to vitamin E in humans. In rats supplemented with α-T, significantly reduced concentrations of both PK and MK-4 were observed in extra-hepatic tissues compared to rats with adequate or reduced dietary vitamin E [16]. The

mechanism of vitamin K loss, however, was not explored. Thus, the reason why vitamin E supplementation results in inadequate vitamin K status is unknown.

Introduction to Vitamin K

Tissue vitamin K forms

Vitamin K refers to family of compounds with a common 2-methyl-1,4-napthoquinone head group. Phylloquinone (PK) is the major dietary form and is used to synthesize the tissue-specific form, menaquinone-4 (MK-4). In the body, the phytyl side chain of PK is cleaved via an unknown mechanism to release the intermediate menadione (MN). MN is subsequently prenylated, catalyzed the enzyme UBAID1, to form MK-4 [17] (**Figure 1.1**).

Many tissues accumulate significantly more MK-4 compared with PK, but whether the vitamin K substrate used in these tissues is PK or MN is unknown. The tissue(s) responsible for MN release is also unknown but it is theorized to occur in a central tissue, i.e. the intestines or the liver, where MN is then transported in the bloodstream to be taken up by extra-hepatic tissues. However, the low circulating MK-4 levels and its rapid elimination from circulation relative to PK [18], suggest that PK is the transport form for MK-4 synthesis. However, there is also evidence that MN, not PK, is the vitamin K transport form required for tissue-specific uptake for MK-4 synthesis. Okano et el. [17] demonstrated that oral PK, but not parenteral administration of PK, resulted in MK-4 synthesis in tissues of mice, suggesting PKs side chain is cleaved in the intestine, freeing MN for transport to specific tissues. It was also observed that MK-4 was generated in multiple cell culture lines, representing different tissues, following addition of MN but MK-4 synthesis was not detectable after addition of PK, suggesting many tissues lack the ability to efficiently cleave PKs side chain [19]. However, cerebral tissue slices have been shown to convert PK to MK-4 [17], which leaves the question of whether tissues are reliant on PK or MN transport for MK-4 synthesis controversial.

Function of vitamin K and vitamin K-dependent proteins

Interest in optimal vitamin K status has been stimulated by evidence demonstrating vitamin K's role as a cofactor for γ-glutamyl carboxylase (GGCX) extends far beyond that of coagulation [20]. GGCX catalyzes the γ-carboxylation of glutamate residues in vitamin K-dependent proteins, which is a post-translational modification that allows the proteins

to bind calcium and is required for their activity. There are 16 known vitamin K-dependent proteins involved in a wide variety of physiological functions that not only include coagulation (e.g. factors II, VII, IX, and X), but also include bone mineralization (e.g. osteocalcin), and prevention of arterial calcification (e.g. matrix GLA protein) [20]. Although PK is the major vitamin K form in the liver and heart, MK-4 is preferentially accumulated other tissues, such as the brain [21]. Both PK and MK-4 can act as a cofactor for GGCX [22], thus the synthesis of a tissue-specific form of vitamin K has led to theory that MK-4 has a different function unrelated to GGCX [23]. Although a tissue specific function for MK-4 has not been demonstrated, it is clear that vitamin K, as a cofactor for GGCX, is involved in important short-term (blood-clotting) and long-term (bone mineralization and prevention of calcification) physiological functions. Because of difficulty assessing optimal concentrations of vitamin K necessary for synthesis of vitamin K-dependent proteins not related to coagulation, concerns have been raised about the long-term consequences of drugs, such as warfarin, that antagonize vitamin K status [24, 25].

Vitamin K recycling

Vitamin K epoxide reductase (VKOR) catalyzes the reduction of the vitamin K quinone to its hydroquinone form, which is the form used by GGCX to catalyze γ-carboxylation of vitamin K-dependent proteins [26] (**Figure1.2**). VKOR is a multi-subunit protein ubiquitously expressed in tissues [26] and is the only enzyme known to reduce both the vitamin K quinone and epoxide, thereby fulfilling both steps required to recycle vitamin K [27].

Metabolic Pathways of Vitamins E and K

Intestinal Absorption

Dietary vitamin E and PK are lipid-soluble vitamins that can be solubilized along with lipids in the intestine by bile acids to diffuse through the brush border membrane of the enterocyte via micelles. In the enterocyte they are incorporated into chylomicrons, which pass into the lymphatics and then into the circulation via the thoracic duct [25, 28]. Vitamin E and K can be taken up by peripheral tissues or travel via chylomicron remnants (CR) to the liver following recognition of the CR by LDL-like receptors (LRP).

The liver preferentially secretes α-T, facilitated by the α-TTP, into the plasma for distribution to tissues. Triglyceride-rich fractions are the major carriers of PK [18]. Mechanisms of MN and/or MK-4 transport following oral intake of PK have not been elucidated.

Metabolism and excretion

Like xenobiotics, lipophilic compounds, such as vitamins E and K, are metabolized in processes involving: 1) phase I enzymes, or cytochrome P450s (CYPs), responsible for ω-oxidation of these compounds, 2) phase II enzymes responsible for their conjugation to enhance water-solubility, and 3) phase III transporters responsible for movement of these compounds into and out of tissues and their excretion in the bile or urine. The first step in the metabolism of both vitamins E and K involves their ω-hydroxylation by the phase I enzyme human CYP4F2 [29, 30]. Although, in vitro data demonstrate that CYP3A is not important for vitamin E metabolism [30], CYP3A family members have been shown to metabolize vitamin E, as well [31-34] and there may be some overlap in substrate specificity. Following the initial ω-hydroxylation of their side chains, they then undergo several rounds of β-oxidation and conjugation to glucuronide or sulfate to yield their respective urinary metabolites, carboxy ethyl hydroxy chroman (CEHC) for vitamin E [35] and 5C- and 7C-aglycone for vitamin K [35, 36]. Alternatively, vitamins E and K, and/or their metabolites, can excreted via the bile [37, 38].

Vitamin E tissue regulation

Xenobiotic pathways, that mediate gene regulation of enzymes and transporters involved in the detoxification of drugs, can be altered by vitamin E [39] and this is thought to be a result of vitamin E acting as a ligand for the pregnane xenobiotic receptor (PXR, also known as SXR in humans) [40, 41]. PXR regulates expression of various xenobiotic detoxifying enzymes (e.g. CYP3A) and transporters (e.g. MDR1, BCRP1, MRP1, and OATP) [42] and the expression of these genes were modified in response to α-T [43]. PXR activation was also found to reduce vitamin E metabolism, demonstrating PXR regulation can modify vitamin E catabolism and excretion [44]. Additionally, studies with vitamin E demonstrated that altered expression of CYP3A and MDR1 was associated with more efficient elimination of vitamin E and its metabolites from plasma and tissue of rats [45, 46]. Vitamin E also up-regulated the protein expression of MDR1 transporter in the lung [46] suggesting it could stimulate xenobiotic

pathways in extra-hepatic tissues as well. Moreover, troglitazone, which shares the chromanol head group common to α-T and its metabolites, is transported by OATP and BCRP1, two transporters that were also modified in response to excess α-T in rats [43]. Thus, their modified expression in liver following α-T administrations support speculation that they are involved in regulating liver concentrations of vitamin E. Some ABC transporters have been shown to mediate efflux of vitamin E [37] [47] and vitamin K [48] out of tissues, and the predominant expression of many of these transporters on the apical membrane of bile canaliculi [49] would mean they may also facilitate their excretion via bile. Moreover, xenobiotic uptake transporters, such as the organic anion-transporting polypeptide (OATP), are essential in mediating the entry of amphipathic compounds, which may include vitamin K, into tissues. All of transporters are also considered highly important in regulating drug transport in multiple tissues including the brain [49].

Hypothesis and Aims

This dissertation presents studies aimed at determining how vitamin E is reducing vitamin K status. The central hypothesis was that vitamin E leads to reduced vitamin K status by modifying its xenobiotic metabolism and/or transport to potentiate its excretion. Alternatively, it was proposed that a metabolic pathway involved in MK-4 synthesis may also be disrupted in response to α-T. In Aim 1, the metabolism of vitamins E and K were studied in vitro and the excretion of vitamin E and K catabolites in response to excess α-T was investigated in vivo. Aim 2 investigated whether α-T interferes with the conversion of PK or MN to MK-4 in vivo. In Aim 3 the biliary excretion of vitamins E and K were examined in vivo as well as the gene expression of enzymes and transporters in liver and extra-hepatic tissues as mechanisms involved in regulating their concentrations in these tissues.

Contents of the Dissertation

The studies described in the following chapters were conducted to elucidate mechanisms of reduced vitamin K status in response to high concentrations of vitamin E. Chapter 2 of this dissertation compared the in vitro metabolism of vitamins E and K by human CYP4F2 and investigated potential enzyme-substrate interactions that may stimulate vitamin K metabolism in the presence of α-T. Chapters 3-5 investigated vitamin K status in response to vitamin E using an established rat model. Vitamin K concentrations and modified expression of xenobiotic pathways in response to α-T were examined in rat tissues (Chapters 3 and 5) and evidence of increased metabolism and/or excretion of vitamin K in response to vitamin E were examined in urine (Chapters 3-4) and bile (Chapter 4) of rats. Whether vitamin E targeted MK-4 synthesis was also investigated by examining MK-4 concentrations in tissues of α-T-injected rats fed PK or MN (Chapter 3); and by feeding labeled PK to rats to observe its conversion to labeled MK-4 in tissues in response to α-T (Chapter 5).

Figure 1.1: PK Conversion to MK-4

Dietary PK is converted to MK-4 by side chain cleavage to release the MN intermediate followed by geranylgeranylation, catalyzed by UBIAD1, to generate the tissue-specific vitamin K form, MK-4

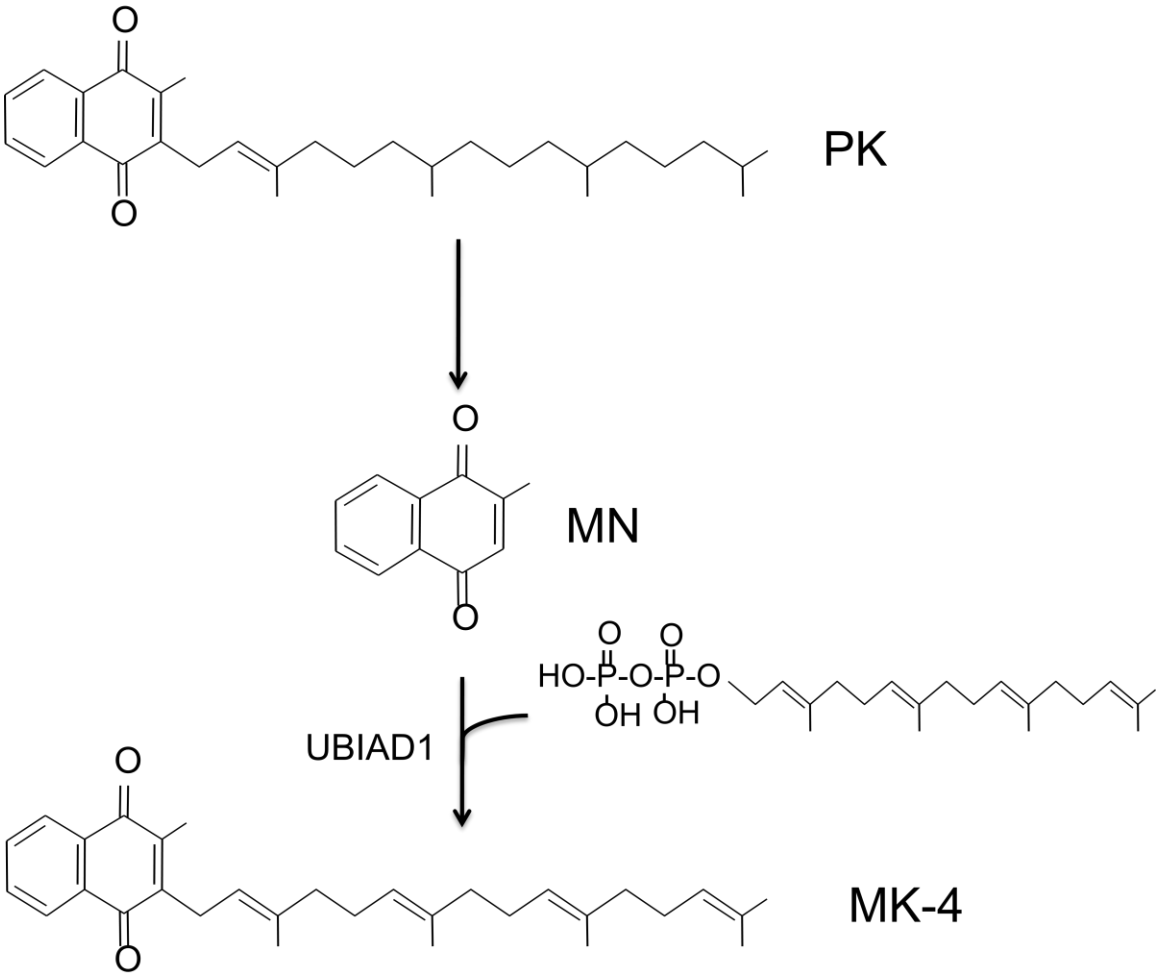
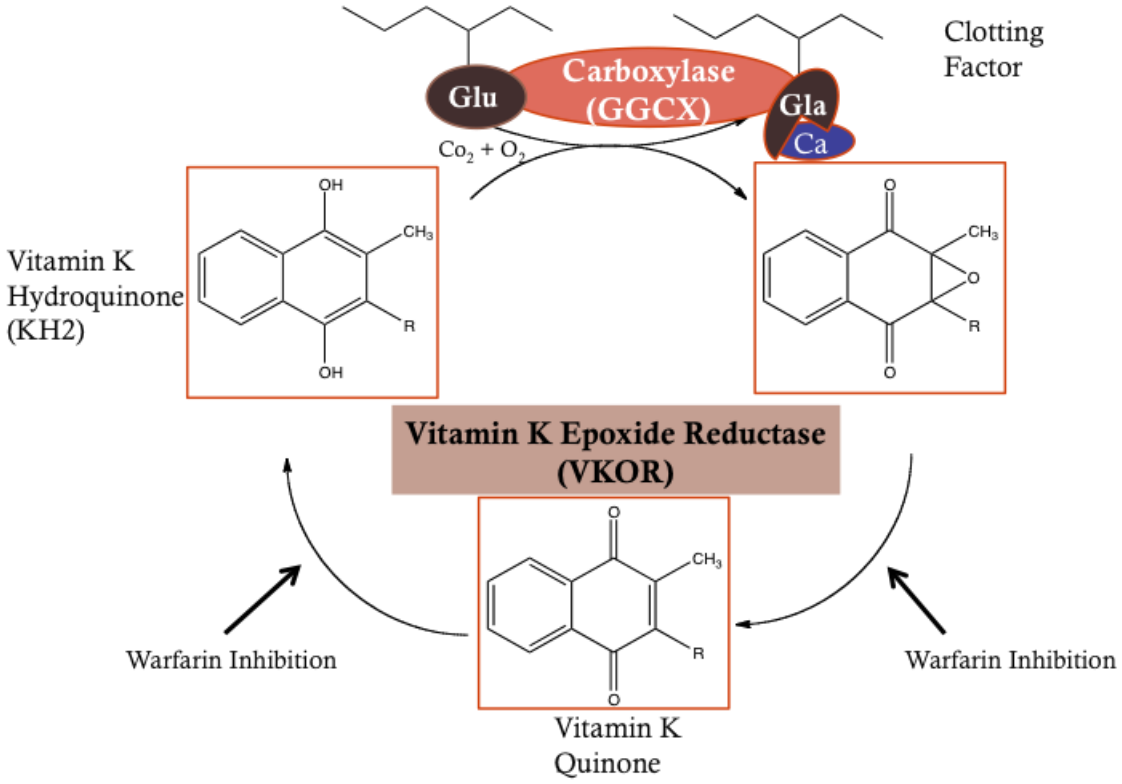


Figure 1.2: Vitamin K Recycling

Vitamin K quinone is converted to the vitamin K hydroquinone, which is used by the carboxylase (GGCX) to γ-carboxylate glutamate residues in vitamin K-dependent proteins, e.g. clotting factors. Vitamin K is recycled by vitamin K epoxide reductase, which can recycle the epoxide back to the quinone form. VKOR is the only enzyme known to reduce the epoxide and the quinone and is inhibited by warfarin.



Chapter 2:

ω-Hydroxylation of phylloquinone by CYP4F2 is not decreased by α-tocopherol

Sherry M. Farley, Scott W. Leonard, Alan Taylor, Marc Birringer, Allan E. Rettie, Maret G. Traber

Submitted to: Molecular Nutrition and Food Research

Abstract:

Scope:

The objective of this study was to investigate the initial catabolic step of vitamin E and K metabolism, the ω-hydroxylation by human cytochrome P450 4F2 (CYP4F2).

Methods and Results:

Tocopherol (T) metabolism was compared using rat liver slices incubated with deuterated (d_6)-*RRR*-α-T (d_6-α-T), racemic 2*S*-α-T (2*S*, 4'*RS*, 8'*RS* α-T, 2*S*-α-T), or d_2-γ-T (d_2-γ-T). Following comparable uptake of each T by liver slices, twice as much 13'-OH-T was produced from 2*S*-α-T or d_2-γ-T (39 ± 15 or 42 ± 5 pmol/g liver, respectively) as from d_6-α-T (17 ± 2, $p<0.01$). Kinetic studies were conducted using insect microsomes expressing human CYP4F2 incubated with d_4-phylloquinone (d_4-PK), d_6-*RRR*-α-T, d_3-*SRR*-α-T, or d_2-γ-T. CYP4F2 demonstrated similar maximal velocities (Vmax) when d_2-γ-T or either of the α-Ts were used as substrates, all of which were less than the d_4-PK Vmax ($p<0.0002$), while the CYP4F2 catalytic efficiency towards d_4-PK (15.8 Vmax/Km) was 4 times greater than for any of the Ts. Vitamin K had no effect on vitamin E catabolism, while vitamin E had slightly decreased d_4-PK Vmax.

Conclusions:

CYP4F2 discriminates between Ts and PK in vitro, but α-T does not appreciably increase vitamin K catabolism by this mechanism.

Introduction

Human cytochrome P450 4F2 (CYP4F2) is an enzyme localized in the ER that catalyzes the ω-hydroxylation of various endogenous compounds in humans [50]. CYP4F2 is the only known human P450 shown to effectively ω-hydroxylate vitamin E, which is the first step in its catabolism to the excreted water-soluble metabolite, carboxy ethyl hydroxy chroman (CEHC) [30]. In addition, Bardowell et al. [51] demonstrated that disrupting expression of the mouse CYP4F2 ortholog, CYP4F14, decreased vitamin E metabolism; indicating that CYP4F14 plays a significant role in its metabolism. Other endogenous CYP4F2 substrates include leukotriene B4 (LTB4) and long chain fatty acids, such as arachidonic acid [50]. CYP4F2 is also the major vitamin K hydroxylase [29]. Importantly, CYP4F2 polymorphisms are determinants of warfarin dose in humans emphasizing the critical role this enzyme plays in vitamin K status [52-54]. Thus, CYP4F2 may be an important regulator of both vitamin E and K tissue concentrations in humans.

α-Tocopherol (α-T) is the vitamin E form preferentially maintained in human plasma and tissues, as a result of the function of the hepatic α-T transfer protein (α-TTP); whereas enhanced hepatic metabolism promotes the excretion of other vitamin E forms, such as γ- T [55, 56] or tocotrienols [57]. The naturally occurring α-T form, *RRR*-α-T, has three chiral centers at positions 2, 4′, and 8′ in the side chain, all in the *R* configuration. Chemical synthesis of α-T produces a racemic mixture of eight stereoisomers *(RRR, RSR, RRS, RSS, SRR, SSR, SRS,* and *SSS)*, which differ in the configuration around these three chiral centers and is referred to as *all-rac*-α-T. The forms with the *R* configuration at the 2 position (2*R*-α-T) are preferentially retained, as reviewed [4]. Consequently, 2*R*-α-T have longer half-lives while 2*S*-α-Ts, similar to the non-α-T forms, are more readily metabolized and excreted [58-60]. Thus, the Food and Nutrition Board

defined vitamin E as 2*R*-α-T and gave it the distinction of being the only form of vitamin E that meets human requirements [61].

Although α-TTP selectivity plays an important role in the preferential enrichment of non-hepatic tissues with *RRR*-α- T, hepatic metabolism of the different vitamin E compounds by CYP4F2 also plays a critical role. Sontag and Parker [30, 62] reported an enhanced ω-oxidation of non-α-Ts compared with α-T in rat and human liver microsomes and in insect microsomes expressing human CYP4F2, and demonstrated that the number and position of methyl groups on the chromanol ring influence vitamin E metabolism. They also demonstrated that while differences in the side-chain saturation of the vitamin Es are important, based on the observation that tocotrienols are metabolized more rapidly than are the Ts, the side-chain stereochemistry, as assessed using *all-rac*-α-T relative to *RRR*-α-T, was not important [30, 62]. However, since *all-rac*-α-T contains equimolar concentrations of 2*R*- and 2*S*-, the metabolism of 2*S*-stereoisomers remains an open question.

CYP4F2 is not only important in vitamin E metabolism, but as noted above, CYP4F2 is the major ω-hydroxylase that initiates vitamin K metabolism [29]. Similar to vitamin E metabolism [35, 63], vitamin K's side chain is ω-hydroxylated, followed by multiple rounds of ß-oxidation to yield the vitamin K major urinary metabolites, 5C- and 7C-aglycones (**Diagram 1**) [36].

Vitamin K functions as a co-factor for the carboxylation of glutamic acids converting them to γ-carboxy-glutamic acids in specific vitamin K-dependent proteins [22]. These proteins are required for a number of functions, including hemostasis, apoptosis, bone mineralization, calcium homeostasis, growth control, and signal transduction [22]. Vitamin K is most notably needed in the coagulation cascade for γ-carboxylation of

glutamic acids in a number of proteins. Therefore, it is generally agreed that vitamin E and K interactions are the cause of bleeding observed with vitamin E supplements [64]. Wheldon et al. [65] demonstrated that extraordinarily high dietary vitamin E causes increased bleeding in rats, a phenomenon that was reversed with increased vitamin K supplementation. Additionally, the rationale cited in the Dietary Reference Intakes for the upper tolerable limit (UL) for vitamin E was set based on the data showing that rats fed high levels of dietary vitamin E experienced increased bleeding tendencies [4]. Although the mechanism for the vitamin E and K interactions is unknown, there is growing evidence that vitamin E antagonizes vitamin K status. An increase in a biomarker for poor vitamin K status, PIVKA-II (protein induced by vitamin K absence-II), was observed in human subjects supplemented with α-T [15]. Vitamin E's negative influence on vitamin K status has also been demonstrated in rats where α-T supplementation resulted in reduced extra-hepatic tissue concentrations of vitamin K [16, 66]. Thus, considering their similar metabolic pathways, we hypothesized that elevated levels of vitamin E may interfere with the bioavailability of vitamin K by increasing vitamin K catabolism and excretion.

To test this hypothesis, the ω-hydroxylation of vitamins E and K was studied in vitro to examine possible discriminatory processes involved in the initial step of their catabolism. Vitamin E was investigated using two systems: rat precision-cut liver slices and insect microsomes expressing human cytochrome P450s. Liver slices have been used successfully to study metabolism [67, 68] and are especially touted because the liver slice, as compared with the isolated hepatocyte, maintains the architecture and cell heterogeneity of the liver. This system was used to assess vitamin E hydroxylation but technical problems for measuring vitamin K hydroxylation in tissues precluded their use. Therefore, the ω-hydroxylation of the different vitamin E forms along with vitamin K, as phylloquinone (PK), was examined in the absence of extraneous factors, using human

CYP4F2 expressed in insect microsomes to assess differences in the specificity of CYP4F2 for the vitamin E substrates and PK.

Materials and Methods

Materials

2*R*,4′*R*,8′*R*-α-5,7-(C^2H_3)$_2$ tocopheryl acetate (d_6-α-T) was a gift from Dr. James Clark of Cognis Nutrition and Health. γ-3,4-(^{2}H) tocopheryl acetate (d_2-γ-T) was a gift from Dr. Jeffrey Atkinson, Brock University (ON, Canada). Unlabeled synthetic 2*S*-α-T (2*S*-α-T), which is a racemic mixture of *SRR-, SRS-, SSR,* and *SSS*-α-T, was a gift from Dr. Brent Flickinger (Archer Daniels Midland, Decatur, IL). 2*S*,4′*R*,8′*R*-α-5,7-(C^2H_3)$_2$ tocopheryl acetate (d_3-*SRR*-α-T) was a gift from DSM Nutritional Products (Dr. Thomas Netcher, Basel, Switzerland). Phylloquinone (ring-D4, d_4-PK) was obtained from Buchem BV (Apeldoorn, The Netherlands). The internal standard, α-tocotrienol, was a gift from Dr. Tomohiro Saito of Eisai Food and Chemical Co., LTD. The 13′-OH-α-T used for a standard has been described previously [69].

Liver slice preparation and incubation

The Oregon State University (OSU) Institutional Animal Care and Use Committee approved all procedures (HHS Animal Welfare Assurance Number: A3229-01). Male Sprague-Dawley rats (Charles River, 250-300g) were housed in plastic cages with hard wood chips, kept on a 12-h light/dark schedule, and maintained on a AIN-93G diet (Harlan Teklad) and water ad libitum for at least 1 week to acclimate to the OSU animal facility. Rats were anesthetized by intraperitoneal (ip) injection of sodium pentobarbital (70 mg/kg), then the organs were perfused with 0.9% saline containing 2 U/ml heparin. The liver was excised and placed into ice-cold Krebs-Hensleit buffer (Sigma, St. Louis, MO) containing 2.5 mM $CaCl_2$-H_2O and 25 mM $NaHCO_3$. The liver was immediately cored using a Tissue Coring Press and sectioned into 200-300 µm-thick slices approximately 8 mm in diameter using a Krumdieck Tissue Slicer (Alabama Research

and Development Corporation, Munford, AL). Slices were floated onto titanium mesh rollers (2 per roller) and placed into 20 ml scintillation vials containing 2 ml Waymouth's MB 752/1 medium (Applichem, Ottoweg, Germany), with 25 mM $NaCO_3$, 50 µg/ml gentamycin (Sigma) and 10% horse serum. The slices were incubated in the rotating Vitron Dynamic Incubator (Vitron, Inc., Tucson, AZ) at 37˚C in an atmosphere of 95% O_2/ 5% CO_2 for a 1 hour recovery period then incubated in vitamin E supplemented medium (in triplicate with d_6-α-T, d_2-γ-T, unlabeled synthetic 2*S*-α-T, or vehicle, as described in the next paragraph) and were harvested after 24 hours. Liver slices were washed on ice 3 times in a buffer containing 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 2 mg/ml bovine serum albumin (BSA) (Sigma A9647) followed by 3 more washes in the same buffer minus the BSA. The wet weight was obtained and the slices were stored in 100% ethanol at -80˚C until analysis.

Prior to incubation, tocopheryl acetates were saponified in alcoholic KOH in the presence of ascorbic acid, extracted with hexane, dried under nitrogen and resuspended in ethanol and the concentrations determined by UV spectroscopy, as described [70]. To prepare the vitamin E supplemented medium, deuterium-labeled d_6-α-T, d_2-γ-T, or unlabeled synthetic 2*S*-α-T was dissolved in 100% ethanol, then the ethanol solutions were added drop-wise to horse serum while gently stirring. The supplemented serum was incubated at 4˚C overnight then diluted 1:10 with Waymouth's MB 752/1 medium to obtain a final concentration of ~10 µM Vitamin E (< 0.5% ethanol).

ATP assay

Liver slices were collected before and after incubation to determine slice viability. Slices were blotted dry, then weighed. Slices were placed into 0.5 ml 2.5% trichloroacetic acid (TCA) on ice, homogenized, then centrifuged at 12,000 rpm for 10 minutes. The

supernatant was transferred to another tube, snap frozen in liquid nitrogen, and stored at -20˚C until analysis.

Adenosine triphosphate (ATP) was quantified using ENLITEN Luciferase/Luciferin Reagent (Promega) following the manufacturer's protocol. Supernatant samples were thawed and diluted 1:5000 in 200 µM Tris-HCl, pH 7.7. ATP standard was diluted in the same buffer as samples (200 µM Tris-HCl, pH 7.7 with 0.0005% TCA) to prepare a standard curve for analysis of the samples. Assay was performed using Spectramax L Luminometer (Molecular Devices).

Cytochrome P450 expressed by insect microsomes

Insect microsomes expressing recombinant human CYP4F2 (CYP4F2 Supersomes, Gentest BD Biosciences, Woburn, MA), hereafter called S-CYP4F2, were incubated with vitamin E as d_6-α-T, d_2-γ-T, 2*S*-α-T or d_3-*SRR*-α-T, vitamin K as d_4-PK, or vehicle (ethanol). Reactions consisted of 1-100 µM vitamin substrate (using ethanol as vehicle) incubated with 25 or 50 pmol S-CYP4F2 in 100 mM KH_2PO_4, pH 7.4, then incubated for 10 minutes at 37˚C (total volume of reactions were 0.5 ml or 1 ml, < 2% ethanol). The reaction was initiated by adding 1 mM nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma) and incubated another 20-30 minutes at 37˚C. In some experiments NADPH was omitted to demonstrate the specificity of the reaction.

For optimal reaction conditions, it was important to add the lipid-soluble vitamin substrates in ethanol directly to the S-CYP4F2 for a 10-minute pre-incubation on ice prior to adding the aqueous 100 mM KH_2PO_4 buffer. This procedure, theoretically, gives the substrate time to incorporate into the S-CYP4F2. In initial experiments the substrates were added to the aqueous reaction system prior to addition to the microsomes. This protocol was problematic because, even though OH-d_2-γ-T was produced under these

reaction conditions, there was very little production of the OH-α-Ts (OH-d_6-*RRR*-α-T and OH-d_3-*SRR*-α-T) or the OH-d_4-PK metabolites (**Diagram 2**).

Using the optimized incubation conditions with the lipophilic substrates in ethanol added directly to the S-CYP4F2, we conducted the kinetic experiments using a range of final substrate concentrations (1, 5, 10, 25, 50, and 100 μM). The reactions were terminated by extraction with 1:1 volume ethanol and hexane. The extract was retained for analysis of the hydroxylated metabolites.

HPLC-MS/MS analysis of vitamins E, K and their hydroxylated metabolites

For the liver slice and insect microsome experiments, Ts and 13'-OH-Ts were extracted as described [45]. They were measured using a Shimadzu HPLC coupled to a triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex API 3000, Foster City, CA, USA) with a turbo ion spray (TIS) source set to negative mode. A Synergi Hydro-RP column (150 x 2 mm, 4 μm, Phenomenex, Torrance, CA) was used with 100% methanol mobile phase for separations. The hydroxylated α-T (13′OH-α-T) peak was identified using an authentic 13′-OH-α-T standard [69]. Quantitation of 13′OH-α-T was found to be linear over 3 orders of magnitude (25-25,000 pg on column, R^2 = 0.998), with a lower limit of quantification (signal/noise = 10) of 25 pg on column, and a lower limit of detection (signal/noise = 5) of 12.5 pg on column.

Because vitamin K is poorly ionized using TIS, an atmospheric pressure chemical ionization (APCI) source set in negative mode was used for experiments that included both vitamins. The APCI source is capable of ionizing both vitamin E and vitamin K and their oxygenated metabolites. Addition of an oxygen atom to substrates was monitored by the following SRM transitions: d_6-α-T (mass/charge *(m/z)* 451.4 to 168.9), d_2-γ-T (*m/z* 433.4 to 150.0), α-T (*m/z* 445.4 to 162.9), d_3-α-T (*m/z* 448.4 to 166.0), and d_4-PK (*m/z*

470.4 to 189.0). α-Tocotrienol was used as an internal standard (*m/z* 423.4 to 162.9) with a retention time of 3.6 minutes. Typical retention times for the initial metabolites of d_6-α-T, 2*S*-α-T, d_3-*SRR*-α-T were 2.4 minutes, while that of d_4-PK metabolite was 3.9 minutes.

To confirm the identity of the compounds of interest, accurate masses of d_2-γ-T, d_4-PK, and products of oxygen (O) addition to the molecules were measured on an AB Sciex 5600 quadrupole-time-of-flight mass spectrometer (TOFMS) (Foster City, CA, USA) coupled to a Shimadzu Nexera UPLC using the same Synergi Hydro-RP column, as described above. Both TOFMS and product ion scans were analyzed using Peakview version 1.1.0.1 (AB Sciex) The d_2-γ-T had a measured *m/z* of 417.3707 (calculated 417.3702), and its product ion had a measured mass of 150.0677 (calculated 150.0665). The precursor of the d_2-γ-T metabolite had a measured *m/z* of 433.3659 (calculated 433.3651) with a measured product ion *m/z* of 150.0671 (calculated 150.0665), consistent with the addition of an O atom on the d_2-γ-T side chain and the expected d_2-γ-T cleavage pattern in negative mode, which results in the loss of 1 of the 2 deuterium atoms. Thus, the accurate mass measurements of d_2-γ-T and its putative metabolite are consistent with the addition of a single O atom to the side chain of the molecule.

d_4-PK had a measured *m/z* of 454.3754 (calculated 454.3749), and the product ion had a measured *m/z* 189.0863 (calculated 189.0854), consistent with cleavage between the first and second carbon atoms on the side chain, as proposed by the Peakview software. The measurements of d_4-PK and its putative metabolite are consistent with the addition of a single O atom in the d_4-PK side chain. Evidence that PK is ω-hydroxylated includes the presence of the tail-shorted aglycone metabolites in human urine [71] that are postulated to arise by ω-hydroxylation followed by ß-oxidation.

Statistics

To evaluate statistical differences, the data were analyzed using one-way ANOVA with Bonferroni post-tests using Prism 5 statistical software (Graphpad Software, Inc). Where variances were unequal between test conditions, the data was logarithmically transformed prior to statistical evaluation. Michaelis-Menten enzyme kinetics was also performed using non-linear kinetics to estimate Vmax and Km, using Graphpad's non-linear kinetics. Statistical analysis of kinetic parameters was assessed using one-way ANOVA with Bonferroni post-tests.

Results

Vitamin E and Liver Slices

Liver slices incubated with the various forms of vitamin E were found to similarly accumulate d_6-α-T, d_2-γ-T, and 2*S*-α-T substrates (**Figure 1A**). The latter is a racemic mixture of *SRR-, SRS-, SSR-,* and *SSS*-α-T. Since the 2*S*-α-T could not be separately identified from the endogenous α-T in the slices, the unlabeled α-T concentrations in all liver slices analyzed for a given experiment were averaged and then the average subtracted from the total α-T measured in the 2*S*-α-T-supplemented slices. This provided an approximate value of 2*S*-α-T accumulated by the liver slices. The 2*S*-α-T concentrations accumulated were comparable to those of d_6-α-T or d_2-γ-T (**Figure 1B**). It should be noted that the presence of metabolically active cells was also determined by measuring ATP in the liver slices. Following 24 hours incubation, ATP production was ongoing and there were no differences between treatments (data not shown).

To assess the initial degree of metabolism, we measured hydroxylated vitamin E metabolites (13′-OH-d_6-α-T, 13′-OH-d_2-γ-T, or 13′-OH-2*S*-α-T) that were produced from vitamin E accumulated by the slices. After averaging the unlabeled endogenous 13′-OH-α-T in slices and subtracting this value from the total 13′-OH-α-T in the 2*S*-α-T-supplemented samples, the ω-hydroxylation of the different vitamin E forms was assessed. The 13′-OH-d_2-γ-T and 13′-OH-2*S*-α-T concentrations were nearly double that of 13′-OH-d_6-α-T (**Figure 1C).**

Insect microsomes

To investigate whether CYP4F2 discriminates between Ts, natural and synthetic α-T or between α-T and γ-T, and to compare the Ts' rate of metabolism to vitamin Ks, we examined the CYP4F2-mediated hydroxylation of labeled vitamin substrates using S-

CYP4F2. For better accuracy in our measurements and to further investigate the preferential metabolism of *all-rac*-α-T in humans [58] we obtained the labeled isomer d_3-*SRR*-α-T. Although the previous method utilizing the TIS ionization source is suitable for vitamin E metabolite measurements, it was inadequate for vitamin K ionization. Therefore, we developed a different analytical technique using APCI (see methods), which allowed for the simultaneous analysis of all the vitamin E and K substrates and their metabolites. Using this detection method, significant production of the putative ω-hydroxylated metabolites following incubation of the substrates (T or d_4-PK) with S-CYP4F2 was observed, but only in the presence of NADPH, which is required for CYP4F2 activity (**Diagram 2B**). It should be noted that the hydroxylated α-T (13′OH-α-T) peak was identified using an authentic 13′-OH-α-T standard [69]. Additional mass spectrometer testing (see methods) was used to identity the OH-d_2-γ-T and OH-d_4-PK metabolites.

CYP4F2 enzyme kinetic studies were carried out by incubating each of the labeled vitamin substrates with S-CYP4F2 individually. It should be noted that for the calculations the substrate concentrations used are the vitamin concentrations in the medium and thus the kinetics are an approximation based on the assumption that the uptake into the microsomes was equivalent to the concentration in the media. Similar to the findings by Sontag and Parker [62], there were no significant differences in the estimated Michaelis-Menten constant, Km, or in maximal velocity, Vmax, between d_6-*RRR*-α-T or d_3-*SRR*-α-T (**Figure 2, Table**). Of the vitamin E forms, d_2-γ-T showed a significantly higher Vmax compared with either of the α-Ts (**Figure 2, Table**). Under the same in vitro conditions, d_4-PK incubated with S-CYP4F2 demonstrated a higher Vmax compared with the vitamin Es (P=0.0002), but the Km estimated for d_4-PK was not statistically different between the vitamins (Table). Thus, the catalytic efficiency

(Vmax/Km) of S-CYP4F2 for the vitamin K substrate was about 4 times greater than for the vitamin E substrates.

To investigate the effect of the presence of one vitamin on the other's omega-hydroxylation, we examined S-CYP4F2-mediated catabolism of both vitamins in the presence of other. Thus, d_4-PK (0, 10, 20 and 50 μM) was incubated with S-CYP4F2 in the presence of d_6-*RRR*-α-T (0, 10, 20 and 50 μM) or d_3-*SRR*-α-T (0, 10, 50, and 100 μM). The d_4-PK Vmax decreased from 198 ± 23 to 137 ± 16 ($p = 0.0333$) in the presence of α-T, but no effects on Km were observed with addition of the competing α-T substrates (**Table 1**). There were no differences in the formation of OH-α-T metabolites when incubated with S-CYP4F2 in the presence of d_4-PK (**Table 1**).

Discussion

Our studies show contradictory evidence concerning the metabolism of non-α-Ts depending on whether the study was done with rat liver slices or with insect cells expressing the human vitamin E hydroxylase CYP4F2. In liver slices, similar amounts of 13′-OH-d_2-γ-T and 13′-OH-2*S*-α-T (racemic mixture of *SRR-, SRS-, SSR-,* and *SSS*-α-T) were produced from equal substrate concentrations. The concentrations of these metabolites were both significantly greater than that of 13′-OH-d_6-*RRR*-α-T. We next investigated whether the discrimination observed with the liver slices between natural and synthetic α-T isomers and between α-T and γ-T was CYP4F2-specific using insect microsomes that express only CYP4F2. Our results are consistent with those Parker's group [62], with a tendency for a greater Vmax for the γ-T substrate with CYP4F2 compared to its *RRR*- and *all rac*-α-T substrates, which did not differ from each other. Our findings with regard to vitamin E are consistent with the outcomes reported by Sontag and Parker [62] suggesting that structural differences between α-T and γ-T in the chroman ring, but not differences in side chain stereochemistry, influence ω-hydroxylation of the vitamin E forms by CYP4F2.

The mechanism of discrimination between stereoisomers of α-T observed in vivo [58], and as we observed with the liver slices may not be at the CYP4F2 hydroxylation step. For example, α-TTP function or the architecture of hepatocytes, may be more influential in differentiating between side chain stereoisomers of α-Ts, leading to accumulation of substrate for catabolism. Given the increased excretion of α-CEHC (terminal vitamin E catabolite), when *all rac*- compared with *RRR*-α-T is administered to humans [58], the liver slice experiments more accurately mimic the in vivo situation.

The same regulatory mechanisms responsible for vitamin E's metabolism and excretion may also regulate vitamin K concentrations [29, 36, 72]. Knowing that elevated vitamin E concentrations lead to reduced vitamin K status [16], we hypothesized that excess vitamin E may up-regulate metabolic pathways, especially ω-hydroxylation by CYP4F2 and thus stimulate the catabolism and excretion of vitamin K. Therefore, we examined the enzymatic efficiency of CYP4F2 towards vitamin E relative to vitamin K. Our results demonstrate PK had a higher Vmax than did the vitamin E forms (Table). Moreover, the in vitro catalytic efficiency of CYP4F2 for PK was significantly greater compared to the vitamin E substrates, including γ-T, which suggests PK may be a better substrate for CYP4F2 compared to T. We also investigated whether vitamin E could stimulate CYP4F2-specific hydroxylase activity as a possible mechanism for vitamin E and K interactions. However, the presence of α-T in the vitamin K-CYP4F2 enzyme incubations did not stimulate vitamin Ks metabolism. Characterizing how the enzyme and substrates interact in the microsomal membranes is necessary to fully understand the CYP4F2-substrate interactions that may exist between vitamins E and K in vivo. Regardless, these studies indicate that enhanced CYP4F2 activity is not how excess α-T might cause *increased* catabolism of vitamin K and thus decrease vitamin K status in vitamin E supplement takers.

CYP4F2 is the only P450 enzyme demonstrated to be capable of vitamin E and K ω-hydroxylation, which is the initial step in their metabolism and excretion [29, 30]. A common CYP4F2 variant, V433M (rs2108622), was reported to have reduced ability to metabolize vitamin E [73] and vitamin K [29] and was associated with elevated hepatic vitamin K [29] and serum α-T concentrations [74] in those carrying the allele. CYP4F2 polymorphisms are also associated with altered fatty acid metabolism [75] and, in regard to arachidonic acid hydroxylation, the V433M is correlated with elevated blood pressure and may predispose individuals to hypertension, which is speculated to be related to

diminished 20-hydroxyeicosatetraenoic acid (20-HETE) production [76]. Importantly, the V433M variant is also linked to negative health outcomes related to ischemic stroke [76, 77] and myocardial infarction [78]. CYP4F2 polymorphisms also contribute to the wide variability in individual response to warfarin [52, 78], which demonstrates the importance of CYP4F2 in regulating vitamin K concentrations. These associations warrant further investigation in order to determine which CYP4F2-specific metabolite(s) are influencing disease outcome; and how vitamin supplementation may modify metabolism of competing endogenous substrates and what that means for disease outcome.

In summary, our data show that both γ-T and 2*S*-α-T are more readily hydroxylated than is *RRR*-α-T, when the metabolizing system is the whole liver slice. These data suggest that intracellular trafficking is critical for the preferred retention of *RRR*-α-T and necessary for the increased excretion of both γ-T and 2*S*-α-T. This is in contrast to what is observed in isolated insect microsomes expressing human CYP4F2 where the differences in the chroman ring contribute to varying rates of metabolism but side chain stereochemistry has no influence. Furthermore, vitamin K is more readily catabolized by CYP4F2 than is any of the vitamin E forms but vitamin E did not augment vitamin Ks catabolism. These data indicate that the decreased vitamin K status observed in subjects taking vitamin E supplements is not caused by stimulation of CYP4F2 activity by vitamin E to result in increased vitamin K hydroxylation.

Acknowledgements

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Figure 2.1: Metabolic pathway of vitamins E and K

The metabolic pathway of vitamins E and K involve their initial ω-hydroxylation by CYP4F2 followed by multiple rounds of β-oxidation to yield their respective urinary metabolites, carboxy ethyl hydroxy chroman (CEHC) for vitamin E and 5C- and 7C-aglycones for vitamin K.

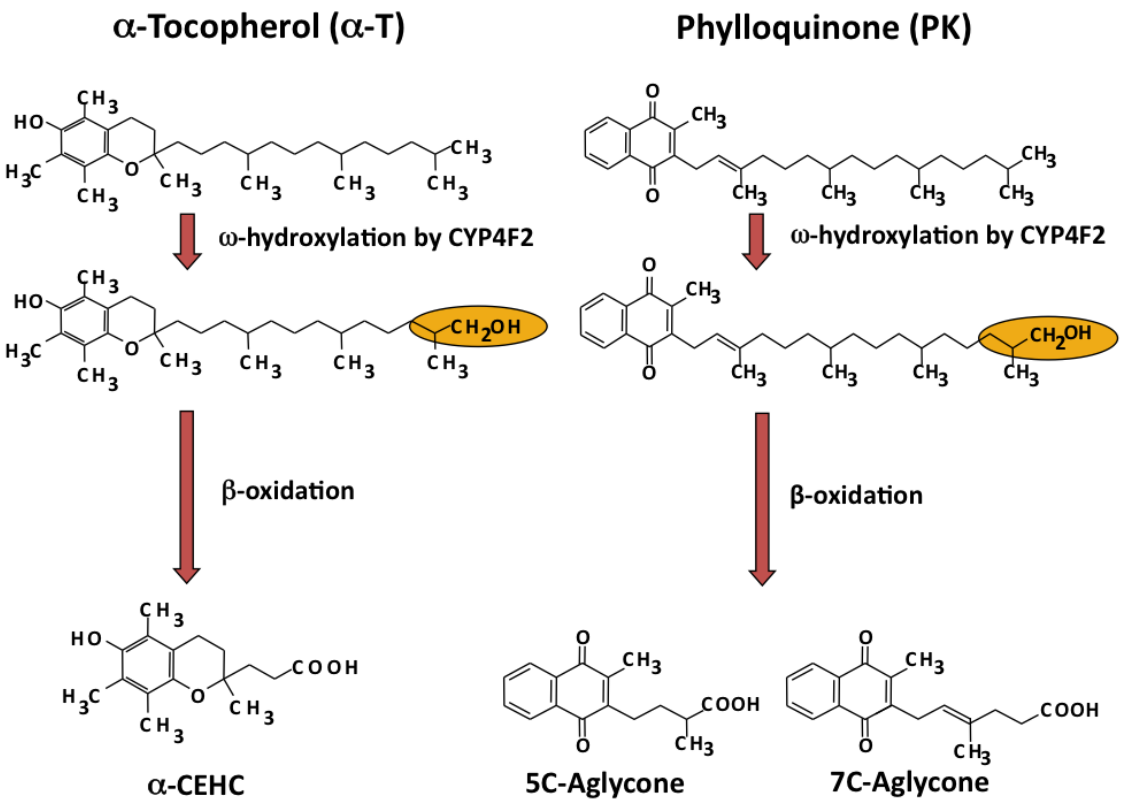

Figure 2.2: Chromatogram of vitamins E and K metabolites

The chromatograms of the measured vitamin metabolites, OH-d_6-*RRR*-α-T, OH-d_3-*SRR*-αT, d_2-γ-T, or d_4-PK with and without NADPH (see Methods). (A) Addition of substrates to aqueous reaction conditions without pre-incubation with S-CYP4F2. (B) Pre-incubation of vitamin substrates with S-CYP4F2 on ice 10 minutes prior to addition of aqueous buffer.

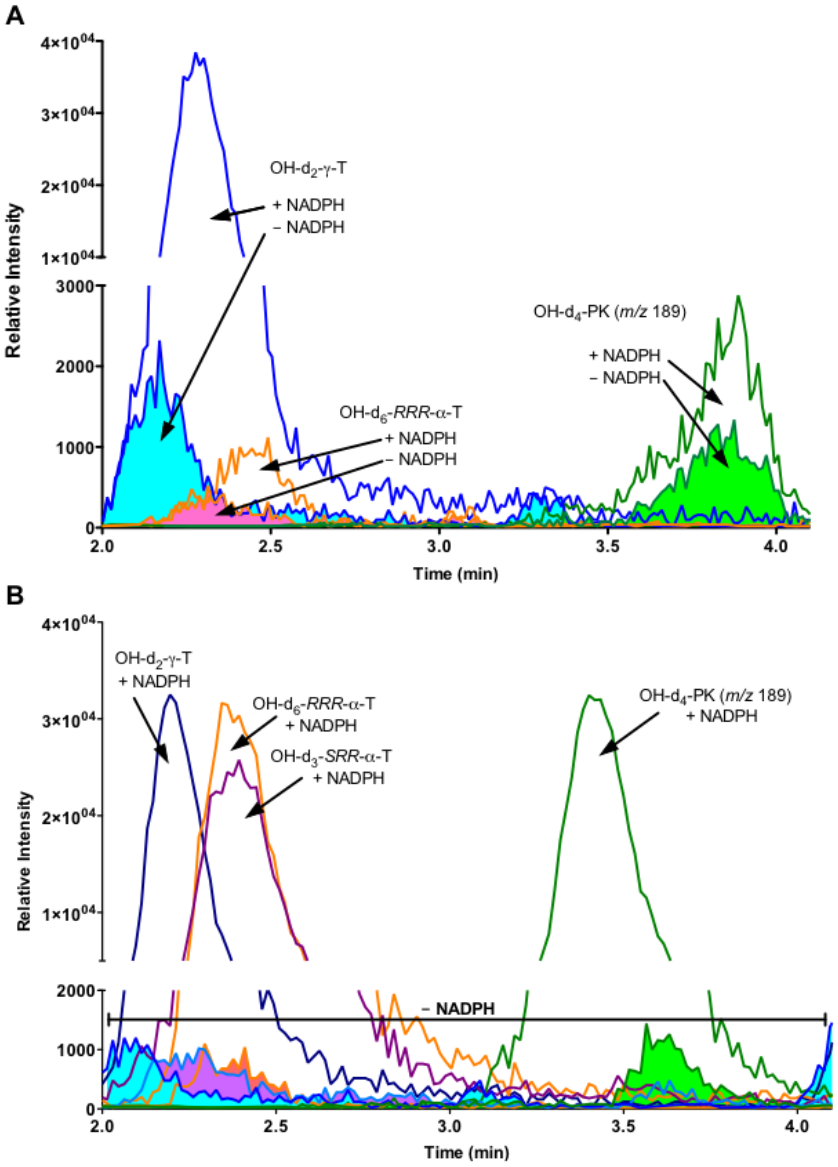

Figure 2.3: Vitamin E metabolism by liver slices

Liver slices were incubated with d_6-α-T, 2*S*-α-T, or d_2-γ-T for 24 hours. A) Unlabeled α-T measured in the liver slices. B) Measured exogenous T (d_6-α-T, 2*S*-α-T, or d_2-γ-T) taken up by the liver slices. 2*S*-α-T was approximated by subtracting the average unlabeled α-T (shown in A) measured in the liver slices from liver slices supplemented with 2*S*-α-T. C) 13'-OH-T produced from the exogenous vitamin E taken up by the liver slices. 13'-OH-2*S*-α-T was estimated by subtracting the average unlabeled OH-α-T from the 13'-OH-2*S*-α-T measurements. Data are means ± SEM, n=6 for d_6-α-T, n=4 for 2*S*-α-T, and n=3 for d_2-γ-T. Means without a common letter differ, $p<0.01$.

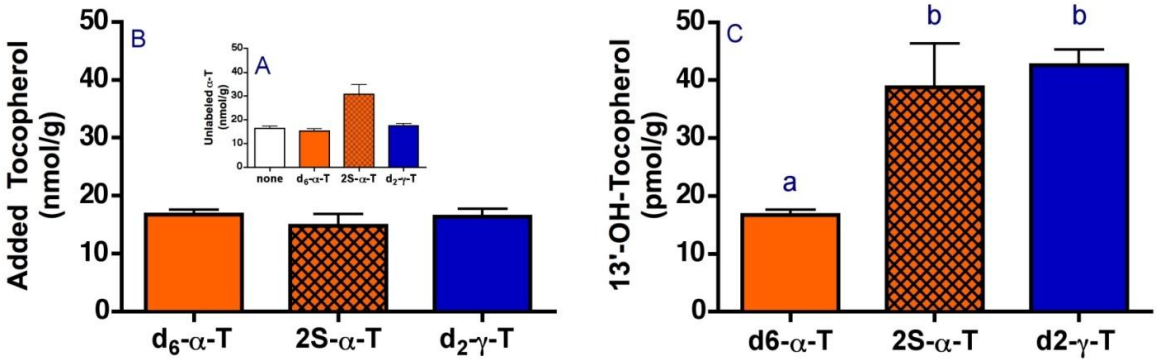

Figure 2.4: Vitamin E and K metabolism by CYP4F2

A. Concentrations of CYP4F2-mediated metabolite formation following 30-minute incubations of 1, 5, 10, 25, 50, and 100 μM concentrations of each vitamin substrate (d_2-γ-T, d_3-*SRR*-α-T, d_6-*RRR*-α-T, or d_4-PK) with 25 pmol S-CYP4F2, and estimated non-linear curve fits as described in Table 1. The Michaelis-Menten curves were generated, compared and found not to be statistically different for the α-Ts; thus, all data was combined to generate a single α-T Michaelis-Menten curve. Kinetic parameters were calculated based on the assumption of vitamin concentrations in the microsomes equaled that in the medium and that the two vitamins were competitive inhibitors of the other. The concentrations and curves for the kinetic parameters are shown in B for PK vs (PK+ vitamin E) and C for vitamin E vs (vitamin E+ PK). *Each vitamin E substrate was investigated separately.

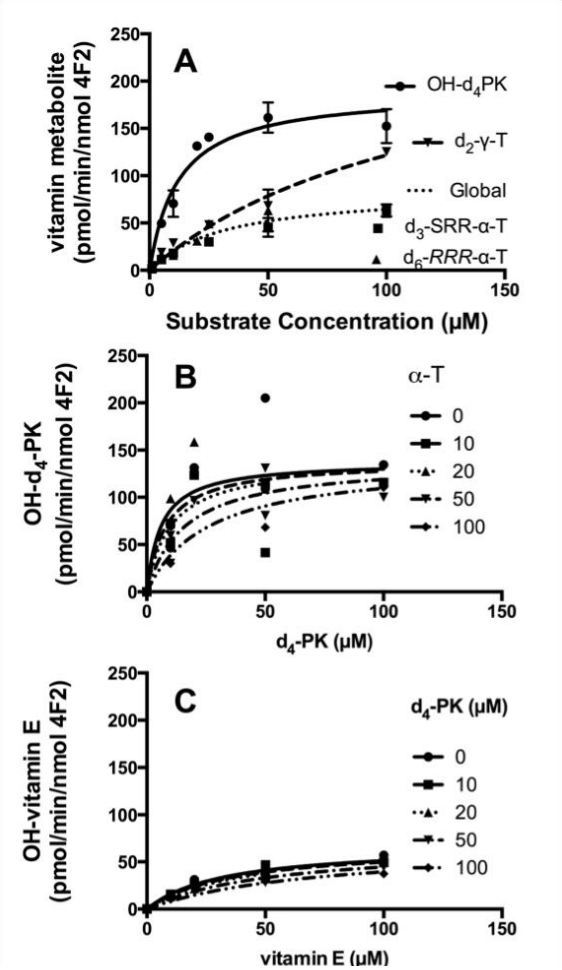

Table 2.1: CYP4F2-mediated metabolism of vitamin substrates

Substrate	Km ± SE (μM)	Vmax ± SE (pmol catabolite / min / nmol CYP4F2)	Catalytic Efficiency (Vmax/Km)
Vitamin E* (d_6-*RRR*-α-T and d_3-*SRR*-α-T)	32 ± 12	85 ± 13[a]	2.6
d_2-γ-T	25 ± 4	100 ± 7[a]	4.0
d_4-PK	14 ± 6	198 ± 23[b]	15.8
ANOVA	NS	P=0.0002	
Interactions between vitamins			
d_4-PK plus Vitamin E* (d_6-*RRR*-α-T or d_3-*SRR*-α-T) KI = 30 ± 27 μM	6 ± 4	137 ± 16	22.8
t-test, PK vs (PK+ vitamin E)	NS	P=0.033	
Vitamin E* plus d_4-PK (d_6-*RRR*-α-T or d_3-*SRR*-α-T) KI = 82 ± 20 μM	32 ± 5	68 ± 4	1.9
Vitamin E vs (vitamin E+ PK)	NS	NS	

Kinetic parameters were calculated based on the assumption of vitamin concentrations in the microsomes equaled those in the medium and that the two vitamins were competitive inhibitors of the other. The concentrations and curves for the kinetic parameters shown in the table are shown in Figure 2A for individual vitamins, Figure 2B for PK vs (PK+ vitamin E), and Figure 2C for vitamin E vs (vitamin E+ PK). Michaelis-Menten model for d_4-PK concentrations was R^2=0.8358 with df=15, and for d_2-γ-T was R^2=9716 with df=4. *Each vitamin E substrate was investigated separately. The Michaelis-Menten curves were generated, compared and found not to be statistically

different for the α-Ts, so all data was combined to generate a single Michaelis-Menten curve, for which the Km, Vmax and catalytic efficiency are shown, the global (shared) Michaelis-Menten model for all concentrations was R^2=0.8358, with df=18. Statistical significance was assessed using ANOVA on the means ± SE for the parameters generated; for values in a column that bear different letters, $p<0.05$. To test the effects of vitamin E and vitamin K on the other's catabolism, increasing concentrations of either d_6-*RRR*-α-T or d_3-*SRR*-α-T were incubated with increasing concentrations of PK. For the effect of vitamin E (both RRR and SRR data were pooled) on vitamin K catabolism kinetics, the global (shared) competitive inhibition model for all concentrations was R^2=0.9682, with df=29; for the effect of vitamin K on vitamin E (both RRR and SRR data were pooled) catabolism kinetics, the global (shared) competitive inhibition model for all concentrations was R^2=0.7363, with df=29. Note that the presence of the other vitamin had no statistically significant effect on the Km, but vitamin E did decrease the estimated PK Vmax.

CHAPTER 3:

Vitamin E Decreases Extra-Hepatic Menaquinone-4 Concentrations in Rats Fed Menadione or Phylloquinone

Sherry M. Farley, Scott W. Leonard, Edwin M. Labut, Hannah F. Raines, David J. Card, Dominic J. Harrington, Debbie J. Mustacich, and Maret G. Traber

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Abstract

The mechanism for increased bleeding and decreased vitamin K status accompanying vitamin E supplementation is unknown. We hypothesized that elevated hepatic α-tocopherol (α-T) concentrations may stimulate vitamin K metabolism and excretion. Furthermore, α-T may interfere with the side chain removal of phylloquinone (PK) to form menadione (MN) as an intermediate for synthesis of tissue-specific menaquinone-4 (MK-4). In order to investigate these hypotheses, rats were fed phylloquinone (PK) or menadione (MN) -containing diets (2 μmol/kg) for 2.5 weeks. From day 10, rats were given daily subcutaneous injections of either α-T (100 mg/kg) or vehicle and were sacrificed 24 hours after the 7th injection. Irrespective of diet, α-T injections decreased MK-4 concentrations in brain, lung, kidney, and heart; and PK in lung. These decreases were not accompanied by increased excretion of urinary 5C- or 7C-aglycone vitamin K metabolites, however the urinary α-T metabolite (α-CEHC) increased ≥100 fold. Moreover, α-T increases were accompanied by down-regulation of hepatic cytochrome P450 expression and modified expression of tissue ATP binding cassette transporters. Thus, in rats, high tissue α-T depleted tissue MK-4 without significantly increasing urinary vitamin K metabolite excretion. Changes in tissue MK-4 and PK levels may be a result of altered regulation of transporters.

Introduction

Studies showing that high doses of vitamin E lead to impaired vitamin K-dependent coagulation processes have been reported since the 1940's [5-9]. In humans, high-dose vitamin E supplements (1000 IU) led to increased serum levels of under-γ-carboxylated prothrombin (protein induced by vitamin K absence or antagonism-factor II, PIVKA-II), a functional biomarker of inadequate vitamin K status [15]. Potential health benefits of vitamin E supplementation arise from its role as an anti-coagulant. Specifically, the Women's Health Study tested the efficacy of 600 IU vitamin E or placebo taken every other day for ten years by nearly 40,000 women aged 45 years and older and demonstrated that vitamin E supplements decrease the risk of thromboembolism [11]. Furthermore, in 2000, the Food and Nutrition Board set the vitamin E upper tolerable limit (UL) for humans based on rat studies demonstrating its hemorrhagic affects, which were prevented by increased vitamin K intake [4]. To date, however, the mechanism by which vitamin E supplementation effects vitamin K activity is unknown. Studies in animals have demonstrated that increased vitamin E intake alters vitamin K status in tissues. Tovar *et al*. [16] reported that rats fed 460 μg phylloquinone (PK) /kg diet with vitamin E as *all-rac*-α-tocopherol at 30 mg/kg diet for 3 months decreased both kidney and brain PK and menaquinone (MK-4) concentrations compared to rats fed a restricted vitamin E diet at 10 mg *all-rac*-α-tocopherol/kg diet. The mechanism by which α-T reduces tissue vitamin K concentrations, however, was not identified.

Interest in optimal vitamin K status and concerns about compounds that antagonize its function have been stimulated by recent evidence demonstrating that the role of vitamin K as a cofactor for γ-glutamyl carboxylase (GGCX) extends beyond that of coagulation [20]. Vitamin K refers to a family of compounds with a common 2-methyl-1,4-napthoquinone head group. PK is the dietary form and is used to synthesize the tissue-specific form, menaquinone-4 (MK-4) [17]. In nature, the phytyl side chain of PK is cleaved to release the intermediate, menadione (MN), followed by geranylgeranylation, to form MK-4 [17, 79]. Although there is evidence that the enzyme UbiA prenyltransferase domain-containing protein 1 (UBIAD1) is involved in the geranylgeranylation step, the mechanism of side chain removal and replacement is not yet fully established [79]. The site of MN release is also unknown and has been

theorized to occur in the intestines [17], or in the liver as we propose, where MN is then transported via the bloodstream to the extra-hepatic tissues for MK-4 synthesis. Alternatively, the simultaneous side chain cleavage and geranylgeranylation of PK to form MK-4 could occur within tissues [17].

The term vitamin E refers to a family of structurally related compounds that vary in biological activity. α-Tocopherol (α-T), is the form with greatest bioavailability that is preferentially maintained in the plasma and tissues due to its hepatic secretion into the blood by α-tocopherol transfer protein (α-TTP) [4]. There are also regulatory mechanisms that prevent accumulation of excess vitamin E that involve xenobiotic pathways, which are responsible for the transport, metabolism and excretion of the vitamin [80]. In humans vitamins E and K are catabolized and excreted by a similar mechanism and therefore it follows that the rates of excretion of both vitamins are metabolically linked [36, 72]. As with xenobiotics, lipophilic compounds, such as vitamins E and K, are metabolized in processes involving: 1) phase I enzymes, or cytochrome P450s (CYPs), responsible for ω- and β-oxidation; 2) phase II enzymes responsible for conjugation where increased polarity enhances water-solubility; and 3) phase III transporters responsible for movement of these compounds into and out of tissues and their excretion in to bile or urine. The first step in the metabolism of vitamins E and K involves ω-hydroxylation by the phase I enzyme human CYP4F2 [29, 30]. Subsequently the vitamins undergo recurrent rounds of ß-oxidation, shortening the side chain by two carbon units per round prior to conjugation forming glucuronides or sulfates to yield their respective urinary metabolites. In the case of vitamin E the major metabolite is carboxy ethyl hydroxy chroman (CEHC) [35] and for vitamin K the 5 and 7 carbon chain aglycone metabolites predominate [36] (**Figure 3.1**). Additionally, xenobiotic transporters may facilitate the biliary excretion of vitamins E and K and their metabolites. The ATP binding cassette (ABC) gene family of transporters have a broad range of substrate specificity and tissue distribution and may be involved in removal of vitamins E and K from tissues resulting in their ultimate excretion from the body. These include multidrug resistance protein (MDR1, or p-glycoprotein), breast cancer resistance protein 1 (BCRP1), and multidrug resistance-associated protein 1 (MRP1). Moreover, xenobiotic uptake transporters, such as the organic anion-transporting polypeptide (OATP), which is expressed in many tissues as well as at the level of the blood-brain barrier, are essential in mediating the entry of amphipathic compounds into tissues [81]. Previously, using a

model system of subcutaneous vitamin E injections in rats to elevate plasma, hepatic and extra-hepatic α-T concentrations we have demonstrated that transporter gene expression is effectively increased within 7 days [43].

Given that α-T and PK share a similar metabolic pathway and that α-T modulates xenobiotic factors [43, 45, 46], we hypothesized that α-T up-regulates CYPs, increasing the metabolic turnover of vitamin K and therefore, the excretion of urinary vitamin K metabolites. Additionally, exploring the mechanism of the conversion of PK to MK-4, we postulated that high α-T concentrations interfere with the as yet unknown process for side chain removal of PK to form the intermediate MN for MK-4 synthesis. MN is a reliable source of vitamin K for synthesis of MK-4 by rodents [17]. Therefore, we hypothesized that our model system of vitamin E injections in rats would decrease MK-4 tissue concentrations only if the dietary source of vitamin K was PK as opposed to MN.

Methods

Animal study design

The Oregon State University (OSU) Institutional Animal Care and Use Committee approved all procedures. Male Sprague-Dawley rats (Charles River, 250-300 g) were housed in plastic cages with hard wood chips, kept on a 12-hour light/dark schedule, and maintained on an AIN-93G diet (Harlan Teklad, Madison, WI) and water ad libitum for 5 days to acclimatize to the OSU animal facility. Rats were then randomly divided into groups that were fed set diets for 10 days (Harlan Teklad TD.10198 and TD.10199, respectively) that contained vitamin E (60 IU *all-rac*-a-tocopheryl acetate/ kg) and contained equimolar concentrations (2 µmol/kg) of either PK or MN (menadione sodium bisulfite). After 10 days, rats were moved to metabolic cages, maintained on their assigned diets, and for 1 week received daily subcutaneous (sq) injections of α-T (100 mg *RRR*-α-tocopherol/kg body weight, Emcelle, Stuart Products, Bedford, TX) or the same volume of vehicle, which was the same emulsion without addition of α-T (Stuart Products, Bedford, TX). Twenty-four hour urine collections were carried out during the week of injections. Twenty-four hours after the last injection (including a 12-hour fast), rats were anesthetized by intraperitoneal (ip) injection of sodium pentobarbital (70 mg/kg). Blood was collected in 10-mL vacutainer tubes containing 1 mg/ml EDTA (Tyco Healthcare Group LP, Mansfield, MA). Plasma was obtained by centrifugation (1500 X *g*, 15 min) and stored at -80ºC until analysis. Immediately following blood collection a 21-gauge perfusion catheter was inserted into the left ventricle of the heart and a small incision made in the right atrium to allow systemic perfusion of organs with 0.9% saline containing heparin (2 U/ml). Liver, lung, kidney, heart, brain, and muscle were excised and aliquots were frozen in liquid nitrogen and stored at -80ºC until analysis. Additionally, during tissue collection, aliquots of liver were stored in RNA*later* (Ambion, Austin, TX) for measurement of xenobiotic enzyme and transporter gene expression.

Vitamin E Measurements

Plasma and tissue α-T concentrations were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) as previously described [70]. Briefly, tissue or plasma was saponified with ethanolic KOH, extracted with hexane, dried under nitrogen, resuspended in ethanol:methanol (1:1) injected to the HPLC system. α-T was detected electrochemically in oxidizing mode with 500 mV potential. Peak areas were integrated using Shimadzu Scientific 4.2 Class VP software package, and α-T was quantitated using calibration standards prepared using pure compounds.

Urinary α-CEHC concentrations were determined by high-performance liquid chromatography with mass spectrometry (HPLC-MS) as previously described [82]. Sample extracts were detected using a Waters Micromass ZQ2000 (Milford, MA) single-quadropole mass spectrometer with an electrospray ionization probe. α-CEHC concentrations were calculated using calibration standards prepared using pure compounds (Cayman Chemical Company, Ann Arbor, Michigan) and an internal standard (Trolox, from Sigma).

Vitamin K Measurements

Plasma PK and MK-4 were extracted using a modification of the method of Podda et al. [70] for extraction. Briefly, 0.3 mL of plasma was added to 2 mL of ethanol and mixed thoroughly followed by addition of 0.7 mL SDS (0.1 M). After addition of internal standard (d_4-phylloquinone (d_4-K1), (Buchem BV, The Netherlands)), 4 mL of hexane was added. Samples were mixed by repeated inversion for one minute. An aliquot of the organic phase was dried under nitrogen and resuspended in 100 μL of ethanol-methanol mixture (1:1 v/v) prior to injection to the LC-MS (see below). For tissue PK and MK-4 measurements, samples were homogenized in 66% isopropanol, extracted with hexane, and loaded onto SPE cartridges, as described previously [83]. PK and MK-4 were eluted with 4% ether in hexane, dried under nitrogen and resuspended in 100 μL ethanol:methanol mixture (1:1 v/v) for analysis using LC-MS.

For quantification of PK and MK-4, the extracts were injected to a Waters HPLC system (Milford, MA), consisting of a 2695 Separations Module containing a cooled auto injector (10°C), a 50 µl sample loop, and a column oven set at 30°C. Separation was achieved using an analytical HPLC column (Synergi Hydro-RP,150 mm L × 4.6 mm I.D., 4 µm particle size, Phenomenex, Torrance, CA) fitted with a precolumn (AQ C18, 4 × 3 mm I.D. SecurityGuard, Phenomenex). The mobile phase consisted of 100% methanol (flow = 1 mL/minute for 13 minutes increased to 1.5 mL/min for 5 min and returned 1 mL/minute for 2 minutes). Typical retention times for MK-4, PK and d_4-K1 were 8.0, 13.3 and 13.3 minutes respectively.

The HPLC was coupled to a Micromass ZQ 2000 single-quadrupole mass spectrometer (Manchester, UK) with an atmospheric pressure chemical ionization (APCI) source operating in negative mode. The corona voltage was set to 20 µA, and the sample cone voltage to -25 V. The source temperature was set to 120°C and the probe temperature to 400°C. The desolvation gas (nitrogen) was set to 350 L/hr, and the cone gas (nitrogen) at 25 L/hr. For vitamin K analysis, single-ion recording (SIR) data were obtained for PK (*m/z* 450) and MK-4 (*m/z* 444), and the internal standard, d_4-K1 (*m/z* 454). The dwell time for each of the ions was set to 0.20 seconds. Instrument control and acquisition were performed using Waters Masslynx software version 3.4. Plasma and tissue MK-4 and PK were calculated using peak area, calibrated against a standard curve and corrected for the internal standard concentration. The lower limit of quantification (LLOQ) for plasma K1 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.

Urinary 5C- and 7C-aglycone vitamin K catabolites were determined according to methods published previously [36, 71]. Five hundred microlitres of urine sample was desalted with deionised water using a SPE cartridge (Isolute C18, 100 mg, 1 mL) followed by addition of the internal standard and elution with methanol. The methanolic extract was collected and dried under nitrogen, with conjugates hydrolyzed overnight in methanolic HCl. The aglycone catabolites were then extracted in to chloroform and dried under nitrogen. The endogenously occurring carboxylic acid forms of the catabolites were stabilized by conversion to their methyl ester derivatives using 1-methyl-3-nitro-1-nitrosoguanidine. Further purification was carried out using SPE cartridges (Waters SEP-PAK Silica) eluted with diethyl ether (3%) in hexane. Sample extracts were dried

under nitrogen, reconstituted in methanol and injected to the HPLC-ECD system. Isocratic reversed-phase chromatographic separation utilized a mobile phase consisting of a methanol-water sodium acetate buffer. The derivatized 5C and 7C aglycone catabolites and internal standard were firstly reduced using an upstream dual coulometric electrode (ESA 5011) set to -1.2V and then oxidized at the downstream amperometric wall jet electrode (Antec VT-03) set at +0.3V. Cells were operated by Coulochem II and Decade II controllers, respectively. Chromatograms were generated using the current generated from the wall jet electrode. Waters Empower was used for data capture. Quantification was carried out using a calibration curve of methanolic solutions containing the derivatized catabolites and internal standard.

qPCR

Total RNA was isolated from tissue samples stored in RNA*later* (Ambion, Austin, TX) using a Trizol Reagent assay per the manufacturer's instructions (Invitrogen, Carlsbad, CA). cDNA was prepared using SuperScript III cDNA kit (Invitrogen). The following mRNA-specific primers were designed using Primer-BLAST (www.ncbi.nlm.nih.gov): 1) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5'-TAAAGGGCATCCTG-3' and reverse 5'-TTACTCCTTGGAGG, 2) CYP4F1 forward 5'-GGAACTTTCGTCGCCTCCGTGG-3' and reverse 5'-CGCAACGGCAGCTGAGGCAT-3', 3) CYP4F4 forward 5'-TGCCTCAGCTTCGGTTGCCC-3' and reverse 5'-TGCCTCCTGAGGCCAGGTCC-3', and 4) CYP3a23/3a1 (CYP3A) forward 5'-CCTGGCAGTCGTCCTGGTGC-3' and reverse 5'-ACTGGGCCAAAATCCCGCCG-3'. The following previously published rat xenobiotic transporter primer sets were also utilized: 1) ATP-binding cassette, subfamily G, member 2 (Abcg2, alias BCRP1), 2) solute carrier organic anion transporter family, member 1a4 (Slco1a4, alias OATP2), 3) ATP-binding cassette, subfamily C, member 1 (Abcc1, alias MRP1), and 4) ATP-binding cassette, subfamily B member 1B (Abcb1b, alias MDR1) [80]. Quantitative real time RT-PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA) and the 7900HT Real Time PCR System (Applied Biosystems). All genes were run in triplicate; the average transcript expression for each gene of interest was determined for each rat and normalized to the average transcript expression of the housekeeping gene, GAPDH. The fold-change in transcript expression for transporter genes, BCRP1 and OATP, were determined relative to vehicle-injected PK-fed rats

using the $2^{-\Delta\Delta C_T}$ method. The fold-change in transcript expression for CYP genes, CYP4F4, CYP4F1, and CYP3A were determined relative to vehicle-injected PK-fed rats using plasmids to generate an absolute copy number standard curve for real-time PCR quantification as described previously [84].

Immunoblotting

Rat microsomes were prepared from liver samples as previously described [45]. Equal concentrations of microsomal protein were resolved by SDS-PAGE electrophoresis and transferred to PVDF membranes. Membranes were probed with Abcg2 (D-20, sc-25156), CYP4F2 (H-40, sc-67156), or Actin (I-19, sc-1616-R) primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary antibodies were either anti-goat or anti-rabbit-HRP-conjugated antibodies (Santa Cruz Biotechnology, Inc.). The proteins were visualized with enhanced chemiluminescence (Perkin Elmer, Inc., Waltham, MA). Expression levels in each sample were normalized to their respective actin protein concentrations using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011).

Statistics

Statistical analysis was performed using JMP Statistical Software (SAS, Cary, NC). Data were analyzed by two-way ANOVA and Tukey's posttests were performed when overall group effects were significant. When necessary, data were log-transformed to equalize variances. Data are reported as means ± SD (n=5).

Results

Plasma and liver α-tocopherol and vitamin K concentrations

For 7 days, Emcelle (100 mg α-T/kg body weight), or vehicle was subcutaneously injected into rats fed diets containing either PK or MN. On day 8, 24 hours after their final injection, rats were sacrificed and blood and tissues were collected. As previously reported [46], this study showed 7 days of vitamin E injections more than doubled plasma α-T concentrations and increased liver α-T concentrations more than 10-fold (**Figure 3.2**). There were no differences in plasma or liver α-T concentrations between the two dietary vitamin K regimens (PK or MN).

Plasma PK increased ($p<0.0013$) in PK-fed rats that received vitamin E injections, while it had no effect on MN diet fed rats (**Figure 3.3A**). In contrast, α-T-injections significantly decreased plasma MK-4 concentrations, irrespective of dietary vitamin K form fed to the rats (**Figure 3.3C**).

Hepatic PK concentrations were altered in response to both diet ($p<0.0001$) and vitamin E injections ($p<0.0006$) (**Figure 3.3B**). The hepatic PK concentrations of rats fed the PK diet were higher relative to those fed the MN diet and hepatic PK concentrations increased further in both groups post vitamin E injections. Both diet ($p<0.0001$) and vitamin E injections ($p<0.0010$) altered the hepatic MK-4 concentrations (**Figure 3.3D**). Livers from rats consuming the PK diet injected with vehicle had the lowest MK-4 concentrations, while vitamin E injections significantly increased hepatic MK-4 concentrations. The highest MK-4 concentrations were observed in rats fed the MN diet that received vitamin E injections.

Extra-hepatic vitamin K concentrations

Vitamin E injections had little effect on PK tissue concentrations, irrespective of diet. Although PK was not detected in the MN-based diet (data not shown), PK was detectable, albeit significantly reduced ($p<0.05$) in extra-hepatic tissues, especially the heart, of MN-fed rats (**Figure 3.4A**). Moreover, in MN rats, PK concentrations were unchanged by the α-T- injections. Similarly, in PK fed rats, vitamin E injections had little

effect on PK concentrations, except in the lung, where reduced PK concentrations were observed ($p<0.05$).

Tissue MK-4 concentrations were decreased in response to vitamin E injections in all extra-hepatic tissues examined (brain, kidney, lung, and heart) (**Figure 3.4B**). Extra-hepatic tissues from MN-fed rats had higher concentrations of MK-4 compared to those fed PK ($p<0.0010$). In regards to the brain, not only did it exhibit significant reduction in MK-4 levels following vitamin E injections but also MK-4 predominated over PK, with concentrations at least 3 times higher than PK. Lung and kidney tissues had equal PK and MK-4 concentrations while heart and liver had higher PK concentrations relative to MK-4.

Urinary excretion of vitamin E and vitamin K metabolites

Urinary excretion of the 5C- and 7C-aglycone vitamin K metabolites, and α-CEHC, were examined on day 1 (baseline, prior to injections), day 4 (post 3 days of injections) and day 8 (post 7 days of injections) (**Figure 3.5**). Urinary α-CEHC excretion increased nearly 100-fold post Vitamin E injections from day 1 to day 8 ($p<0.0001$, **Figure 3.5A**), while there were no differences in α-CEHC excretion in either PK or MN fed rats. Neither the type of dietary vitamin K nor the vitamin E injections had any significant effect on the urinary excretion of vitamin K metabolites. In this sample of rats the 7C catabolite predominated over the 5C (molar ratio = ~ 5:1) whereas in humans the 5C catabolite has been found to be the major form (molar ratio = ~ 4:1) [36].

Cytochrome P450 enzymes and xenobiotic transporters

To investigate the mechanism of vitamins E and K metabolism and redistribution in tissues, we examined hepatic gene expression of cytochrome P450 enzymes (CYPs) and transporters reported to be involved in vitamin E and K metabolism and transport. As vitamins E and K share the key catabolic mechanism stage of ω-oxidation by CYP4F2 [29, 30], the mRNA expression of CYP4F1 and CYP4F4 (rat homologues of human CYP4F2 [85]), and CYP3A were evaluated. All CYPs were down-regulated in the liver of vitamin E injected rats compared to vehicle, irrespective of form of dietary vitamin K given (**Figure 3.6**). Human CYP4F2 has over 95% homology with rat CYP4F1 and CYP4F4 therefore human anti-CYP4F2 was used in western blots to estimate CYP4F

protein expression in microsomes isolated from the rat tissues. CYP4F expression was observed at the expected molecular weight of 52 kD and results were consistent with the mRNA results demonstrating decreased expression of the enzyme in the liver (**Figure 7 A, C**).

Hepatic mRNA expression of the efflux transporters BCRP1 and MDR1, and the uptake transporter OATP were altered in response to vitamin E injections; diet had no effect on these transporters (**Figure 3.8**). Specifically, OATP was found to be highly down-regulated by vitamin E injections compared to vehicle (**Figure 3.8D**), while MDR1 and BCRP1 mRNA expression was up-regulated. (**Figures 3.8A & 3.8B**). In contrast, neither dietary vitamin K form nor vitamin E injections had any significant effect on MRP1 (ABCC1) regulation.

Protein expression of transporters in hepatic membranes and microsomes was also evaluated. Using commercially available antibodies, BCRP1 was the only transporter detected. The molecular weight calculated for this transporter (~60 kDa) corresponds to the unglycosylated but fully functional form [86] (**Figure 3.7A & B**). Additionally, BCRP1 protein expression was only observed in rats receiving the vitamin E injections and only in microsomes, not membranes.

Discussion

α-T injections reduced plasma and extra-hepatic MK-4 concentrations. Although both PK and MK-4 can act as a cofactor for GGCX, tissue-specific synthesis of MK-4 suggests that it has an important role in these tissues. Moreover, studies indicate that MK-4 is also the major form of vitamin K in rat [87] and human [21] brain. We observed that MK-4 is not only preferentially accumulated in the brain relative to PK but that MK-4 concentrations were more than halved in response to 7 days of α-T injections. In response to vitamin E supplementation MK-4 dependent brain functions in particular may be impacted, although there are likely adverse consequences for loss of vitamin K activity in other tissues.

As for hepatic levels of vitamin K, both MK-4 and PK were elevated in α-T-injected rats compared to vehicle. Because we hypothesized that the metabolism and excretion of vitamin K would be increased, we had expected that vitamin K concentrations would be reduced in the liver as well. However, very little is known about vitamin K tissue distribution following vitamin E supplementation. In a 3 month feeding study, Tovar et al. [16] reported reduced extra-hepatic tissue concentrations of PK and MK-4 in rats fed high concentrations of vitamin E compared to low vitamin E, but they found no significant differences in vitamin K concentrations in the liver of rats. It is theorized that coagulation is a more essential function of vitamin K and is spared during periods of vitamin K deficiency over other vitamin K-dependent proteins that have more long-term adverse consequences [88-90]. If this is correct, it may take longer for vitamin K to be depleted in the liver where clotting factors are synthesized. Elevated hepatic PK and MK-4 observed in our study may be evidence that the 7 days of vitamin E injections stimulated vitamin K return to the liver from extra-hepatic tissues, in the case of MK-4, and from the circulation as a result of dietary intake, in the case of PK. And it may follow that since vitamins E and K share metabolic pathways, vitamin K metabolism was less efficient in rats that also had high concentrations of hepatic vitamin E.

To assess the mechanism for MK-4-specific loss in extra-hepatic tissues, rats were fed either PK or the intermediate to MK-4 synthesis, MN. The purpose of the two diets was to study whether vitamin E interfered with the conversion of PK to MN. MK-4 synthesis

would be expected to decrease if MN production from PK was limited by α-T injections, as demonstrated in brain, lung, kidney and heart (**Figure 3.4B**). However, although tissue MK-4 concentrations were significantly higher in rats with MN diets, they were not vastly different from the concentrations found in the PK rats. Moreover, it has been suggested that UBIAD1 may have dual enzymatic roles involving side chain cleavage of PK in addition to geranylgeranylation of MN to form MK-4 [79]. If this is correct then the targeted MK-4 reductions in extra-hepatic tissues of rats fed either vitamin K diet suggests that α-T may be interfering with UBIAD1-mediated synthesis of MK-4 at either step.

A second mechanism of vitamin E and K interaction examined in this study was the reduced bioavailability of vitamin K caused by vitamin E induced increases in metabolic turnover of vitamin K as assessed by measurement of the urinary excretion of vitamin K catabolites. Although highly elevated hepatic concentrations of vitamin E resulted in increased urinary excretion of α-CEHC, this was not accompanied by increased excretion of urinary vitamin K catabolites. These data could suggest that there is no increase in rate of vitamin K metabolism in response to vitamin E injections. Conversely if the rate of vitamin K metabolism was increased it is possible that the metabolites were excreted via the bile duct. Alternatively, excretion of vitamin K in the form of PK, MK-4, and/or MN, which is also reportedly a metabolite of vitamin K [91], could also be increased in the bile.

The xenobiotic enzymes CYP3A and CYP4F, which are implicated in vitamin E and K metabolism, were also examined. As previously observed [43], hepatic gene expression of CYP3A decreased with vitamin E injections. However, contrary to expectation, we observed that gene and protein expression of CYP4F was also reduced in response to vitamin E injections. In vitro studies have shown that CYP4F2 is the major vitamin E and K hydroxylase [29, 30] although other as yet undefined CYPs may also be involved in their metabolism in vivo. As discussed in greater detail in another study by our lab [43], it is still inconclusive how vitamin E mediates it effects on expression of xenobiotic proteins. Reduced expression of CYP enzymes, however, supports evidence that increased rate of metabolism of vitamin K may not be the mechanism of vitamin E-mediated loss of vitamin K in tissues.

Vitamin E modulates the expression of xenobiotic transporters that are involved in influx and efflux of compounds in tissues and their excretion into urine or bile [43, 45]. In this study, the hepatic gene expression of the efflux transporters, MDR1 and BCRP1, were increased whereas hepatic gene expression of the uptake transporter, OATP, was significantly decreased in rats receiving vitamin E injections; these responses occurred irrespective of the form of vitamin K given in the diet. Moreover, we observed a major induction of the unglycosylated form of BCRP1 protein expression in the liver microsomes of rats that received α-T injections. Like other ABC transporters, BCRP1 is expressed in the plasma membranes of many tissues and in the liver it functions to facilitate excretion of various compounds into bile [92]. Additionally, ABC transporters, such as BCRP1, are distributed intracellularly where they may accumulate and transport compounds including nutrients into vesicles [93]. Thus, elevated expression of unglycosylated BCRP1 in the microsomal fraction of the liver of rats receiving α-T injections suggests its synthesis in the ER may have been up-regulated for eventual localization to the plasma membrane to facilitate biliary excretion and/or to aid in intracellular transport of vitamin E. Although it has been observed that glycosylation of BCRP1 is not necessary for activity or localization [86], it should be noted that conflicting in vitro evidence suggest that post-translational glycosylation of BCRP1 may be critical for stabilization of BCRP1 for its subsequent localization to the plasma membrane [94]. Enhanced degradation of BCRP1 would explain why expression in the membrane fraction was not observed; alternatively, conditions for detection by western blot using commercial antibodies (for all transporters) was not optimal.

The identity and nature of transporters specific to vitamins E and K and their metabolites are currently poorly defined, but may be critical to understanding of how vitamins E and K interact.

Our data show that extra-hepatic MK-4 concentrations were decreased but that both hepatic PK and MK-4 were elevated following vitamin E injections. This supports evidence for redistribution of vitamin K, which may be facilitated by modified expression of xenobiotic transporters in response to vitamin E injections. Currently, the circulatory form of vitamin K that is taken up by tissues for synthesis of MK-4 is unknown. It has been hypothesized that synthesis of MK-4 is tissue-specific resulting from uptake of either PK or MN [17, 79]. We propose that the side chain of PK is cleaved in the liver

and MN is transferred into circulation to be delivered to extra-hepatic tissues for MK-4 synthesis. If transporters, such as OATP, are necessary for MN uptake, this may explain why extra-hepatic MK-4 concentrations (e.g. brain) are more substantially reduced following α-T injections in relation to PK. OATP, similar to the efflux transporters BCRP1 and MDR1, can bind a variety of substrates. These transporters are highly expressed in multiple tissues including the liver, kidneys, placenta, and along the blood-brain barrier [81, 95, 96]. Moreover, tissue culture studies have provided evidence that MN is a substrate for the efflux transporter BCRP1 [48]. It is likely that there is overlap in substrate specificity among uptake and efflux transporters that regulate movement and distribution of compounds among tissues. Although measurement of transporter activity is beyond the scope of this study these data reflect the need to further evaluate the role transporters play in nutrient transport.

In conclusion these data demonstrate that high doses of α-T lead to decreased tissue MK-4 concentrations in rats. Decreased tissue vitamin K, resulting from α-T is not a result of increased catabolism and urinary excretion of vitamin K. More targeted reductions in MK-4 rather than PK indicate that vitamin E may be interfering with synthesis of MK-4. Additionally, α-T injections in rats alter regulation of proteins involved in xenobiotic pathways, which could influence vitamin K concentrations in tissues. More studies into vitamin K transport including excretion via the bile duct, as well as the urine, and how vitamin E modulates these pathways are necessary to further our understanding of how vitamins E and K interact.

Figure 3.1: Metabolic pathway of vitamins E and K

The metabolic pathway of vitamins E and K involve their initial ω-hydroxylation by CYP4F2 [29, 30] followed by multiple rounds of ß-oxidation to yield their respective urinary metabolites, carboxy ethyl hydroxy chroman (CEHC) for vitamin E [35] and 5C- and 7C-aglycone for vitamin K [36].

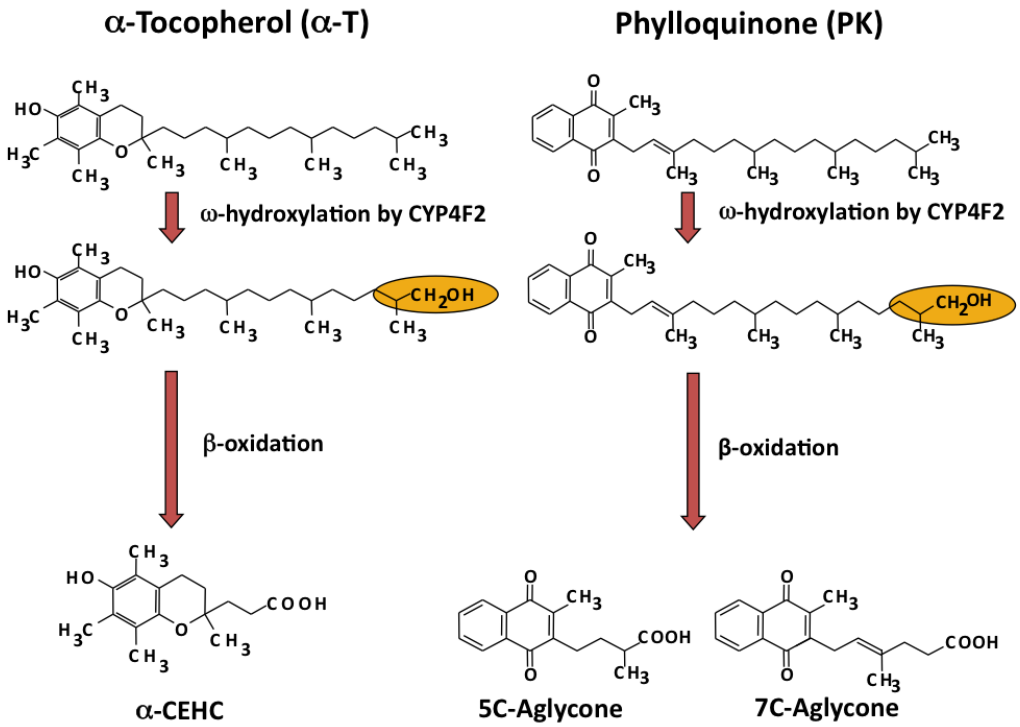


Figure 3.2: Plasma and liver α-T

Subcutaneous vitamin E injections markedly increase plasma and hepatic α-tocopherol concentrations. Rats fed diets containing either PK or MN received subcutaneous injections of α-T (100 mg/kg) or vehicle for 7 days. Mean values ± SD of (A) plasma and (B) liver α-T concentrations are shown. Type of vitamin K given in the diet (PK or MN) had no significant effect on α-T concentrations in liver or plasma. Vitamin E injections on plasma α-T or liver α-T was significant ($p<0.0001$). Bars showing vehicle and α-T injections in each panel (plasma or liver) not bearing the same letter are significantly different, $p<0.001$ ($n=5$).

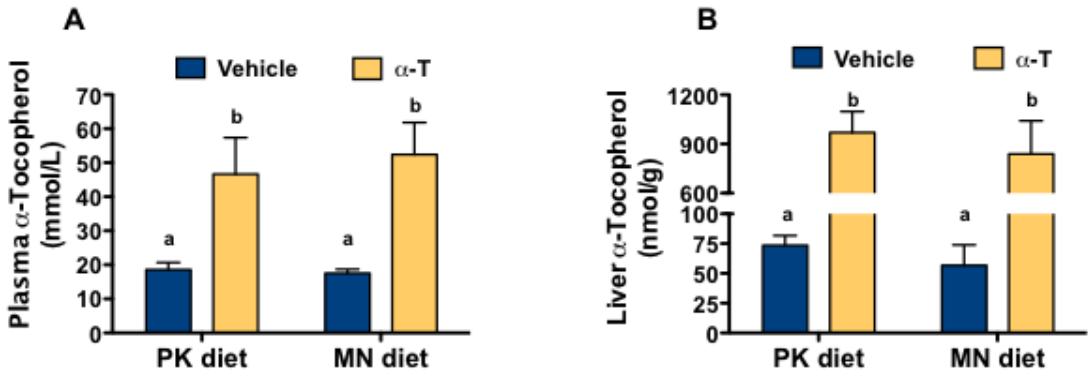


Figure 3.3: Subcutaneous vitamin E injections increased plasma and hepatic PK concentrations in PK and MN fed rats

The vitamin K concentrations in plasma and liver are shown from the same rats in figure 2. (A) PK concentrations in plasma (effect of diet, p=0.0020, effect of injections, p=0.0408), (B) PK concentrations in liver (effect of diet, p<0.0001, effect of injections, p=0.0006), (C) MK-4 concentrations in plasma (effect of diet, p=0.3620, effect of injections, p<0.0001), and (D) MK-4 concentrations in liver (effect of diet, p<0.0001, effect of injections, p=0.0010). Data are mean values ± SD. Bars in each panel not bearing the same letter are significantly different, p<0.05 (n=5).

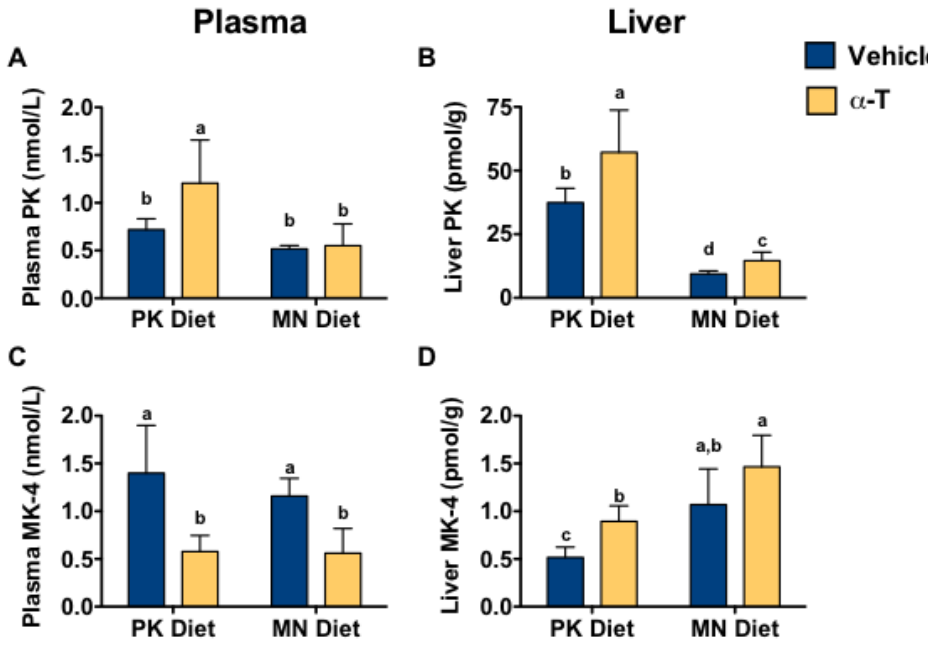


Figure 3.4: Subcutaneous vitamin E injections decreased extra-hepatic MK-4 concentrations

PK and MK-4 concentrations in extrahepatic tissues are shown from the same rats in figure 2. (A) PK concentrations were reduced in all extra-hepatic tissues of rats fed the MN diet ($p<0.0100$); α-T injections decreased PK only in lung ($p=0.0045$). (B) MK-4 concentrations were higher in all extra-hepatic tissues of rats fed the MN diet relative to rats fed the PK diet ($p<0.0010$). α-T injections decreased MK-4 in all extra-hepatic tissues examined ($p<0.0001$). Means $\pm$ SD are given. Bars not bearing the same letter for each tissue are significantly different, $p<0.05$ ($n=5$).

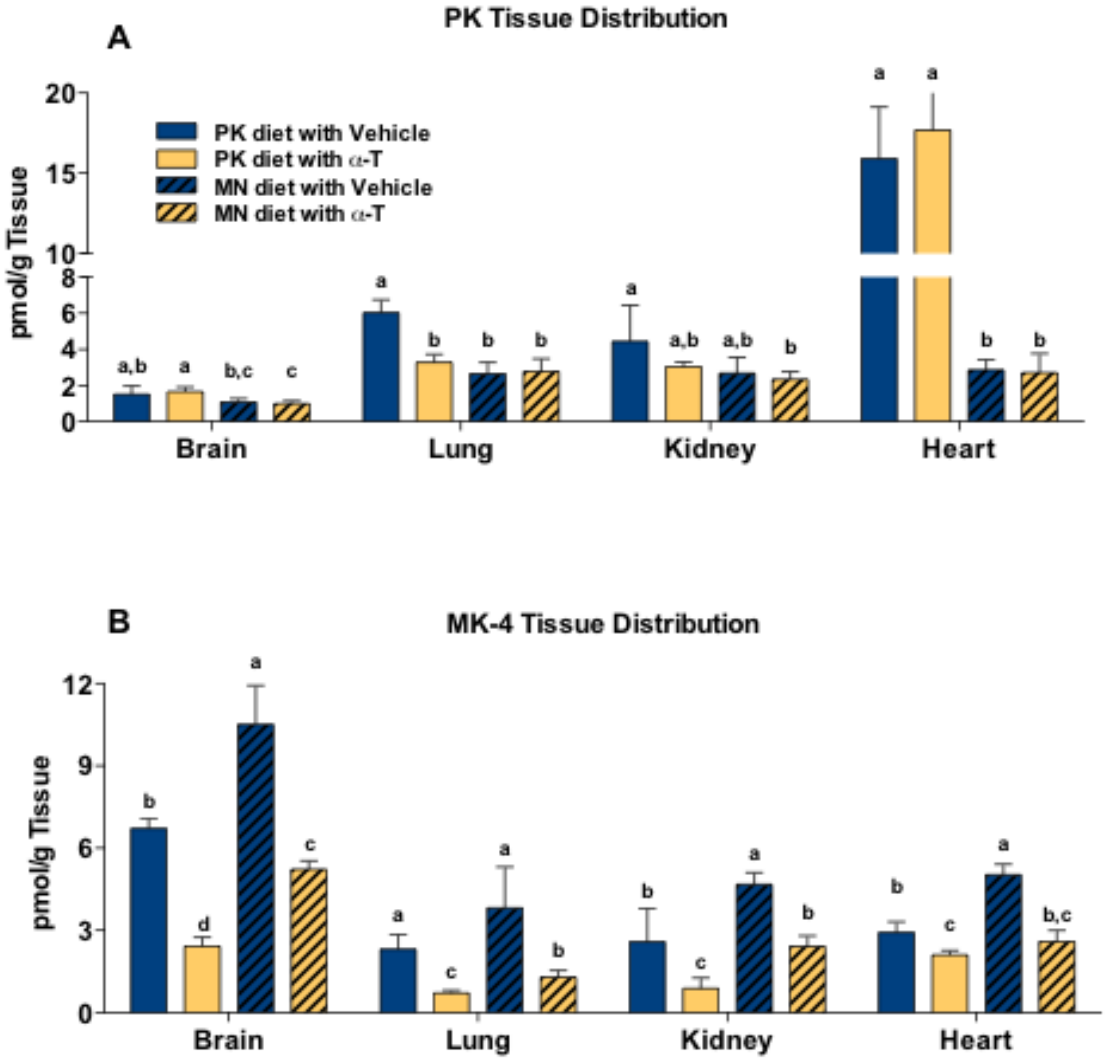


Figure 3.5: Vitamin E injections had no effect on vitamin K metabolite excretion

Urinary metabolite excretion is shown from the same rats in figure 2. α-CEHC excretion (A) increased significantly over time with vitamin E injections (effect of injection, $p<0.0001$). Although 5C-Aglycone excretion (B) decreased over time (effect of time, $p<0.0001$), neither 5C-aglycone or 7C-aglycone excretion (C) changed with respect to α-T or vehicle injection.

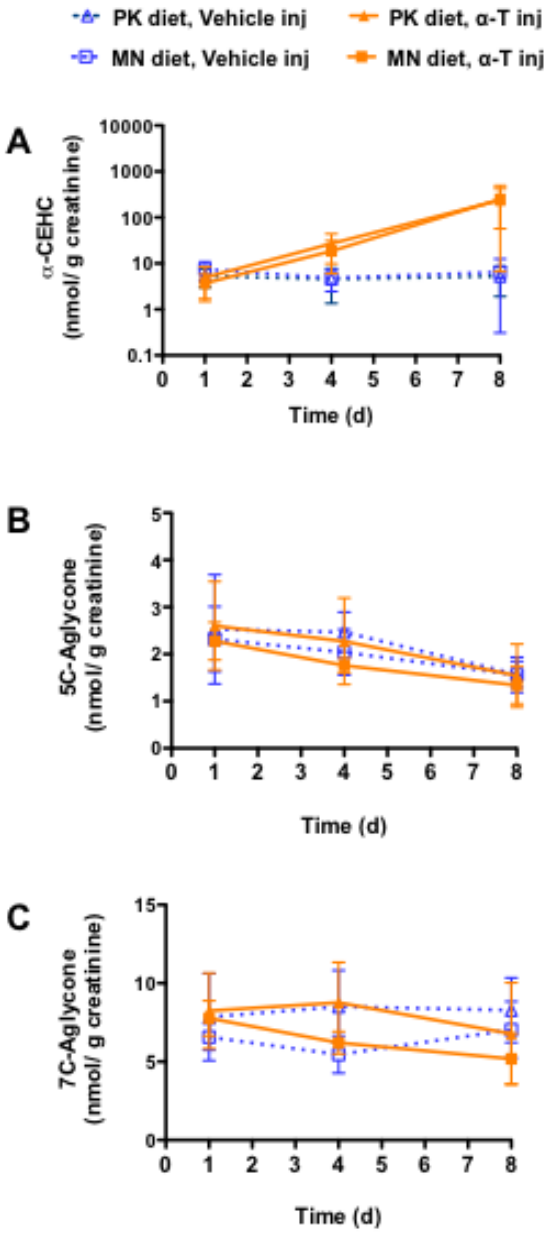


Figure 3.6: Vitamin E injections decreased CYP mRNA

The hepatic mRNA expression of the following CYPs: (A) CYP3A (effect of injection, p=0.0001), (B) CYP4F4 (effect of injection, p=0.0039), and (C) CYP4F1 (effect of injection, p<0.0001) are shown from the same rats in figure 2. The expression of mRNA are shown relative to averaged PK-fed, vehicle-injected measurements (control). Data are mean values ± SD. Bars in each panel not bearing the same letter for each tissue are significantly different, p<0.05 (n=5).

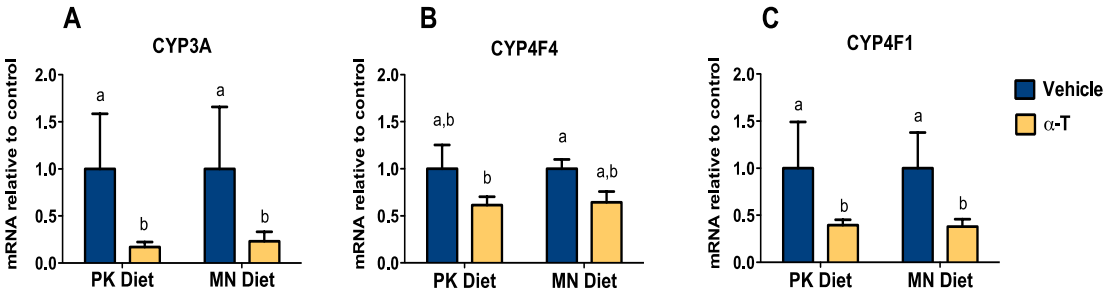


Figure 3.7: Protein expression of CYP4F and BCRP1

Protein expression of CYP4F and BCRP1 in liver microsomes are shown from the same rats in figure 2. (A) Western blot of BCRP1, CYP4F, and actin. Expression of (B) BCRP1 and (C) CYP4F normalized to actin are shown relative to PK-fed, vehicle-injected measurements (control). There was no effect of diet on protein expression of these factors, but vitamin E injections reduced expression of CYP4F ($p=0.0002$) and increased expression of BCRP1 ($p<0.0001$). The expression of mRNA are shown relative to averaged PK-fed, vehicle-injected measurements (control). Data are means ± SD. Bars not bearing the same letter for each tissue are significantly different, $p<0.05$ ($n=5$).

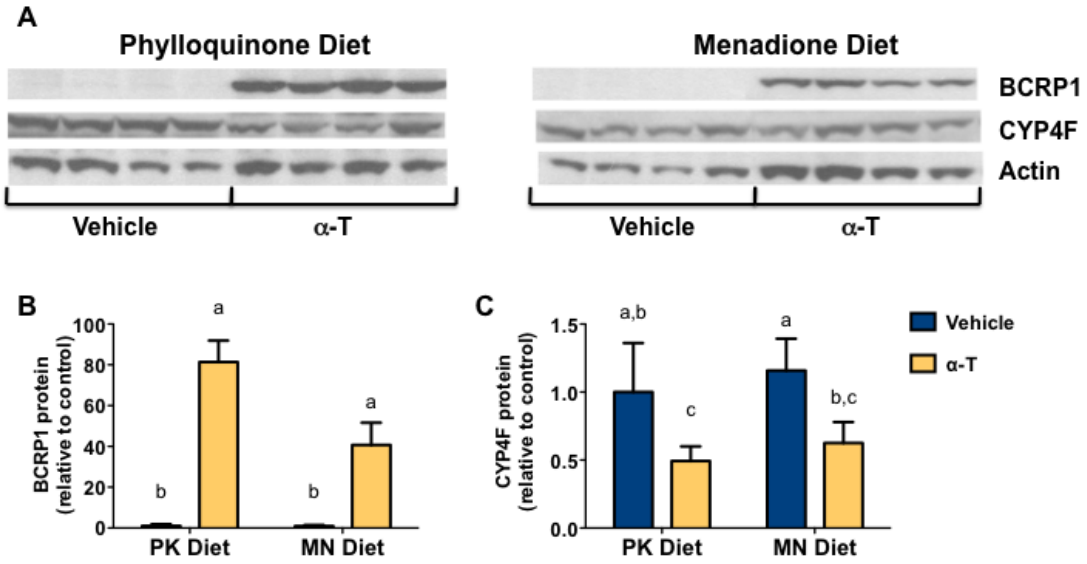
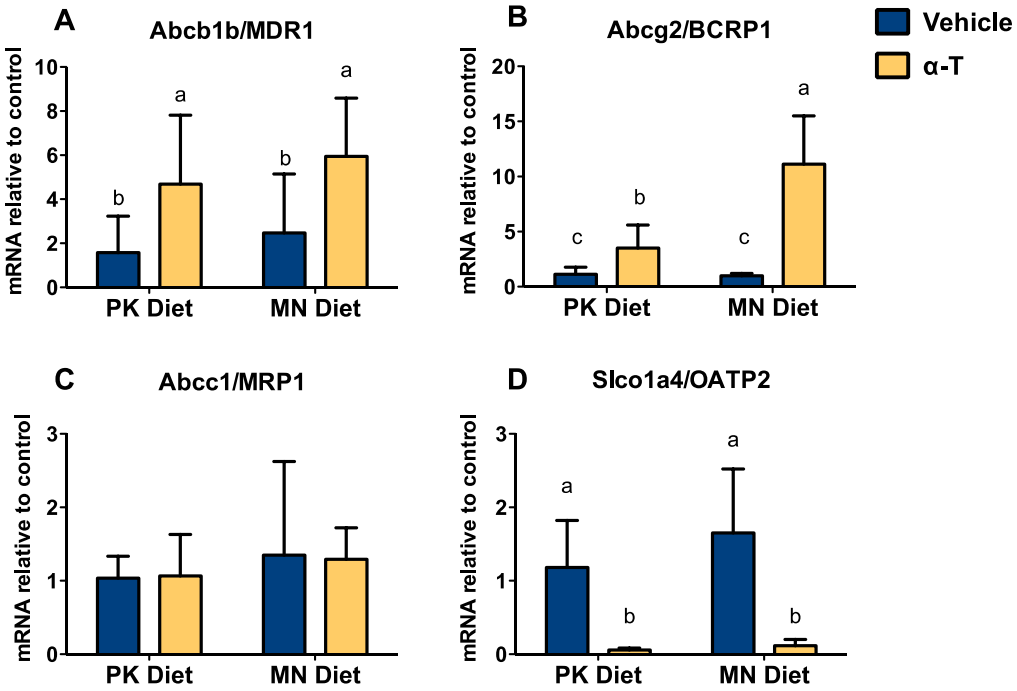


Figure 3.8: Vitamin E increased BCRP1 and MDR1 and decreased OATP gene expression

Hepatic mRNA expression is shown from the same rats in figure 2 for the following xenobiotic transporters: (A) Abcb1b/MDR1 (effect of injection, p=0.0208) (B) Abcg2/BCRP1 (effect of injection, p=0.0005), (C) Abcc1/MRP1 and (D) Slco21a4/OATP2 (effect of injection, p<0.0001). Data are means ± SD. Bars not bearing the same letter for each tissue are significantly different, p<0.05 (n=5).



Chapter 4:

Biliary excretion of vitamin E and K and their catabolites by rats during subcutaneous vitamin E administration

Sherry M. Farley, Scott W. Leonard, David J. Card, Dominic J. Harrington, Edwin M. Labut, Maret G. Traber

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Abstract

To test the hypothesis that high dose vitamin E administration potentiates biliary excretion of vitamin K, ~1 h bile and 24-h urine samples were collected daily from bile cannulated rats (n=5), prior to and following daily subcutaneous injections with α-tocopherol (100 mg/kg body weight injections) at 0, 1, 3, 5 and 7 days. α-Tocopherol, phylloquinone and menaquinone-4 (MK-4) were measured in bile; the vitamin E catabolites, carboxyethyl hydroxychromanol (CEHC) and carboxymethylbutyl hydroxychroman (CMBHC), and the 5C-and 7C-aglycone vitamin K catabolites were measured in both bile and urine. Following 7 daily injections, biliary α-tocopherol concentrations increased ~4-fold, while both biliary and urinary CEHC concentrations increased more than ~10-fold. Urinary excretion of 5C- and 7C-aglycone vitamin K catabolites and biliary phylloquinone excretion were unchanged in response to α-tocopherol administration. Biliary 5C-aglycone concentrations were approximately doubled following 5 and 7 days of α-tocopherol injections, while biliary MK-4 and 7C-aglycone were below levels of detection. Thus, in times of vast hepatic α-tocopherol excess, α-tocopherol's major route of excretion is as biliary α-CEHC. The sum of the biliary and urinary 5C-aglycone excretion rates (pmol/h) was unaffected by α-tocopherol administration, leaving the mechanism for how high dose vitamin E administration impairs vitamin K status unanswered.

Introduction

Vitamin E refers to 8 molecules with antioxidant activity with structural similarity to α-tocopherol (α-T) [35, 97]. The pathway for vitamin E catabolism involves an initial ω-hydroxylation of the side chain by the human cytochrome P450 4F2 (CYP4F2) [30], which is followed by ω-oxidation and multiple rounds of β-oxidation to yield the major urinary α-T catabolites, α-CEHC [2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman, or 3-(6-hydroxy-2,5,7,8-tetramethylchroman-2-yl) propanoic acid], and α-CMBHC [2,5,7,8-tetramethyl-2(2'-carboxymethylbutyl)-6-hydroxychroman, or 5-(6-hydroxy-2,5,7,8-tetramethyl-chroman-2-yl)-2-methyl-pentanoic acid] [98-100].

Phylloquinone (PK) (also known as vitamin K_1) is the major dietary form of vitamin K; like vitamin E, plants synthesize PK. In animals, the PK side-chain is hydrolyzed to form the napthoquinone ring intermediate, menadione (MN) (also known as vitamin K_3), which undergoes geranylgeranylation to form menaquinone-4 (MK-4) (also known as vitamin K_2), which is the tissue-specific vitamin K form, which has an unsaturated side chain [17]. The enzyme, UbiA prenyltransferase domain-containing protein 1 (UBIAD1), mediates the conversion from PK to MK-4 [79].

Vitamin K follows a similar catabolic pathway as that of vitamin E. Both PK and MK-4 are catabolized to 5C- and 7C-aglycone, a process that is initiated with an ω-hydroxylation by CYP4F2 [29], followed by ω-oxidation and multiple β-oxidations to yield the 5C- and 7C-side chain catabolites [36]. The intermediate of vitamin K, MN, is also a urinary catabolite associated with vitamin K intakes [91].

Plasma concentrations of under-gamma carboxylated factor II (proteins-induced by vitamin K absence II, PIVKA II) have been used as a functional marker of vitamin K

status [15]. Vitamin E supplementation in humans increased PIVKA II [15]. Increased dietary vitamin E also decreased concentrations of both PK and MK-4 in the extra-hepatic tissues of rats [16]. To investigate the mechanisms for decreased extra-hepatic tissue PK and MK-4 concentrations in response to “excess” vitamin E, we fed rats diets containing exclusively PK or MN and gave subcutaneous α-T injections (100 mg/kg body weight). Irrespective of the dietary vitamin K form consumed, subcutaneous α-T administration resulted in increased hepatic α-T concentrations, as well as PK and MK-4 concentrations, significant reductions in extra-hepatic tissue MK-4 concentrations, and a reduction in pulmonary PK concentrations, but no detectable changes in the urinary vitamin K catabolites [101]. These data suggested that α-T did not alter vitamin K catabolism; however, neither biliary secretion of vitamin K nor its catabolites were determined. Previously, in studies using radiolabeled PK in humans conducted by Shearer et al [38], the major excretory route of PK and its catabolites was bile.

Membrane transporters, such as members of the ATP-binding cassette (ABC) family, may be involved in tissue regulation and excretion of both of these vitamins. Vitamin E up-regulates hepatic ABC transporters involved in the tissue efflux [45, 101]. Two of these transporters, multidrug resistance protein 1 (MDR1) and breast cancer resistance protein 1 (BCRP1), are ABC transporters and are highly expressed in many tissues. In the liver they are localized in the canalicular membrane of hepatocytes to aid in biliary excretion [49]. Both MDR1 and BCRP1 transporters are promiscuous with regard to their ligand recognition [102]. For example, BCRP1 reportedly transports MN, the vitamin K intermediate [48], as well as a sulfated metabolite of troglitazone, which contains α-T, as part of its structure [47].

Given that α-T injections into rats fed either the PK and MN-containing diets led to decreases in extrahepatic tissue MK-4 concentrations and that no changes in urinary

excretion of vitamin K catabolites were observed [101], we hypothesized that α-T injections in rats causes up-regulation of biliary secretion of both vitamin E and its metabolites, as well as vitamin K and its metabolites. To investigate this hypothesis, the biliary and urinary excretion of vitamins E and K and their catabolites was investigated in rats with cannulated bile ducts following subcutaneous α-T injections, according to our established protocols [43, 45, 46, 101].

Methods

Animal study design

The Oregon State University (OSU) Institutional Animal Care and Use Committee approved all procedures. Bile-duct cannulated male Sprague-Dawley rats (9 w old) were obtained commercially (Charles River, Wilmington, MA) with cannulas surgically placed in both the bile duct and duodenum. This method of double catheterization allows the bile to be diverted to a catheter, which is routed to the outside of the body, where it is connected to the duodenal catheter, which maintains flow to the duodenum except during the time the bile is diverted for collection. Bile-duct cannulated rats were received within 72 hours following surgery and housed individually in metabolic cages to allow urine collection. The rats were maintained at OSU on the same chow (Purina LabDiet 5L79), as was provided to the rats by the animal supplier (Charles River). Food and water were provided ad libitum and the rats were kept on a 12-hour light/dark schedule (8 AM-8 PM light/ 8 PM-8 AM dark). The diet used in this study contained adequate vitamin K (MN at 3.4 mg/kg diet and soy bean oil, a rich source of PK).

One-hr bile and 24-hr urine collections were obtained daily for the 8-days of the study. Bile sampling occurred at 9 AM for approximately 1 hr/day. Bile sampling entailed manually restraining the animal while a second technician collected the bile into a plastic tube using aseptic techniques. Following sampling, the catheter was flushed with sterile saline, and then replaced to allow enterohepatic circulation of biliary components.

Bile and urine samples were collected for two days (baseline sampling), and then subcutaneous injections of α-T (100 mg *RRR*-α-tocopherol/kg body weight, Emcelle, Stuart Products, Bedford, TX) were carried out daily for 7 days at 4 PM, 17 hrs before the next day's bile sampling. All rats received α-T injections; the baseline bile secretions

and urine collections served as the control samples. On day 8, bile and urine samples were collected, the rats were anesthetized using isoflurane and sacrificed at 10 AM, approximately 18 hours after the previous α-T injection. Blood and tissues were collected as described previously [101]

Due to the crystallization of bile salts in the cannulas, which occurs over time in bile-duct cannulated animals, the study was completed within 12 days of surgery, which is within the 15-day catheter patency recommended by the manufacturer. The catheterization of both the liver and duodenum allowed biliary secretions into the duodenum for absorption of fat and lipid-soluble nutrients by the intestine for 23 hrs/day, which included the dark period (8 PM-8 AM), when the rats are active and predominantly feeding. Because the surgical procedure involved inherent risk of cholestasis, the health of the rats and their bile flow was carefully monitored. One out of the five rats stopped secreting bile within 4 days of arrival, probably due to surgical complications, and was euthanized. A second rat died unexpectedly overnight with hemorrhaging after receiving all 7 α-T injections prior to final bile, urine, and tissue collections. Bile-duct cannulated rats are vulnerable to hemorrhage if bile secretion is inhibited and it is speculated to be caused by impaired vitamin K absorption in the intestines [103]. Previous studies have demonstrated that similar α-T injections in normal, non-surgically altered rats did not result in death [45, 101]. The data presented in this manuscript and the corresponding statistical analyses were carried out only on the 3 rats that successfully completed the study.

Vitamin E measurements

Plasma, tissue and bile α-T concentrations were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ECD), as previously described [70]. Briefly, tissue, bile or plasma was saponified with ethanolic KOH, extracted with hexane, the extract dried under nitrogen, resuspended in ethanol:methanol (1:1) and

injected on to a C18 column (Synergi Hydro-RP,150 mm × 4.6 mm I.D., 4 μm particle size, Phenomenex, Torrance, CA) fitted with a precolumn (AQ C18, 4 × 3 mm I.D. SecurityGuard, Phenomenex). α-T was detected electrochemically in oxidizing mode with 500 mV potential. Peak areas were integrated using Shimadzu Scientific 4.2 Class VP software package and α-T was quantitated using calibration standards prepared using authentic compounds.

Urinary and bile α-CMBHC and α-CEHC concentrations were determined by high-performance liquid chromatography with mass spectrometry (HPLC-MS), as previously described [82]. Briefly, urine or bile samples were added to a 10 ml screw-cap tube containing 0.8 ml Milli-Q® water and 0.5 mL 2% ascorbic acid solution. Samples were hydrolyzed by the addition of 100 mL enzyme solution (1 mg b-glucuronidase in 100 mL 100 mM sodium acetate buffer, pH 5.0). Following incubation (60 min at 37ºC), the samples were extracted with 4 mL diethyl ether and an aliquot of the ether fraction was collected and dried under nitrogen. The samples were resuspended in 1:1 (v/v) water:methanol with trolox, and analyzed using a liquid chromatography-mass spectrometer (LC-MS) with a 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™ RP-18 column (3.0x150 mm, 3.5 m particle; Waters) with a Symmetry-Shield™ Sentry™ RP-18 precolumn (3.9x20 mm, 3.5 m particle; Waters). Single-ion recording mass-to-charge ratio (*m/z*) data were obtained for α-CEHC (*m/z* 277.8), γ-CEHC (*m/z* 263.8) and the internal standard trolox (*m/z* 249.8). 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 trolox (internal standard) peak.

Cholesterol measurements

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

Vitamin K measurements

Plasma, bile, and tissue PK and MK-4 were extracted after addition of internal standard (d_4-phylloquinone (d_4-K_1), Buchem BV, The Netherlands) and analyzed using LC-MS, as described previously [101]. Typical retention times for MK-4, PK and d_4-K_1 were 8.0, 13.3 and 13.3 minutes respectively. Peak areas were integrated using Waters Masslynx version 4.0 software. PK and MK-4 concentrations were quantitated using calibration standards prepared using authentic compounds corrected for the amount of internal standard added. 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.

Bile and urine samples were shipped on dry ice by overnight courier to Nutristasis Unit, Centre for Haemostasis and Thrombosis, GSTS Pathology, St. Thomas' NHS Foundation Trust, London, United Kingdom for analysis. Biliary 5C- and 7C-aglycone vitamin K catabolites were determined according to a modified version of the method published previously [36]. Bile (300 µL) was added to a primed and washed solid phase extraction (SPE) cartridge (Isolute C18, 100 mg, 1 mL) followed by addition of the internal standard. The cartridge was washed with 1.0 mL deionised water, which was collected, the remaining material was eluted with methanol. The aqueous washings were placed on a second primed and washed SPE cartridge and again eluted with methanol. The methanolic extracts were combined and dried under nitrogen, with conjugates hydrolyzed overnight in methanolic HCl. The aglycone catabolites were then extracted in to chloroform and dried under nitrogen. The endogenously occurring

carboxylic acid forms of the catabolites were stabilized by conversion to their methyl ester derivatives using 1-methyl-3-nitro-1-nitrosoguanidine. Further purification was carried out using SPE cartridges (Waters SEP-PAK Silica) eluted with diethyl ether (15%) in hexane. Sample extracts were dried under nitrogen, reconstituted in methanol, and injected to the HPLC-ECD system. Isocratic reversed-phase chromatographic separation utilized a mobile phase consisting of a methanol–water sodium acetate buffer. The derivatized 5C and 7C aglycone catabolites and internal standard were firstly reduced using an upstream dual coulometric electrode (ESA 5011) set to −1.2 V and then oxidized at the downstream amperometric wall jet electrode (Antec VT-03) set at +0.3 V. Cells were operated by Coulochem II and Decade II controllers, respectively. Chromatograms were generated using the current generated from the wall jet electrode. Waters Empower was used for data capture. Quantification was carried out using a calibration curve of methanolic solutions containing the derivatized catabolites and internal standard. The additional step was validated by measurement of catabolites in a second collection of washings from the SPE cartridge in which no catabolites were detectable.

The urinary 5C- and 7C-aglycone vitamin K catabolites were determined according to previously published methods [36, 71] as described previously [101]. Quantification was carried out using a calibration curve of methanolic solutions containing the derivatized catabolites and internal standard.

Statistics

The statistical significance of the comparisons was analyzed using repeated measures ANOVA with Dunnett post-tests (Prism 5 statistical software, Graphpad Software, Inc) by comparing bile (concentration) or urine (total amount excreted per day) samples collected at baseline (0 injections) with samples collected after 1, 3, 5, or 7 α-T

injections. Data presented in log scale were transformed logarithmically prior to statistical evaluation. Significant ANOVA values are presented, then post-hoc tests are also reported.

To determine the major route of α-CEHC and 5C-aglycone excretion, the hourly rate excreted in bile and urine were compared. Biliary α-CEHC and 5C-aglycone secreted per hour were calculated by multiplying concentration (per ml) of respective catabolite in bile samples to bile secretion rate (ml/hr). Urinary excretion per hour was calculated by dividing the total excreted per day by 24h. This comparison makes the assumption that the rate of biliary excretion of catabolites was relatively constant so that the hour of bile sampling reflected average daily biliary secretion. Concentrations of α-CEHC and 5C-aglycone in bile were compared to urine using repeated measures two-way ANOVA and Bonferroni post-tests.

α-T concentrations in plasma, liver and kidney were similar to those previously described using our injection protocol [101], but no statistical comparisons were possible since no tissues were obtained from animals not injected with vitamin E. MK-4 concentrations were higher in liver and kidney than previously observed [101] because the estimated dietary intake of vitamin K was 10 times greater than previously administered.

Results

Rat body weights did not change significantly over the time of the study (**Figure 1A**). Plasma and tissue levels of vitamins E and K were examined after 7 days of α-T injections (**Table 1**). α-T concentrations in bile-duct cannulated rats were comparable to α-T concentrations observed in liver and plasma from previous studies following 7 days of α-T injections [101]. As for vitamin K concentrations in the bile-duct cannulated rats, PK, but not MK-4, was detected in the plasma. PK and MK-4, however, were both detectable in the kidney and liver at similar concentrations relative to each other.

Baseline 1 hr bile secretions and 24 hr urine samples collected before α-T injections (shown as 0 injections in all figures) served as the control condition. α-T injections did not change the rate of bile secreted (mL/h, **Figure 1B**); however, biliary cholesterol secretion increased marginally (ANOVA P=0.0398) after 3 injections of α-T ($p<0.05$) but was no longer different from baseline levels (0 injections) by the 5^{th} and 7^{th} daily injections (**Figure 1C**).

Vitamin E Excretion

α-CEHC was the major vitamin E catabolite excreted in the urine with concentrations approximately 60-fold higher than those of α-CMBHC. Maximal increases of both α-CEHC and α-CMBHC were observed after 3 daily α-T injections (ANOVA P=0004 and P=0.0092, respectively). The increases from baseline to peak concentrations were approximately 100- and 150-fold respectively (**Figure 2**). α-CEHC and α-CMBHC concentrations tended to decrease following the 5^{th} and 7^{th} daily α-T injections; however, urinary excretion of both catabolites after 7 injections remained significantly higher than observed at baseline.

Biliary concentrations of α-T and its major catabolites (α-CEHC and α-CMBHC) also increased in response to α-T injections (**Figure 3**). Biliary α-T concentrations increased linearly over the injection period and its secretion was increased 4-fold between 0 and 7 daily injections of α-T (ANOVA p= 0.0003). The α-T concentration increased, as estimated from the linear regression, by 0.49 µmol/L with every injection (Y = 0.4917X + 4.780, r^2=0.499, p<0.01) (**Figure 3A**). The peak biliary concentrations of α-CEHC and α-CMBHC increased (ANOVA P= 0.0002 and P= 0.0051, respectively) 150-fold and 15-fold respectively relative to baseline following 5 daily injections of α-T (**Figure 3B**). After 7 daily injections, biliary α-CEHC concentrations were approximately 10-fold and 40-fold greater than α-T and α-CMBHC respectively.

Vitamin K Excretion

Routes of excretion of vitamin K and its major aglycone catabolites (5C- and 7C-) were also investigated. Both the 5C- and 7C-aglycones were detectable in the urine but their concentrations were unchanged after 7 days of α-T injections compared to control (**Figure 4**), as was previously observed [101]. The biliary secretion of PK was also unchanged over time in response to the α-T injections (**Figure 5**). Although MK-4 was detected in the liver at concentrations similar to those of PK (**Table 1**), MK-4 was not detected in the bile. Biliary concentrations of 7C-aglycone were also undetectable in all but one of the rats, in which it was measured at 0.3-1 nmol/L (data not shown). In contrast, biliary 5C-aglycone concentrations increased over time (ANOVA P=0.0308); the 0 compared with 5 or 7 daily α-T injections were different by pairwise comparison (P<0.05) (**Figure 5**).

Major Routes of Excretion

To investigate the excretion routes of α-T and vitamin K catabolites, the rates of α-CEHC or 5C-aglycone excretion via bile were compared with the rates into urine (**Figure**

6). Bile was found to be the predominant α-CEHC excretory route with ~30 times more α-CEHC excreted per hour in bile compared with urine. 5C-Aglycone was excreted at pmol/h, not nmol/h as for α-CEHC. 5C-Aglycone was excreted into both the urine and bile; rates were not statistically different on any of the days. The sum of 5C-aglycone rates excreted in bile and urine were not different between baseline and any of the days during the week of α-T injections (ANOVA P=0.6141).

Discussion

The major route of α-T elimination in rats following subcutaneous α-T injections (100 mg/kg body weight, daily) was as α-CEHC. α-CEHC was excreted both via urine and bile, but biliary α-CEHC secretion predominated. Thus, the primary mechanism preventing excess hepatic α-T accumulation is mediated by α-T catabolism and biliary α-CEHC excretion. Urinary and biliary excretion of both α-CEHC and α-CMBHC reached a plateau between 3 and 5 daily injections, which were also associated with a continuous linear increase of biliary α-T secretion. The plateau in α-CEHC and α-CMBHC excretion in response to continued α-T administration indicates that the ability to catabolize α-T may have become overwhelmed, while the direct α-T excretion continued to increase. However, on day 7 the biliary α-CEHC concentration remained 10-fold greater than that of α-T.

We also explored whether increased excretion of vitamin K via bile may be the mechanism by which vitamin E is detrimental to vitamin K status. We previously demonstrated that extra-hepatic vitamin K levels were significantly reduced in rats after 7 days of α-T injections but that the reductions in vitamin K concentrations were not associated with increased urinary excretion of the 5C- and 7C-aglycone catabolites [101]. As reviewed by Shearer and Newman [72], studies conducted in the 1970s using radio-labeled PK in humans demonstrated that approximately 40% of water-soluble vitamin K catabolites were excreted via the bile versus 20% in the urine. Moreover, the biliary transporter BCRP1, which we previously demonstrated was increased by α-T injections in rats [101], can transport MN, the intermediate in the conversion of PK to MK-4 [48]. Thus, we hypothesized that increased secretion of vitamin K into the bile, facilitated by the increased expression of biliary transporters, could cause increased vitamin K losses in response to excess vitamin E. In support of this theory, we observed an increase in the biliary excretion of the 5C-aglycone vitamin K catabolite following 5

and 7 daily α-T injections with no changes in the urinary excretion of either the 5C- or 7C-aglycone catabolites.

Measurements of 5C-aglycone and 7C-aglycone may not account for all of the excreted compounds of vitamin K catabolites in bile or urine. MN, which is the precursor for MK-4 synthesis, was a major dietary component in the rat chow and is also a urinary vitamin K metabolite, and reportedly is correlated to vitamin K intakes [91]. It is also possible that the selected time for bile sampling may not have been optimal for measurement of vitamin K biliary catabolites.

Although hepatic MK-4 concentrations were similar to PK concentrations, only PK was detected in the bile. MK-4 is more rapidly cleared from circulation compared to PK [18] and it has been suggested that MK-4 may be more extensively catabolized relative to PK [72], which may explain why MK-4 was not detected in the bile.

Biliary secretion of cholesterol was increased after 3 days of α-T injections. Although exploring the mechanism of this increase in cholesterol excretion was outside the scope of this study, it has recently been reported that ∂-T stimulates lysosomal exocytosis to prevent cholesterol accumulation and increase cholesterol efflux in cells exhibiting defective intracellular cholesterol trafficking [104]. Although α-T was less potent relative to ∂-T [104], the pharmacological concentrations obtained in α-T-injected rats may have been sufficient to promote hydrolysis of cholesterol esters in lysosomes to liberate cholesterol for its efflux into the bile.

In conclusion, this study demonstrates that the primary route of α-T elimination in rats administered extraordinarily high α-T levels is α-T catabolism to α-CEHC, and its subsequent secretion into the bile. Although increased excretion of vitamin K was not

observed, there was an increase in biliary secretion of 5C-aglycone with continued α-T injections, supporting the hypothesis that pharmacological doses of α-T increases biliary vitamin K excretion, albeit as the catabolite.

Figure 4.1: Rat body weight, bile flow, and biliary cholesterol

Rat body weight (A) are shown at the end of the study. The rates of bile secretion (B) and biliary cholesterol concentrations (C) (mean ± SEM) are shown following 0, 1, 3, 5, and 7 days of α-T injections (100 mg/kg BW), which were given once a day. Bile secretions were collected 17 hrs post-injection for approximately 1 hr. Data was analyzed using repeated measures ANOVA with Dunnett post-tests by comparing samples collected at baseline (0 injections) with samples collected after 1, 3, 5, or 7 α-T injections. Daily biliary secretion rates were not significantly different over the study. Cholesterol *ANOVA (P=0.0424), comparisons to baseline are shown as *p<0.05 (N=3).*

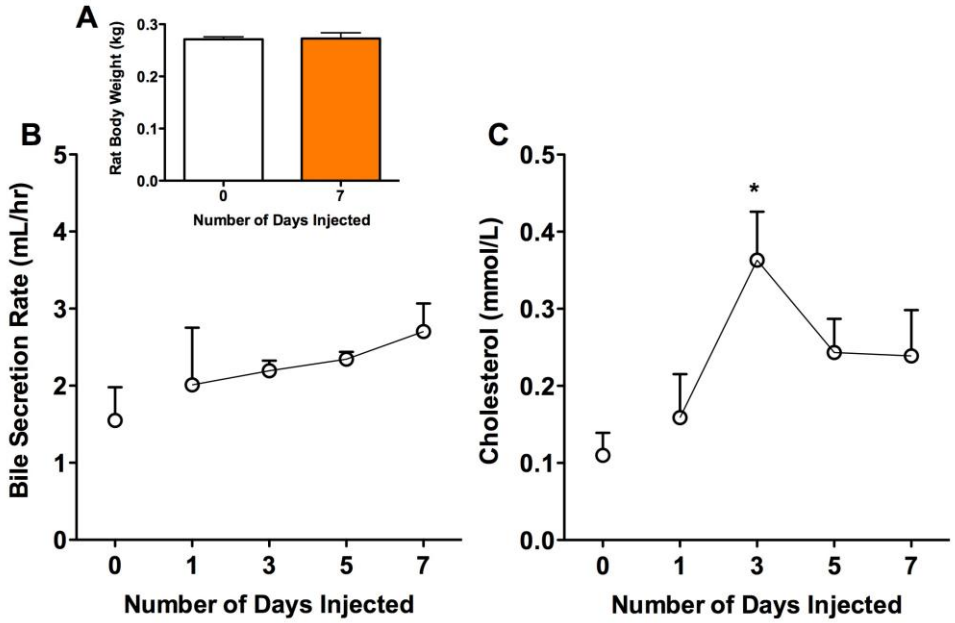


Figure 4.2: Urinary α-CEHC and α-CMBHC

Urinary excretion of α-T's major catabolites, α-CEHC and α-CMBHC (mean ± SEM), following 0, 1, 3, 5, and 7 days of α-T injections (100 mg/kg BW), which were given once daily. Catabolites were measured in 24 h urine collections. Data was analyzed using repeated measures ANOVA with Dunnett post-tests by comparing samples collected at baseline (0 injections) with samples collected after 1, 3, 5, or 7 α-T injections. α-CEHC ANOVA P=0.0001 and α-CMBHC P=0.0146; comparisons for each metabolite are with its respective baseline value: *p<0.05, **p<0.01, ***p<0.001 (N=3).

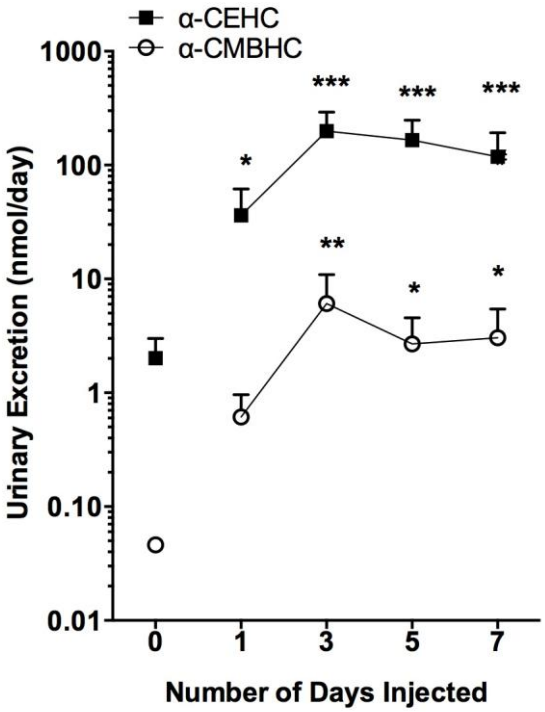


Figure 4.3: Biliary α-T, α-CEHC, and α-CMBHC

Biliary excretion (mean ± SEM) of α-T (**A**) and α-CEHC and α-CMBHC (**B**), following 0, 1, 3, 5, and 7 days of α-T injections (100 mg/kg BW). Bile secretions were collected 17 hrs post-injection for approximately 1 hr. Data was analyzed using repeated measures ANOVA with Dunnett post-tests by comparing samples collected at baseline (0 injections) with samples collected after 1, 3, 5, or 7 daily α-T injections. α-T ANOVA P=0.0003, α-CEHC ANOVA P=0.0002 and α-CMBHC P=0.0051; comparisons for each metabolite are with its respective baseline value: *p<0.05, **p<0.01, ***p<0.001 (N=3).

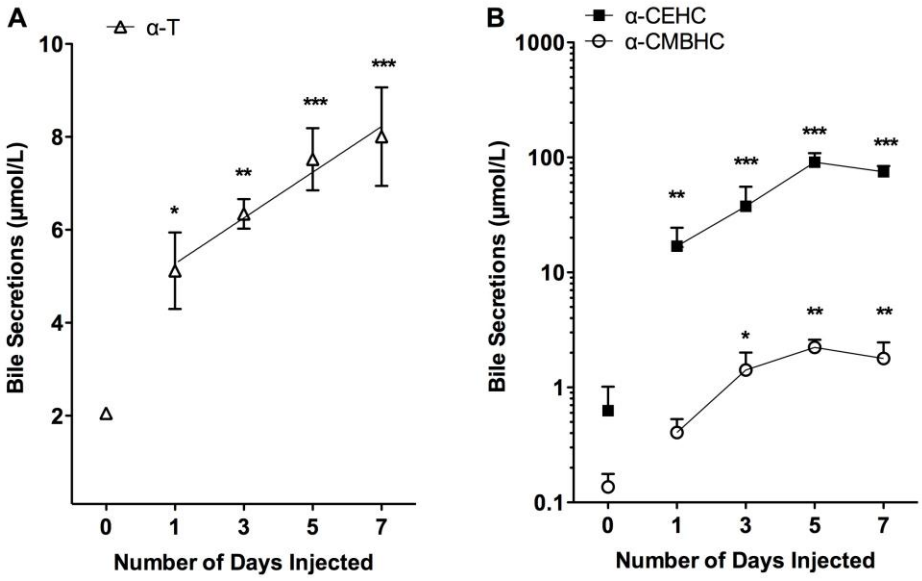


Figure 4.4: Urinary 5C- and 7C-aglycone

Urinary excretion of 5C- and 7C-aglycones (mean ± SEM) following 0, 1, 3, 5, and 7 days of α-T injections (100 mg/kg BW). Catabolites were measured in 24h urine collections. Data was analyzed using repeated measures ANOVA There were no statistically significant differences compared to baseline for either catabolite. (N=3).

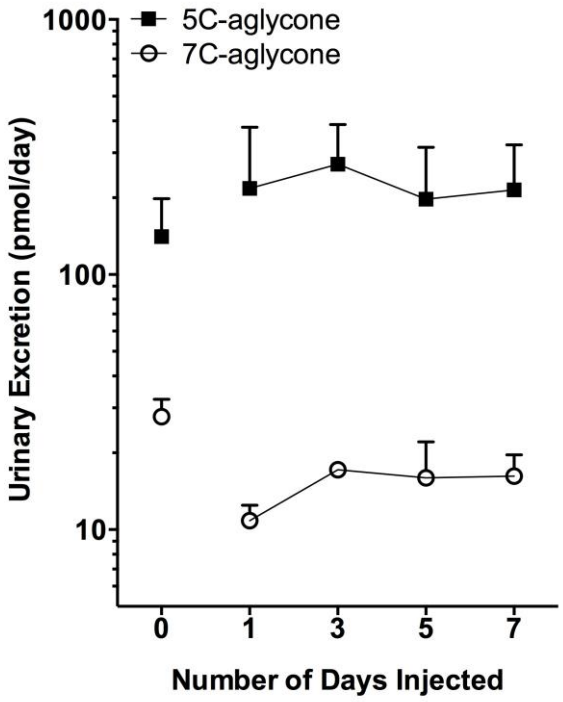


Figure 4.5: Biliary vitamin K

Biliary secretion of vitamin K and 5C-aglycone (mean ± SEM) following 0, 1, 3, 5, and 7 days of α-T injections (100 mg/kg BW). Bile secretions were collected for approximately 1 hr at 17 hrs post-injection. Biliary MK-4 was not detected in bile secretions and 7C-aglycone was detected in only 1 of the rats. Data was analyzed using repeated measures ANOVA with Dunnett post-tests by comparing samples collected at baseline (0 injections) with samples collected after 1, 3, 5, or 7 α-T injections. There were no statistically significant differences in PK concentrations compared to baseline. ANOVA for 5C-aglycone P=0.0477, differences compared to baseline *p<0.05 (N=3).

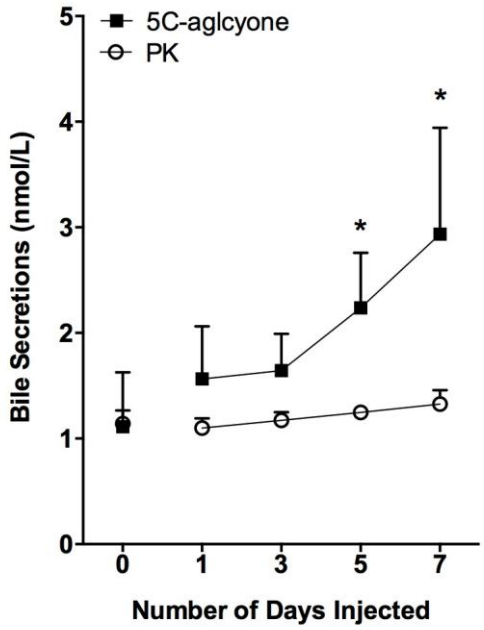


Figure 4.6: α-CEHC and 5C-aglycone in urine and bile

Major excretory routes of α-T and vitamin K were determined by comparing biliary excretion per hour of α-CEHC (**A**) or 5C-aglycone (**B**) to urinary excretion per hour. Concentrations of α-CEHC and 5C-aglycone in bile were compared to urine using repeated measures two-way ANOVA and Bonferroni post-tests. Data is represented as mean ± SEM, Along the lines the comparisons are between baseline and each point for each route of excretion *p<0.05, **p<0.01, ***p<0.001; the values along the x-axis compare the excretion routes each day. Two-way ANOVA for α-CEHC results: main effect of injections p<0.0001, main effect of excretion route, p=0.0457; for 5C-aglycone results: NS for all comparisons.

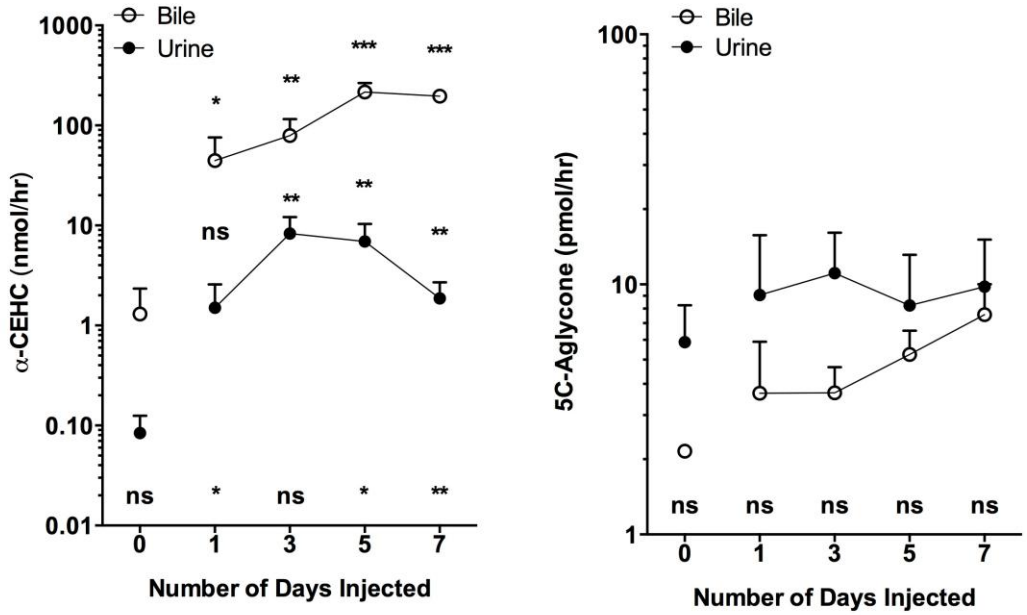


Table 4.1: Plasma, liver and kidney vitamin E and K concentrations after seven daily α-T injections (100 mg/kg BW)

	Vitamin Concentrations (Mean ± SEM)		
	α-T μmol/L or nmol/g	**PK** nmol/L or pmol/g	**MK-4** pmol/g
Plasma	95 ± 14	1.2 ± 0.2	ND
Liver	558 ± 35	11.8 ± 3.5	14.0 ± 1.5
Kidney	68 ± 9	8.7 ± 2.5	13.3 ± 3.2

Samples were collected 17 hr following last injection (N=3). MK-4 was not detected (ND) in the plasma.

Chapter 5:

Deuterium-labeled Phylloquinone Fed to Vitamin E-Injected Rats Demonstrates that α-Tocopherol Decreases Extra-Hepatic Phylloquinone and Menaquinone-4

Sherry M. Farley, Scott W. Leonard, Jay Kirkwood, and Maret G. Traber

Abstract

Following subcutaneous α-T injections (100 mg/kg body weight, BW) in rats for one week, MK-4 concentrations in extra-hepatic tissues are decreased and liver concentrations of PK and MK-4 are increased. To investigate the mechanism for the depleted extra-hepatic tissue concentrations, rats were fed deuterium-labeled (d_4)-PK for 17 days total and starting on day 10, rats were injected with saline, vehicle, or α-T (100 mg/kg BW) for 7 days. On day 8, after an overnight fast, rats were killed and plasma and tissues (liver, brain, lung, kidney, and heart) were collecteded. Brain, lung, and kidney were significantly decreased in total vitamin K by at least 50% in rats injected with α-T compared to saline and vehicle-injected rats. d_4-PK, PK, and d_4-MK-4 were detected in all tissues but unlabeled MK-4 was detected only in brain. Significant reductions in total vitamin K in tissues were accounted for by significant decreases in both d_4-PK and d_4-MK-4. Heart and liver preferentially accumulate PK, by 80-95%, over MK-4 and demonstrated no differences in total vitamin K content as labeled or unlabeled PK or MK-4. High concentrations of α-Ts major catabolite, α-carboxy ethyl hydroxy chroman (α-CEHC) was detected in tissues of α-T-injected rats and it was confirmed that the circulating form of α-CEHC was sulfated. mRNA expression of xenobiotic transporters were changed significantly in liver but many of these changes were not observed in extra-hepatic tissues. These data confirm the deleterious affects of α-T on vitamin K status and the conserved total MK-4 to total vitamin K ratio in tissues that were demonstrated decreased total vitamin K suggests PK, and not MN, is the major substrate for MK-4 synthesis in those tissues. Modified expression of transporters only in liver suggest that extra-hepatic loss of vitamin K is likely a result of reduced PK transport or uptake.

Introduction

Vitamin K's only known function is as a cofactor for γ-glutamyl carboxylase (GGCX), which is important for the γ-carboxylation of vitamin K-dependent proteins. Although dietary vitamin K, phylloquinone (PK), can perform the cofactor function, it is converted to a tissue-specific vitamin K form, menaquinone-4 (MK-4), which can also function as a GGCX cofactor [17, 79]. Synthesis of MK-4 occurs by the cleavage of PK's saturated side chain by an unknown mechanism, to release the intermediate menadione, followed by geranylgeranylation, catalyzed by the UBAID1 enzyme [79], to produce MK-4. It is unclear why synthesis of MK-4 is necessary since both PK and MK-4 can act as the cofactor for GGCX; thus it is theorized that MK-4 may have a different function in tissues [23, 105]. Alternatively, the unsaturated tail of MK-4 hypothetically makes MK-4 more fluid than PK in membranes and may increase vitamin K availability to GGCX. Regardless, vitamin K-dependent proteins chelate calcium and are involved in a number of functions, including coagulation, prevention of vascular calcification, and bone mineralization [20, 106, 107].

Vitamin E supplementation adversely impacts vitamin K status but the mechanism for this interaction is not known. Although there are eight vitamin E forms, α-tocopherol (α-T) is the form preferentially accumulated in human tissues, as a result of the α-tocopherol transport protein (α-TTP), and is recognized as the vitamin E form necessary for meeting the recommended intakes (15 mg or 22 I.U. 2*R*-α-T) [4]. However, setting the upper tolerable limit of vitamin E intakes to 1000 mg, or 1100 I.U. per day was necessary based on studies demonstrating bleeding abnormalities in animals [7, 108, 109], which could be prevented with vitamin K supplementation [7-9]. There is evidence that vitamin E supplementation may also be beneficial in reducing clot formation. The Women's Health Study demonstrated decreased risk of venous thromboembolism in women supplemented every other day for 10 years with 600 IU of vitamin E compared to placebo [11]. However in other studies, vitamin E at 1000 IU per day for 12 weeks reduced a biomarker of vitamin K status in humans, PIVKA-II (protein induced by vitamin K absence or antagonism-factor II) [15], and reduced vitamin K concentrations were observed in extra-hepatic tissues of rats supplemented with vitamin E [16]. Determining how vitamin E and K interact will provide insight into long-term consequences of supplementation with vitamin E on health outcomes related to vitamin K.

We previously hypothesized that “excess” vitamin E may antagonize vitamin K status by increasing metabolism and excretion of both PK and MK-4; or that α-T may be interfering with the conversion of PK to MK-4 to specifically target MK-4 concentrations. We demonstrated that following 1 week of α-T injections (100 mg/kg) in rats, MK-4 concentrations were reduced by approximately 50% in the extra-hepatic tissues, brain, lung, kidney, and heart; lung PK concentrations were also reduced [101]. The lower extra-hepatic vitamin K concentrations were not associated with increased urinary excretion of vitamin K catabolites; moreover expression of cytochrome P450 enzymes that initiate catabolism of vitamin K were *decreased*. In a separate experiment, we found increased biliary secretion of the vitamin K catabolite, 5C-aglcyone, in the α-T-injected rats (Chapter 4). These latter findings support the hypothesis that vitamin E may be stimulating vitamin K biliary excretion, which could ultimately decrease vitamin K status.

We hypothesized that vitamin E increases removal of vitamin K from extra-hepatic tissues or limits its uptake, leading to increased vitamin K delivery to the liver and its metabolism and excretion. To investigate whether “excess” vitamin E antagonizes transport or uptake of vitamin K in extra-hepatic tissues and whether vitamin E targets MK-4 concentrations by inhibiting MK-4 synthesis in tissues, in the present study rats were fed deuterium (d)-labeled-PK, d_4-PK, to allow analysis of vitamin K status at steady state and in response to vitamin E injections. Hepatic xenobiotic transporters, which previously demonstrated significant modified expression in liver in response to α-T, were also examined in extra-hepatic tissues to determine whether they are involved in facilitating vitamin K loss.

Methods

Animal study design

The Oregon State University (OSU) Institutional Animal Care and Use Committee approved all procedures. Male Sprague-Dawley rats (Charles River, 250-300 g) were housed in plastic cages with hard wood chips, kept on a 12-hour light/dark schedule. Upon arrival, rats were fed a defined diet that contained vitamin E (60 IU *all-rac*-α-tocopheryl acetate/ kg) with the source of vitamin K as d_4-PK (Vitamin K1-ring-D4, Buchem BV, Apeldoorn, The Netherlands) at 2 µmol/kg diet (TD.120038**,** Harlan Teklad, Madison, WI) and water ad libitum. After 10 days, rats received daily for 7 days subcutaneous (sq) injections of saline (control), of 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). Twenty-four hours after the last injection (including a 12-hour fast), rats were anesthetized by isofluorane and blood and tissues were collected as previously described [101] and stored at -80ºC until analysis. Additionally, during tissue collection, aliquots of tissues were stored in RNA*later* (Ambion, Austin, TX) for measurement of xenobiotic enzyme and transporter gene expression.

Vitamin E and α-CEHC Measurements

Plasma and tissue α-T concentrations were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ECD), as described previously [70].

Urinary and tissue α-CEHC concentrations were determined by high-performance liquid chromatography with mass spectrometry (HPLC-MS) as described previously [82].

Vitamin K Measurements

Plasma and tissue d_4-PK, PK, d_4-MK-4, and MK-4 were extracted and determined with HPLC was coupled to a Micromass ZQ 2000 single-quadrupole mass spectrometer

(Manchester, UK) with an atmospheric pressure chemical ionization (APCI) source operating in negative mode as previously described [101] and using MK-7 as the internal standard. For vitamin K analysis, 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), and MK-4 (*m/z* 444), and the internal standard, MK-7 (*m/z* 648).

Identification of sulfated α-CEHC

One volume plasma was extracted with 4 volumes ice-cold 50:50 v:v MeOH:EtOH, vortexed 4 seconds, then centrifuged 13 minutes at 15,000 x g. The supernatant was analyzed by LC-MS/MS as previously described [110] with a minor modification, the column temperature was held at 50°C instead of 70°C. Mass error, isotope distribution error, and molecular formula predictions were performed by PeakView software (AB SCIEX). Sulfated-CEHC was identified by mass error (< 5 ppm), isotope distribution error (< 10%) and MS/MS spectra. All fragments had a mass error < 20 ppm.

qRT-PCR

Quantitative real time RT-PCR (qPCR) was performed using SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA) and the 7900HT Real Time PCR System (Applied Biosystems) as described previously [101]. Relative mRNA expression for the following genes were examined: 1) cytochrome P450 (CYP) 4F2 (CYP4F4), 2) CYP4F1, 3) ATP-binding cassette, subfamily G, member 2 (Abcg2, alias BCRP1), 4) solute carrier organic anion transporter family, member 1a4 (Slco1a4, alias OATP2), and 5) ATP-binding cassette, subfamily B member 1B (Abcb1b, alias MDR1) as described previously [101]. All genes were run in triplicate; the average transcript expression for each gene of interest was determined for each rat and normalized to the average transcript expression of the housekeeping gene, GAPDH.

Statistical Analyses

Statistical analyses were performed using Prism 5 statistical software (Graphpad Software, Inc.). Data were analyzed by one-way or two (for time courses) ANOVA and Bonferroni posttests were performed when overall group effects were significant or by

using student t-test where indicated. When necessary, data were log-transformed to equalize variances. Data are reported as means ± SEM (n=5).

Results

Plasma and Tissue Vitamin K

We previously reported reduced concentrations of MK-4 in extra-hepatic tissues and increased liver concentrations of PK and MK-4 following injections of α-T [101]. Using the same α-T injected rat model and the same concentration of PK in the diet, rats were fed d_4-PK for 17 days total and starting on day 10, rats were injected with saline, vehicle, or α-T (100 mg/kg BW) for 7 days to investigate tissue concentrations and forms of labeled and unlabeled vitamin K in response to α-T injections. On day 8, after an overnight fast and 18 days after starting the d_4-PK rat diet, plasma and tissues (liver, brain, lung, kidney, and heart) were collected and vitamin K concentrations measured. An interaction with regard to injection and weight of rats was observed, with significant differences in vehicle and α-T-injected rats comparing weight before injections started (day 0) and following their last injection on day 8 (**Figure 5.1**). As seen previously, α-T was significantly increased following 7 days of α-T injections in liver and plasma [101] (**Figure 5.2**). In contrast, no changes in α-T concentrations were observed in brain after 7 days of α-T injections compared with saline and vehicle-injected rats.

Plasma was found to only contain d_4-PK, not other forms of vitamin K and no unlabeled vitamin K. d_4-PK concentrations were not statistically different between groups (saline 0.4 ± 0.1, vehicle 0.3 ± 0.1, vitamin E 0.2 ± 0.1 nmol/L).

Brain is a unique tissue because it contains very high MK-4 concentrations [17]. Total vitamin K was significantly reduced in brain in α-T-injected rats compared to saline and vehicle-injected (**Figure 5.3**). The decrease in total vitamin K in brain was accounted for by decreased d_4-PK, PK, and d_4-MK-4 concentrations. Brain was the only tissue that had measurable concentrations of unlabeled MK-4, which were also decreased significantly in α-T rats compared to vehicle but not saline-injected rats (ANOVA p=0.0228). 90% of the brain vitamin K was MK-4 (sum of d_4-MK-4 and unlabeled MK-4) (**Figure 5.3E**). The ratio of MK-4 relative to total vitamin K was not changed in response to α-T. When the ratio of d_4-MK-4 to total deuterated vitamin K in the brain was calculated, there were no

differences between treatments. Thus, approximately 90% of the d_4-PK reaching the brain was converted to d_4-MK-4.

Lung contained 75% less vitamin K in response to α-T; this finding was a result of decreased d_4-PK, PK, and d_4-MK-4 concentrations (**Figure 5.4**). MK-4 constituted about 45% of lung total vitamin K. When the ratio of d_4-MK-4 to total deuterated vitamin K in the lung was calculated, there were no differences between vehicle and vitamin E treatments. Thus, in the vitamin E group approximately 58% of total d_4-vitamin K in the lung was converted to d_4-MK-4.

Kidney also demonstrated a greater than 60% decrease of total vitamin K in response to α-T, which was a result of significantly decreased d_4-PK and d_4-MK-4 concentrations (**Figure 5.5A, B, D**). Kidney had comparable concentrations of PK and MK-4, and similar to lung and brain, did not change ratio of MK-4 relative to total vitamin K in response to α-T (**Figure 5.5F**). When the ratio of d_4-MK-4 to total deuterated vitamin K in the kidney was calculated, there were no differences between treatments. Thus, approximately 50% of the total d_4-vitamin K in the kidney was converted to d_4-MK-4.

Heart displayed characteristics of vitamin K status unlike the other extra-hepatic tissues (**Figure 5.6**). Heart, in direct contrast to the brain, preferentially accumulated PK (85-95%) over MK-4 and total vitamin K was slightly elevated in response to α-T. Although differences in total vitamin K concentrations did not reach statistical significance, these findings demonstrate that total vitamin K, as well as d_4-PK and d_4-MK-4 concentrations, were not compromised in the heart by the vitamin E injections. Moreover, somewhat higher unlabeled PK concentrations accounted for the small elevation observed in total vitamin K in α-T-injected rats; thus, the increase in PK was associated with decreased d_4-MK-4 relative to total vitamin K (**Figure 5.6F)**. In contrast to the other tissues discussed, when the ratio of d_4-MK-4 to total deuterated vitamin K in the heart was calculated, vitamin E injections significantly decreased the fraction of total d_4-vitamin K in the heart that was converted to d_4-MK-4 to about 6% compared to 15% in the control injected tissues (**Figure 5.6E**).

Liver was similar to heart in that it did not demonstrate reduced vitamin K status in response to α-T (**Figure 5.7**). The liver also preferentially accumulated PK, ~80% relative to MK-4, moreover there were no significant changes in d_4-MK-4 relative to total deuterated vitamin K.

Plasma and Tissue Vitamin E Metabolites

α-CEHC is the terminal catabolite of α-T and is a major excretion product significantly increased in urine [101] and bile (unpublished data) following α-T administration. α-CEHC was 10 fold and 30 fold higher in the plasma and liver, respectively, after α-T injections compared with saline and vehicle controls (**Figure 5.8**). Moreover, α-CEHC was detected in the brain and kidney only in rats injected with α-T.

Conjugation of α-CEHC with either glucuronide or sulfate increases water-solubility to facilitate excretion; and both conjugated forms have been detected in urine [99]. To identify the conjugated form of circulating α-CEHC, a metabolic profiling experiment was carried out using the rat plasma. Sulfated-α-CEHC, and not glucuronidated-CEHC, was identified in plasma and was 7 fold greater in α-T compared with vehicle-injected rats (**Figure 5.9D**). Although quantitation was not carried out in this experiment, the relationship observed was similar to that shown in Figure 5.8.

Tissue Gene Expression of Transporters

To assess whether tissues had modified expression of transporters and enzymes that regulate the export, uptake, and metabolism of multiple endogenous and exogenous compounds, hepatic and extra-hepatic mRNA expression of transporters, MDR1, BCRP1, OATP, and enzymes, CYP4F4 and CYP4F1, in the liver, kidney, brain, and heart were examined. To confirm that molecules present in the Emcelle E emulsion, which were necessary to solubilize α-T, and present in both vehicle and α-T subcutaneous injections, saline-injection served as a control for the vehicle. As we observed previously [101], α-T injections resulted in significantly altered expression of all genes tested in the liver compared to vehicle and saline-injected controls, which were not different from each other (Figure 5.10). The gene for the uptake transporter, OATP, which is expressed on the basolateral membranes of cells, was significantly decreased in liver and brain in response to α-T injections (Figure 5.10A). The genes for the efflux

transporters, MDR1 and BCRP1, which are expressed on the apical membrane of cells, and in the liver facilitate transport of compounds into the bile for excretion, were significantly increased in rats receiving α-T injections (Figure 5.10B,C). Kidney also demonstrated a significant increase in expression of the gene for MDR1 in response to α-T injections. Expression of the genes for the enzymes, CYP4F1 and CYP4F2, were significantly reduced in the liver after 7 days of α-T injections; their gene expression did not change in other tissues. The heart did not show change in expression in any of the genes for the transporters or enzymes examined following α-T injections (data not shown).

Discussion

One week of subcutaneous injections of α-T in rats significantly decreased total vitamin K concentrations in brain by 56%, lung by 77%, and kidney by 68%. Loss in total vitamin K was accounted for by reductions in both PK and MK-4 in all three tissues. Although in a previous study [101], PK was only statistically reduced in lung in response to α-T, a trend of decreased PK was observed in brain and lung in those rats as well. Total MK-4 relative to total vitamin K was unchanged in tissues in response to injections, demonstrating that MK-4 concentrations were correlated with PK concentrations, regardless of whether vitamin K decreased. This suggests MK-4 concentrations in extra-hepatic tissues are dependent upon PK concentrations, and not MN as was previously proposed [101], for synthesis of MK-4. Inability to detect MN in plasma, which was pursued, also indicated MN is not likely a major source for MK-4 synthesis, when the diet only contains PK.

Xenobiotic transporters were modified in the liver in response to α-T injections as previously reported [45, 101], and may be involved in increasing excretion of vitamin E and K metabolites via the bile. The vitamin E analogue, troglitazone, conjugated to sulfate, is a substrate for the efflux transporter BCRP1 [47] and the uptake transporter OATP [111], and expression of these transporters were up-and down-regulated, respectively, in the liver of α-T injected rats. It is likely that OATP expression was decreased in order to slow uptake of sulfated-CEHC, measured in the plasma, into the liver where its biliary excretion was stimulated by increased expression of biliary transporters, BCRP1 and MDR1, which have overlapping substrates [95]. MDR1 was also increased in the kidney, which would facilitate excretion of α-CEHC in the urine. Although we have not observed increased excretion of 5C- and 7C-aglcyone in the urine [101], we have observed increased excretion of 5C-aglcyone in the bile following α-T injections (Farley et al, unpublished observations). Moreover, the intermediate MN, which is the napthoquinone head group shared by all vitamin K forms and metabolites, is also a substrate for BCRP1 [48]. MN and 5C-aglycone are both metabolites of vitamin K [7, 8]; thus, increased excretion of either would result in loss of both PK and MK-4 and this could ultimately reduce vitamin K status.

In contrast to liver transporter expression, no major differences were found in their expression in extra-hepatic tissues in response to α-T, which suggests it is not likely they were responsible for the dramatic reductions in vitamin K observed in those tissues. It is also improbable that PK transport or receptor-mediated uptake into tissues was inhibited by α-T since dietary MN resulted in reduced concentrations of MK-4 as well [101]. Thus, transport mechanisms may not be the source of vitamin E-mediated tissue-specific loss of vitamin K. However, increased vitamin K metabolism and excreted metabolites, or lack of recycling cannot be ruled out.

Reductions in both PK and MK-4 concentrations in lung, kidney, and brain may support evidence of inhibited vitamin K recycling in response to α-T. Based on the assumption MK-4 synthesis is dependent upon relative PK availability, then losses in PK could be accounted for by its efficient conversion to MK-4, which would accumulate as the MK-4 epoxide if recycling was inhibited. Alternatively, recycling of both PK and MK-4 could be inhibited. Heart and liver, in contrast, did not demonstrate any loss of total vitamin K. Although vitamin E may promote vitamin K recycling [112], there is evidence that vitamin E metabolites may antagonize vitamin K recycling. Previously, vitamin E, as a 6-carbon α-T metabolite, and the PPAR-activator, troglitazone, which contains an α-T moiety in its structure, inhibited the reduction of menadione by dicoumarol-sensitive NADPH:quinone oxidoreductase, whereas α-T in the same concentrations enhanced its activity by 45% [113]. The vitamin E metabolite, CEHC was measured at concentrations 20 fold greater than vitamin K in extra-hepatic tissues, which may detrimental in tissues that do not typically accumulate this form, such as brain, where MK-4 concentrations were approximately 10 pmol/g (Figure 5.8) and α-CEHC concentrations were 200 pmol/g (Figure 5.3). The observed down-regulation of CYP4F enzymes in liver that would initiate catabolism of vitamin E may be the body's way of limiting production of α-T metabolites.

Heart MK-4 relative to total vitamin K was also significantly changed with respect to α-T indicating that unlike tissues that demonstrated vitamin K loss, heart retained more PK than was converted to MK-4. Heart and liver are unique in that they have proportionately greater PK relative to MK-4 compared to all other tissues [21, 114]. Perhaps the greater supply of PK available for synthesis of MK-4 prevented the observation of reduced levels

of MK-4 that would have resulted from inhibited recycling; although it is difficult to reconcile this with decreased conversion of PK to MK-4 unless MK-4 synthesis was also disrupted. The liver did not have any loss of PK or MK-4 in response to α-T. The liver appears to be less at risk for limited vitamin K in general, which may be explained by greater supply of vitamin K not available in extra-hepatic tissues because the liver is involved in the ultimate disposition of the body's vitamin K [26].

In conclusion, vitamin E does not appear to be reducing vitamin K concentrations by interfering with the conversion of PK to MK-4 in extra-hepatic tissues because the fraction of the labeled PK converted was similar in all groups. The decreased fractional conversion of PK to MK-4 in the heart suggests that interference in synthesis is also possible. It should be noted that in the brain, where the most dramatic synthesis and accumulation of MK-4 takes place, there was no differences in α-T concentrations, but α-CEHC was markedly increased to over 200 pmol/g from non-detectable. High concentrations of the vitamin E metabolite α-CEHC could be detrimental to vitamin K recycling by VKOR; however, this has not been demonstrated in vivo and would require further testing.

Figure 5.1: Rat Body Weights

Rats were weighed before starting injections (day 0) and during the injection period. Data was analyzed with two-way ANOVA and bonferroni posttests were performed when overall group effects were significant, **p<0.01. Data is represented as means ± SEM, N=5.

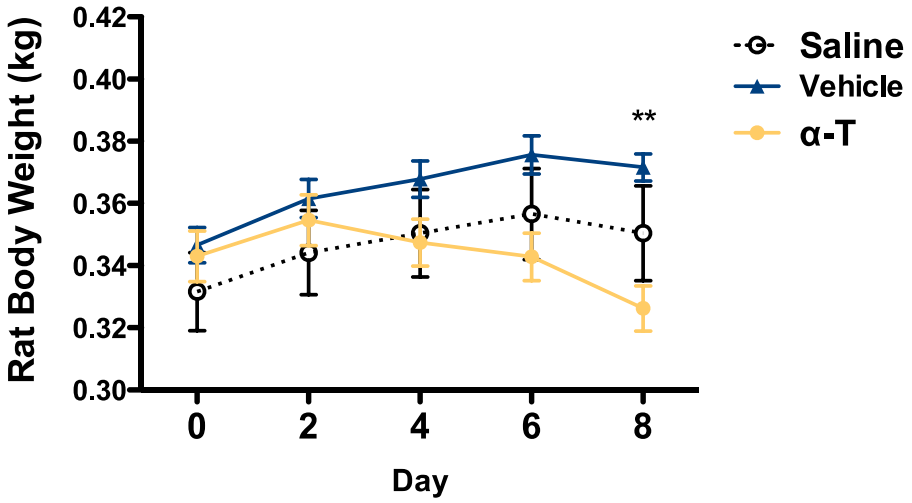


Figure 5.2: α-T in Plasma and Tissue

α-T measured in plasma, liver, and brain following 1 week of injection with saline, vehicle, or α-T and compared by one-way ANOVA; p-value is reported and bonferroni posttests were performed when overall group effects were significant. Columns with a different letter indicate significant differences between treatments within respective tissue, $p<0.05$. Data is represented as means ± SEM, N=5.

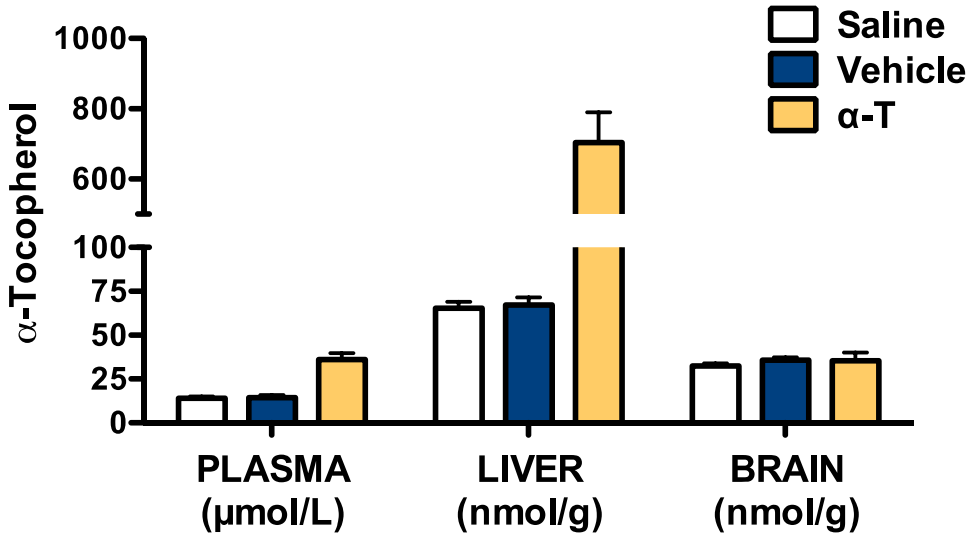


Figure 5.3: Vitamin K in Brain

Labeled and unlabeled vitamin K were measured in brain tissues following 1 week of injection with saline, vehicle, or α-T. Total vitamin K (**A**), d_4-PK (**B**), PK (**C**), d_4-MK-4 (**D**), and MK-4 concentrations were measured and analyzed for differences in vitamin K concentrations between the injection treatments. d_4-MK-4 relative to d_4-vitamin K (**D**) and total MK-4 (sum of d_4-MK-4 and unlabeled MK-4) relative to total vitamin K (**E**) were also examined. Data were analyzed with one-way ANOVA; p-value is reported and bonferroni posttests were performed when overall group effects were significant. Columns with a different letter, within each panel, indicate significant differences between treatments, $p<0.05$. Data is represented as means ± SEM, N=5.

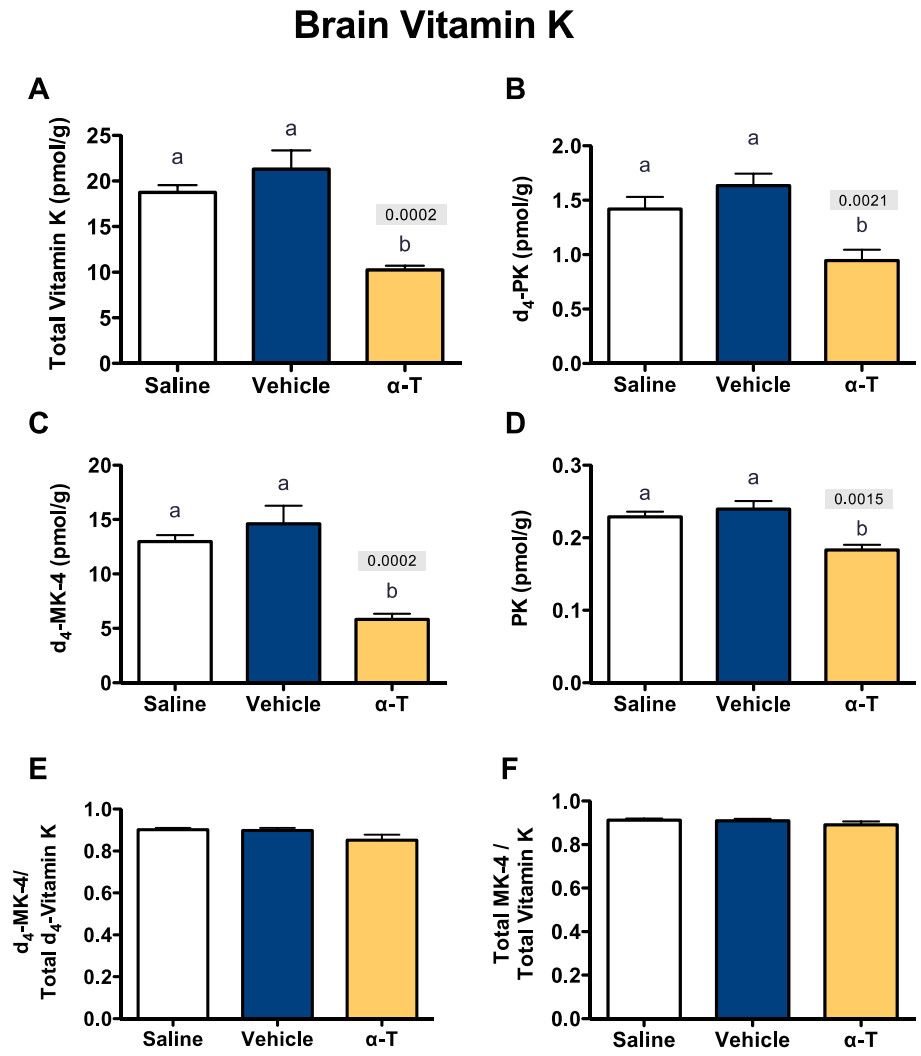


Figure 5.4: Vitamin K in Lung

Labeled and unlabeled vitamin K were measured in lung tissues following 1 week of injection with saline, vehicle, or α-T. Total vitamin K (**A**), d_4-PK (**B**), PK (**C**), and d_4-MK-4 (**D**) concentrations were measured and analyzed for differences in vitamin K concentrations between the injection treatments (saline, vehicle, and α-T). d_4-MK-4 relative to d_4-vitamin K (**D**) and d_4-MK-4 relative to total vitamin K (**E**) were also examined. Data were analyzed with one-way ANOVA; p value is reported and bonferroni posttests were performed when overall group effects were significant. Columns with a different letter, within each panel, indicate significant differences between treatments, $p<0.05$. Data is represented as means ± SEM, N=5.

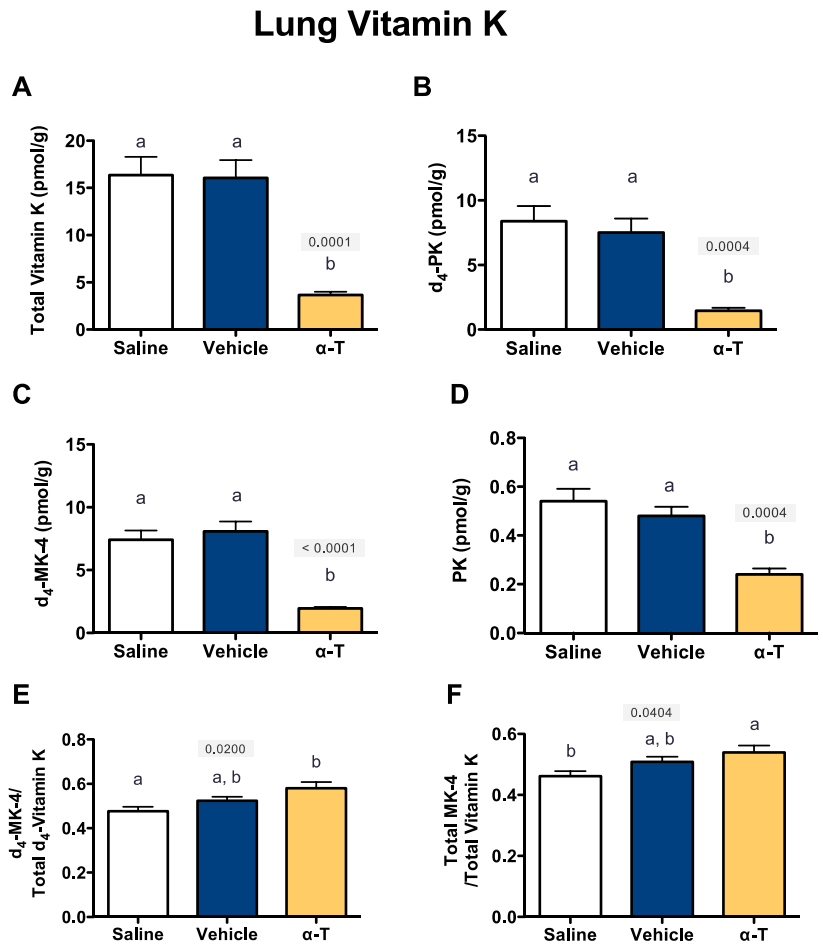


Figure 5.5: Vitamin K in Kidney

Labeled and unlabeled vitamin K were measured in kidney tissues following 1 week of injection with saline, vehicle, or α-T. Total vitamin K (**A**), d_4-PK (**B**), PK (**C**), and d_4-MK-4 (**D**) concentrations were measured and analyzed for differences in vitamin K concentrations between the injection treatments (saline, vehicle, and α-T). d_4-MK-4 relative to d_4-vitamin K (**D**) and d_4-MK-4 relative to total vitamin K (**E**) were also examined. Data were analyzed with one-way ANOVA; p-value is reported and bonferroni posttests were performed when overall group effects were significant. Columns with a different letter, within each panel, indicate significant differences between treatments, $p<0.05$. Data is represented as means ± SEM, N=5.

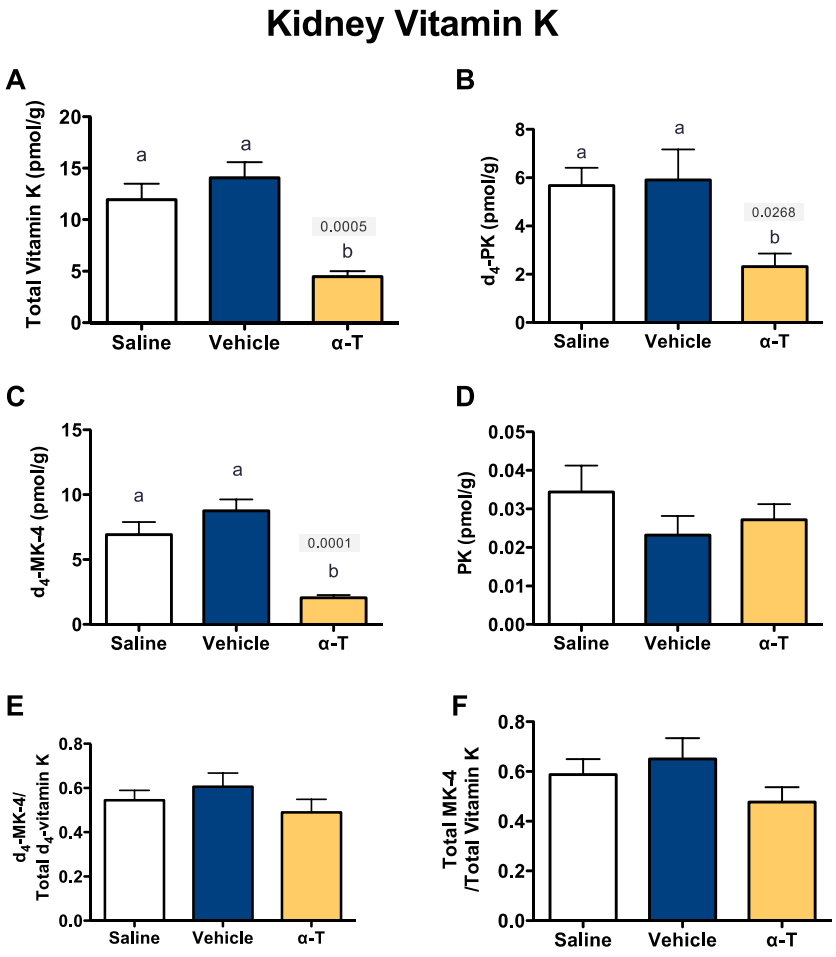


Figure 5.6: Vitamin K in heart

Labeled and unlabeled vitamin K were measured in heart tissues following 1 week of injection of saline, vehicle, or α-T. Total vitamin K (**A**), d_4-PK (**B**), PK (**C**), and d_4-MK-4 (**D**) concentrations were measured and analyzed for differences in vitamin K concentrations between the injection treatments (saline, vehicle, and α-T). d_4-MK-4 relative to d_4-vitamin K (**D**) and d_4-MK-4 relative to total vitamin K (**E**) were also examined. Data were analyzed with one-way ANOVA; p-value is reported and bonferroni posttests were performed when overall group effects were significant. Columns with a different letter, within each panel, indicate significant differences between treatments, $p<0.05$. Data is represented as means ± SEM, N=5.

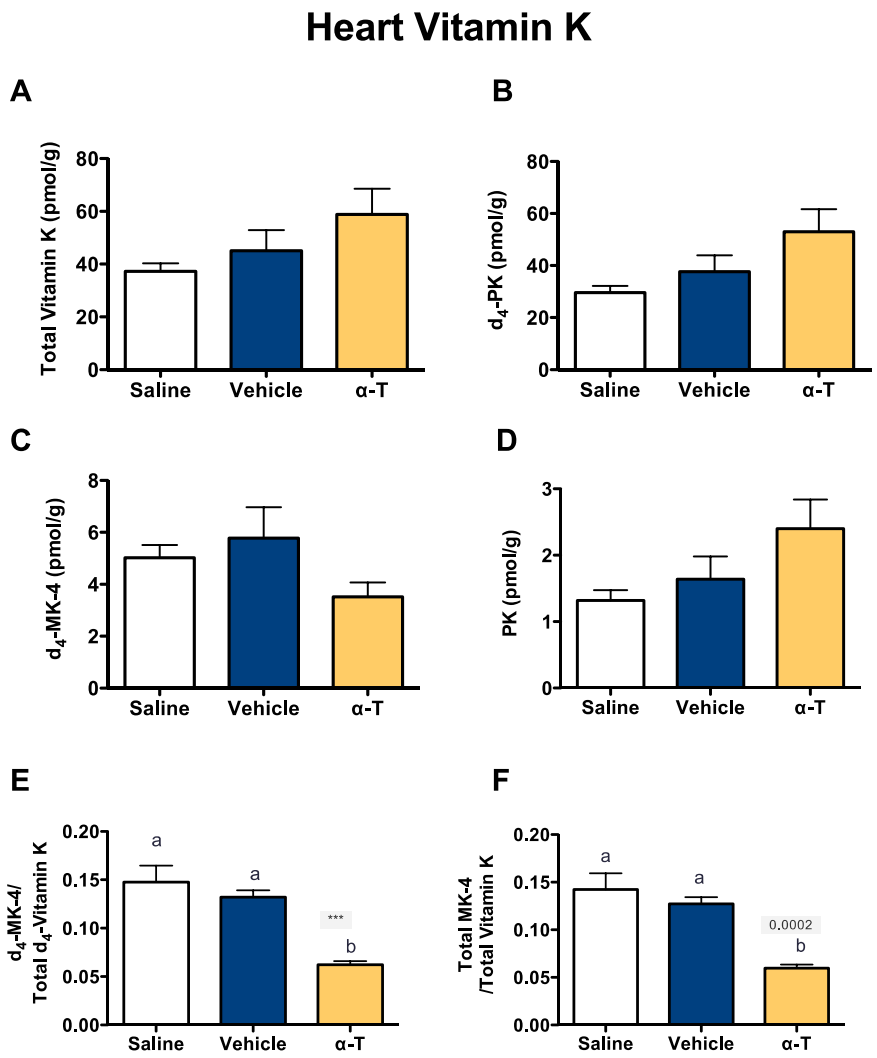


Figure 5.7: Vitamin K in liver

Labeled and unlabeled vitamin K were measured in liver tissues following 1 week of injection with saline, vehicle, or α-T. Total vitamin K (**A**), d_4-PK (**B**), PK (**C**), and d_4-MK-4 (**D**) concentrations were measured and analyzed for differences in vitamin K concentrations between the injection treatments (saline, vehicle, and α-T). d_4-MK-4 relative to d_4-vitamin K (**D**) and d_4-MK-4 relative to total vitamin K (**E**) were also examined. Data were analyzed with one-way ANOVA; p-value is reported and bonferroni posttests were performed when overall group effects were significant. Columns with a different letter, within each panel, indicate significant differences between treatments, $p<0.05$. Data is represented as means ± SEM, N=5.

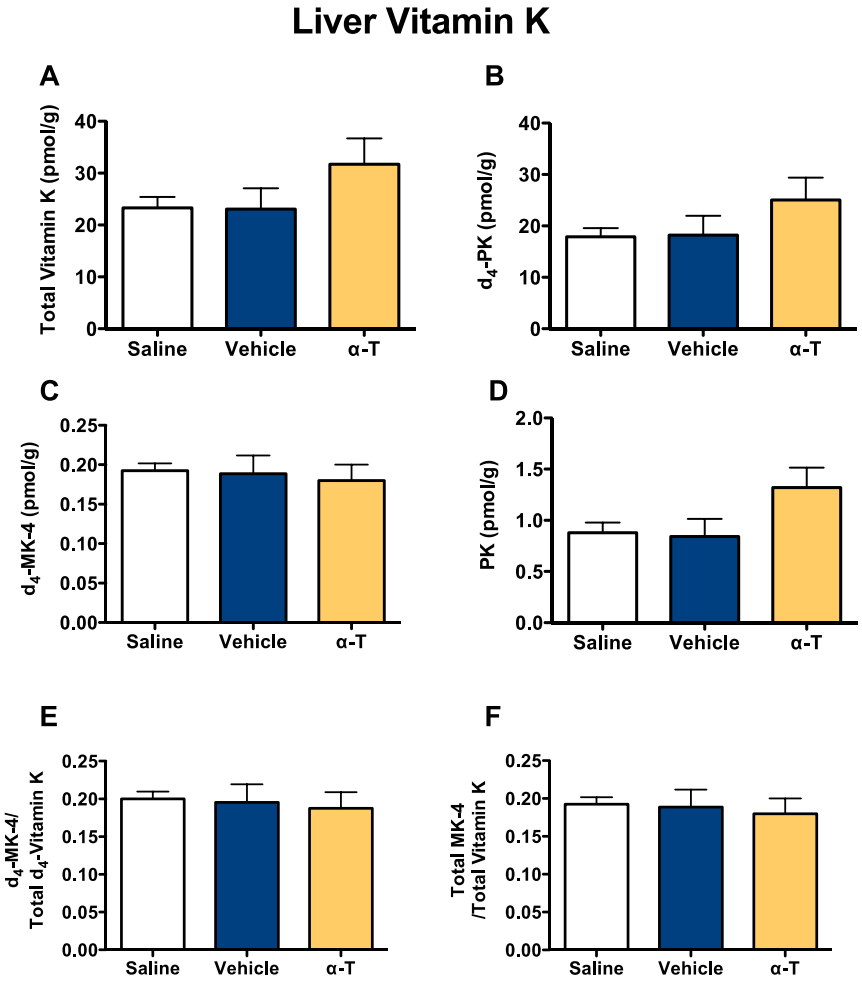


Figure 5.8: α-CEHC in Plasma and Tissues

α-CEHC were measured in liver, brain, kidney and plasma following 1 week of injection with saline, vehicle, or α-T and compared by one-way ANOVA; p-value is reported and bonferroni posttests were performed when overall group effects were significant. Columns with a different letter indicate significant differences between treatments within respective tissue, p<0.05. Data is represented as means ± SEM, N=5.

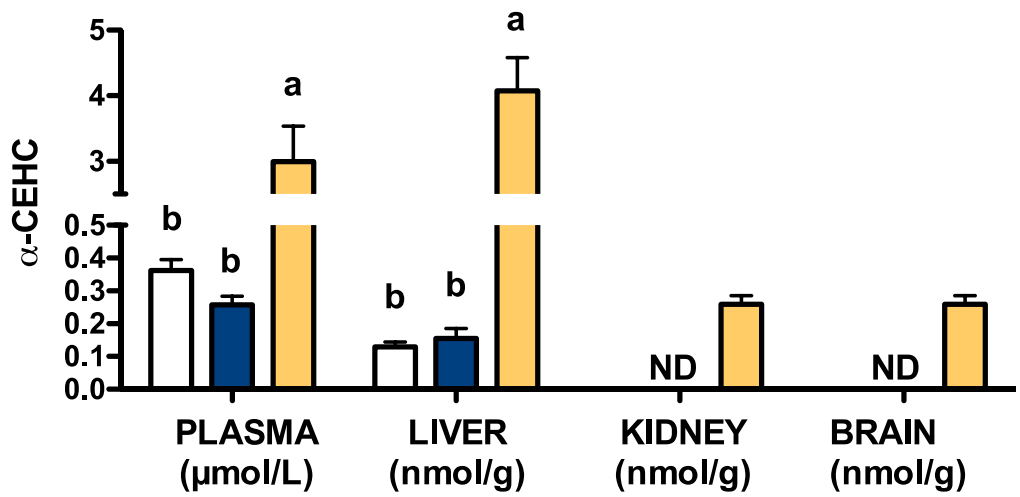


Figure 5.9: Sulfated-CEHC

Identification and quantitation of sulfated-α-CEHC in plasma (**A**) extracted ion chromatogram (XIC) of sulfated-α-CEHC [M-H] ion (**B**) total ion chromatogram (TIC) with theoretical formula based on mass and isotope distribution (**C**) MS/MS spectra of sulfated-α-CEHC (**D**) in vehicle and α-T-injected rats and fold change over vehicle was analyzed by student's t-test. Data is represented as means ±SEM, N=5.

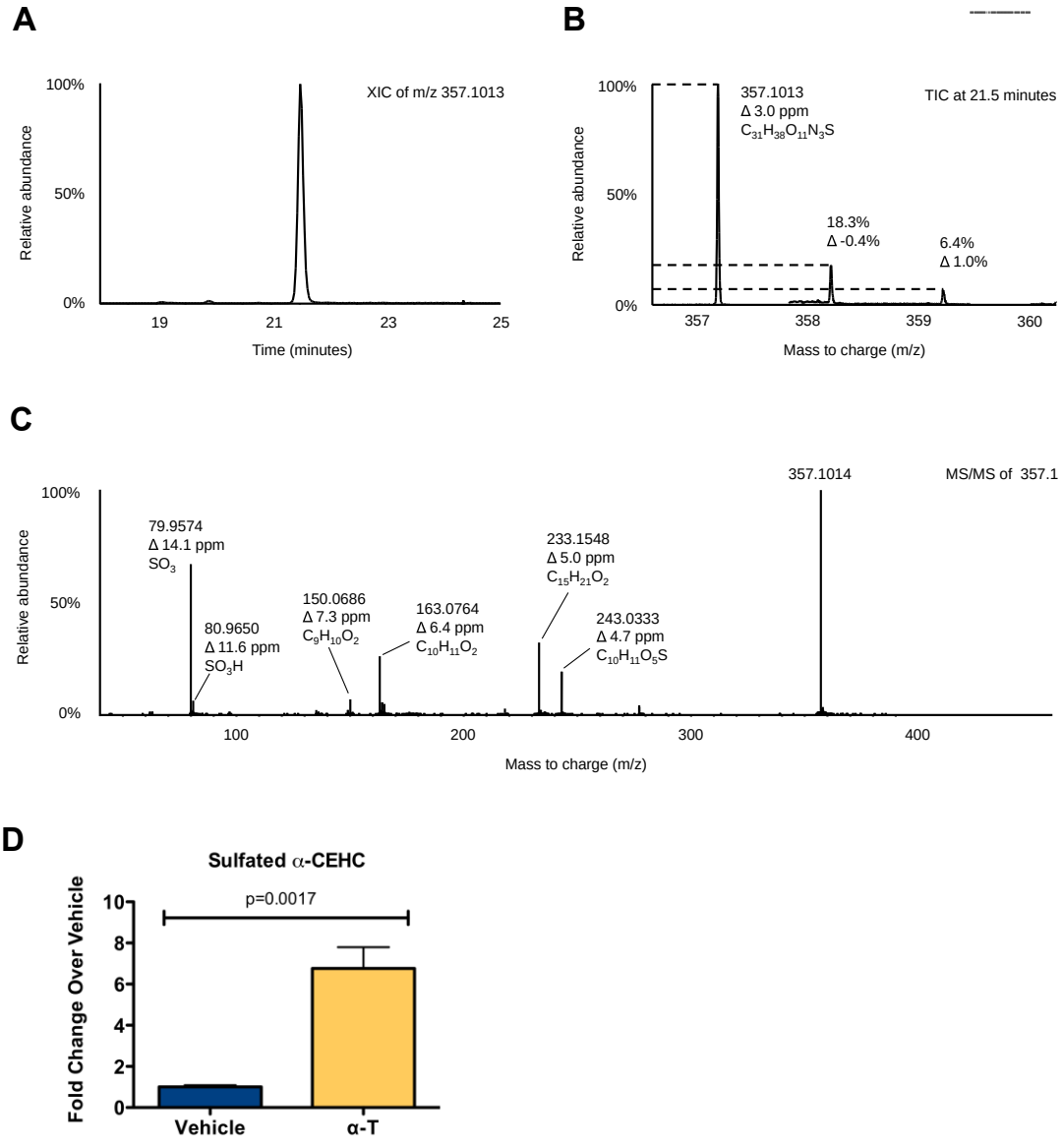
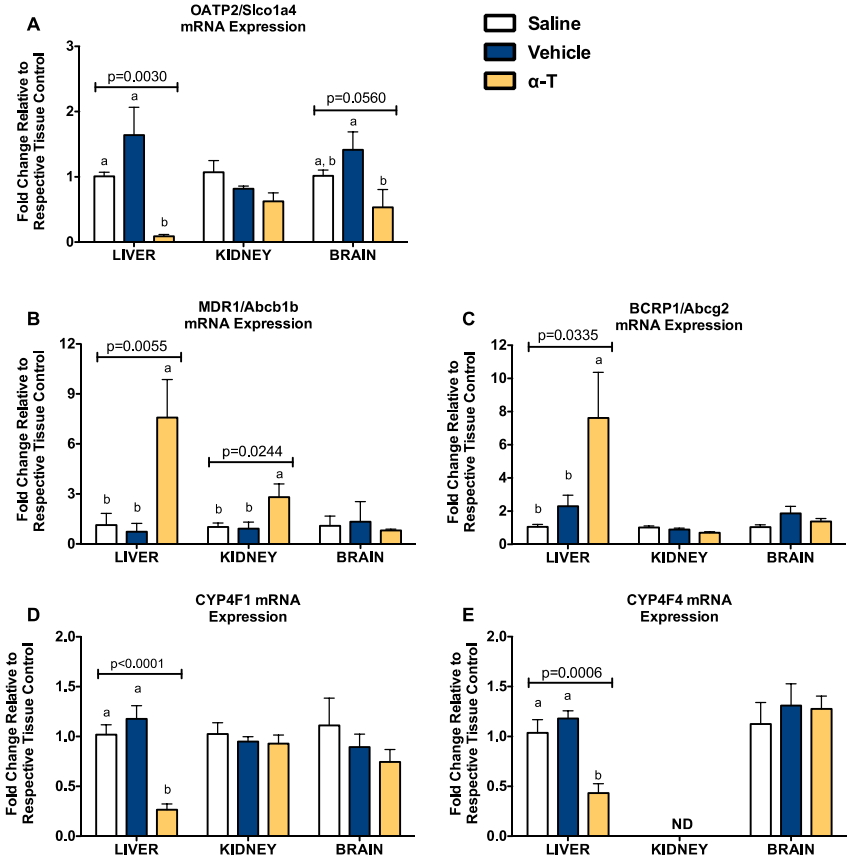


Figure 5.10: mRNA Expression of Xenobiotic Genes

mRNA expression of (A) OATP2 (B) MDR1, (C) BCRP1 (D) CYP4F1, and (E) CYP4F4 were measured following 1 week of injection with saline, vehicle, or α-T and expressed as fold change over control, which were the saline-injected measurements. The differences in the mRNA expression within respective tissues was analyzed by one-way ANOVA; p-value is reported and bonferroni posttests were performed when overall group effects were significant. Columns in each panel not bearing the same letter within respective tissue are significantly different, $p<0.05$ (N=5).



Chapter 6: Conclusion

Mechanism of Reduced Vitamin K Status in Response to Vitamin E

I investigated the mechanism of reduced vitamin K status in response to “excess” vitamin E administration. Previous studies demonstrated that subcutaneous injections of α-T (100 mg/kg BW) in rats resulted in modified expression of xenobiotic pathways, which stimulated vitamin E excretion and metabolism [45, 46, 101]. Thus, initially we hypothesized that metabolic pathways altered in response to excess vitamin E would also potentiate vitamin K loss from tissues. Because the exact mechanism of PK conversion to MK-4 is not entirely understood, we alternatively proposed that a metabolic pathway involved in MK-4 synthesis may also be disrupted in response to α-T. Therefore, conversion of MK-4 from menadione (MN) and PK and the status of MK-4 relative to total vitamin K in response to α-T was also studied.

I found that α-T did not increase the initial step of vitamin K metabolism in vitro. Both vitamin E and vitamin K are ω-hydroxylated by human CYP4F2, which is the rate-limiting initial step in their catabolism [29, 30]. Results from my in vitro experiments demonstrated that PK was hydroxylated by human CYP4F2 in greater amounts than were any of the vitamin E forms tested. Although there is evidence that α-T increased CYP4F2-mediated metabolism of other vitamin E forms [62], α-T does not stimulate CYP4F2-mediated metabolism of vitamin K in vitro. Thus, I have disproven the first hypothesis by showing that CYP4F2-mediated hydroxylation was not increased, or even modified by α-T.

To test the hypothesis that increased metabolism of vitamin K occurs in response to vitamin E in vivo, the terminal vitamin K catabolites, 5C-aglycone and 7C-aglycone, were measured in both bile and urine of rats injected with α-T. Neither vitamin K catabolite increased in the urine in response to α-T injections in rats [101]. Additionally, decreased hepatic expression of cytochrome P450 CYP4Fs and CYP3A were observed after 7 days of α-T injections [101], which is further evidence of a lack of induction of vitamin K metabolism in response to increased hepatic α-T concentrations. The reduced expression of these P450s, however, was surprising considering catabolism is the preferred mechanism of α-T elimination (Chapter 3). It is possible that the decreased expression of P450s is instead a reflection of negative feedback regulation in response

to elevated α-CEHC concentrations to avoid over-accumulation of vitamin E metabolites. Excretion of vitamin K was also examined in the bile. Increased biliary 5C-aglycone concentrations were observed in response to α-T injections, but these were also associated with reduced urinary excretion of 5C-aglycone (Chapter 3). Taken together there is little evidence that increased hepatic α-T concentrations increased vitamin K excretion or metabolism. However, I did not measure vitamin K epoxide metabolites in the urine or bile, and these may have increased if vitamin E, especially elevated α-CEHC concentrations, interfere with vitamin K recycling.

Expression of xenobiotic transporters and clearance of α-T and its metabolites from plasma and tissues of rats were previously studied by the Traber lab [45]. These findings suggested that increased excretion of both vitamin K and its metabolites in bile might be a mechanism by which vitamin E injections were decreasing vitamin K status. The increased biliary 5C-aglycone concentrations in rats injected with α-T supports the hypothesis that “excess” vitamin E may be reducing vitamin K status by stimulating excretion via the bile. The biliary transporter, BCRP1, has also been shown to transport MN. MN and 5C-aglcyone are both metabolites of vitamin K [36, 72]. The increased hepatic expression of BCRP1 in response to vitamin E that was observed in all of my studies may have resulted in loss of MN via the bile, in addition to the increased loss of 5C-aglycone. Increased excretion of either would result in loss of both PK and MK-4 and may ultimately result in reduced vitamin K status following α-T injections. However, the expression of xenobiotic transporters was unchanged in extra-hepatic tissues that demonstrated significant loss in vitamin K, which makes their involvement in vitamin K uptake or removal unlikely.

Hypothetically, a conjugated-MN could be a transport form of vitamin K in the circulation that is delivered as a precursor for MK-4 synthesis in extra-hepatic tissues. Our inability to measure MN, which was extensively pursued, was a major limitation in the evaluation of vitamin K transport and excretion. However, this lack of evidence of circulating concentrations of any MN conjugates supports the hypothesis that tissues use some other form of vitamin K for MK-4 synthesis. Moreover, transporters hypothesized to facilitate MN uptake or efflux were altered in some tissues in α-T injected rats, but these transporters were not altered in all tissues where MK-4 concentrations decreased.

Based on this evidence, it appears likely that MN conjugates are not the plasma transport form of vitamin K.

Vitamin K, as PK and MK-4, were decreased in tissues of rats fed MN or PK [101]; thus, it seems unlikely that the mechanism is specific to transport or uptake of PK or MN. Vitamin E, however, may lead to redistribution of vitamin K from extra-hepatic tissues to the liver where it is excreted as 5C-aglycone, but it is unclear whether this would account for all of the vitamin K loss observed in extra-hepatic tissues. However, only two major vitamin K catabolites were measured; thus it is possible that uncharacterized or undetectable vitamin K metabolites, such as MN or vitamin K epoxides, were present in urine and bile that may also account for the loss of vitamin K in extra-hepatic tissues. Alternatively, it is possible that a metabolite of vitamin E inhibits vitamin K recycling. α-CEHC, for example, was detected in extra-hepatic tissues, including brain, at concentrations 20 fold higher than vitamin K; however, there is no evidence CEHC inhibits vitamin K recycling in vivo. Therefore, the mechanism of vitamin E's antagonism of vitamin K status requires further exploration.

Concluding Remarks

In conclusion, although vitamin E supplementation is a potential therapy providing benefit to some disease states, e.g. thrombosis, its clear antagonism of vitamin K status may put other vitamin-K-dependent processes at risk. It will be important to investigate whether the function of vitamin K-dependent proteins expressed in extra-hepatic tissues are compromised in response to vitamin E as well as the consequences of long-term vitamin E supplementation on their respective functions. These studies will be critical for determining relative intake of both vitamins that will prevent antagonism of vitamin K status and benefit overall health.

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