Published in final edited form as:

Mol Nutr Food Res. 2013 October ; 57(10): . doi:10.1002/mnfr.201200797.

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

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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 - α -T (d_2 - α -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 - α -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 - α -T. CYP4F2 demonstrated similar apparent maximal velocities (V_{max}) when either of the α -Ts were used as substrates, which were less than the apparent d_4 -PK V_{max} ($p < 0.0002$), while the CYP4F2 catalytic efficiency towards d_4 -PK ($15.8 V_{max}/K_m$) was 5-times greater than for α -Ts. Vitamin K had no effect on vitamin E catabolism, while vitamin E slightly decreased the d_4 -PK V_{max} .

Conclusions—CYP4F2 discriminates between Ts and PK in vitro, but α -T does not apparently increase PK ω -hydroxylation by this mechanism.

Keywords

5C- and 7C-aglycones; CEHC; CYP4F2; phylloquinone; tocopherol

Introduction

Human cytochrome P450 4F2 (CYP4F2) is an endoplasmic reticulum enzyme that catalyzes the ω -hydroxylation of various endogenous compounds [1]. CYP4F2 efficiently ω -hydroxylates vitamin E, which is the first step in its catabolism to the excreted water-soluble metabolite, carboxy ethyl hydroxy chroman (CEHC) [2]. Bardowell et al. [3] demonstrated that disrupting expression of the mouse CYP4F2 ortholog, CYP4F14, decreased vitamin E

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There were no conflicts of interest.

metabolism. Other endogenous CYP4F2 substrates include leukotriene B₄ (LTB₄) and long chain fatty acids, such as arachidonic acid [1]. CYP4F2 is also the major vitamin K hydroxylase [4]. Similar to vitamin E catabolism [5,6], vitamin K's side chain is ω -hydroxylated, followed by multiple rounds of ω -oxidation to yield the vitamin K major urinary metabolites, 5C- and 7C-aglycones (Figure 1) [7]. Importantly, CYP4F2 polymorphisms are determinants of warfarin dose in humans emphasizing the critical role this enzyme plays in vitamin K status [8–10]. A common CYP4F2 variant, V433M (rs2108622), was reported to have reduced ability to metabolize vitamin E [11] and vitamin K [4] and was associated with elevated hepatic vitamin K [4] and serum α -T concentrations [12] in those carrying the allele. Thus, CYP4F2 is 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); while enhanced hepatic metabolism promotes the excretion of other vitamin E forms, such as γ -T [13,14] or tocotrienols [15]. The naturally occurring α -T form, *RRR*- α -T, has three chiral centers at positions 2, 4, and 8 in the side chain. Chemical synthesis of α -T produces a racemic mixture (*RRR*, *RSR*, *RRS*, *RSS*, *SRR*, *SSR*, *SRS*, and *SSS*). In humans, the forms with the *R* configuration at the 2 position (2*R*- α -T) are preferentially retained, as reviewed [16], while the all racemic forms are excreted [17].

Hepatic metabolism of the different vitamin E compounds by CYP4F2 plays a critical role in determination of extrahepatic concentrations of the vitamin E forms. Sontag and Parker [2,18] 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 the side-chain stereochemistry, as assessed using *all-rac*- α -T relative to *RRR*- α -T, was not important [2,18]. 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.

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 required for a number of functions [19]. Vitamin K is most notably needed for γ -carboxylation of glutamic acids in a number of proteins in the coagulation cascade; therefore, it is generally agreed that vitamin E and K interactions are the cause of bleeding observed with vitamin E supplements [20]. The mechanism for the vitamin E and K interactions is unknown, but may involve CYP4F2. Sontag and Parker [18] showed that α -T *increases* the ω -hydroxylation of other vitamin E forms. It is now well established that addition of a wide variety of chemicals to P450 metabolic reactions can accelerate oxidative metabolism *in vitro*, and that this phenomenon of heterotropic cooperativity extends across numerous drug metabolizing P450 enzymes, particularly CYP3A4 and CYP2C9 [21]. Interestingly, CYP4F2 appears to be subject to homotropic cooperativity with regards to vitamin E metabolism, as evidenced by the atypical kinetics reported for tocopherol and tocotrienol catabolism [18]. Therefore, we have considered the potential for vitamin Es to heterotropically activate vitamin K catabolism by CYP4F2. 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 hydroxylation 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 [22,23] 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 the typically low tissue vitamin K concentrations relative to vitamin E concentrations precluded

measuring hepatic tissue vitamin K hydroxylation in the tissue slices. Therefore, the hydroxylation of the different vitamin E forms along with vitamin K, as phyloquinone (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*- δ -5,7-(C²H₃)₂ tocopheryl acetate (d₆-T-Ac) was a gift from Dr. James Clark of Cognis Nutrition and Health. δ -3,4-(²H) tocopheryl acetate (d₂-T-Ac) was a gift from Dr. Jeffrey Atkinson, Brock University (ON, Canada). Unlabeled synthetic 2*S*-T (2*S*-T), a racemic mixture of *SRR*-, *SRS*-, *SSR*-, and *SSS*-T, was a gift from Dr. Brent Flickinger (Archer Daniels Midland, Decatur, IL). 2*S*,4*R*,8*R*- δ -5,7-(C²H₃)₂ tocopheryl acetate (d₃-*SRR*-T-Ac) was a gift from DSM Nutritional Products (Dr. Thomas Netcher, Basel, Switzerland). Phyloquinone (ring-D4, d₄-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-T used as a standard has been described previously [24].

Prior to use in any incubation system, the various T-Acs 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 [25]. Only unesterified Ts were used in the described experiments.

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). 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 rodent chow diet (Harlan Teklad 8604, which contained added *all rac*-T-Ac, 120 IU/kg diet, and menadione, 40 mg/kg diet, as menadione sodium bisulfite complex) 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 CaCl₂·H₂O and 25 mM NaHCO₃. The liver was immediately cored using a Tissue Coring Press and sectioned into 200–300 μ m-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 NaCO₃, 50 μ g/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% O₂/5% CO₂ for a 1 hour recovery period then incubated in vitamin E supplemented medium (in triplicate with d₆-T, d₂-T, unlabeled synthetic 2*S*-T, or ethanol-treated medium) and were harvested after 24 hours.

To prepare the vitamin E supplemented medium, deuterium-labeled d₆-T, d₂-T, or unlabeled synthetic 2*S*-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 μ M Vitamin E (< 0.5% ethanol).

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.

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, and 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

Liver slices were abandoned in preference to the use of Insect microsomes expressing recombinant human CYP4F2 (S-CYP4F2) (CYP4F2 Supersomes, Gentest BD Biosciences, Woburn, MA) because the slices did not yield sufficient material to measure vitamin K metabolites. $\text{d}_3\text{-SRR-}^{-}\text{T}$ was used instead of $2\text{S-}^{-}\text{T}$ because $\text{OH-d}_3\text{-SRR-}^{-}\text{T}$ could be measured directly.

The hydrophobicity of the substrates was such that addition directly to the medium led to limited uptake by the microsomes; therefore S-CYP4F2 were pre-incubated on ice for 10-minutes with each of the substrates dissolved in ethanol, prior to the addition of the incubation buffer (100 mM KH_2PO_4). The production of $\text{OH-d}_2\text{-}^{-}\text{T}$ (based on relative peak heights on the chromatograms) did not change whether the ethanol-containing substrate was added directly to the S-CYP4F2 or was added first to the buffer. However, production of the hydroxylated metabolites from the other vitamin E substrates and the vitamin K substrate increased more than 10-fold by the pre-incubation with the ethanol-containing substrates (Figure 2).

Using the optimized incubation conditions with the lipophilic substrates in ethanol added directly to the S-CYP4F2, we conducted kinetic experiments. Reactions consisted of 25 pmol S-CYP4F2 incubated with $\text{d}_6\text{-}^{-}\text{T}$, $\text{d}_2\text{-}^{-}\text{T}$, $2\text{S-}^{-}\text{T}$, $\text{d}_3\text{-SRR-}^{-}\text{T}$, or $\text{d}_4\text{-PK}$, at 1, 5, 10, 25, 50, and 100 μM concentrations (final concentration, based on total reaction volumes), or vehicle (ethanol) on ice for 10 min. Next, 100 mM KH_2PO_4 , pH 7.4, was added and the reaction was incubated for 10 minutes at 37°C . The reaction was initiated by adding 1 mM nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma) and incubated another 30 minutes at 37°C (total volume of reactions were 0.5 ml, $< 2\%$ ethanol). In some experiments NADPH was omitted to demonstrate the specificity of the reaction. Additionally, to investigate influence of vitamin E on vitamin K omega-hydroxylation, 25 pmol S-CYP4F2 was incubated with $\text{d}_4\text{-PK}$ (0, 10, 20 and 50 μM) in the presence of $\text{d}_6\text{-RRR-}^{-}\text{T}$ (0, 10, 20 and 50 μM) or $\text{d}_3\text{-SRR-}^{-}\text{T}$ (0, 10, 50, and 100 μM) using the same conditions described above. Each $\text{d}_4\text{-PK}$ concentration was incubated with the full range of the vitamin E concentrations. 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 [26]. The extracts 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 × 2 mm, 4 μm, Phenomenex, Torrance, CA) was used with 100% methanol mobile phase for separations. The hydroxylated -T (13 OH- -T) peak was identified using an authentic 13 -OH- -T standard [24]. Quantitation of 13 OH- -T was found to be linear over 3 orders of magnitude (56–56,000 fmol on column, $R^2 = 0.998$), with a lower limit of quantification (signal/noise = 10) of 56 fmol on column, and a lower limit of detection (signal/noise = 5) of 28 fmol 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. Addition of an oxygen atom to substrates was monitored by the following SRM transitions: 13 -OH-d₆- -T (mass/charge (m/z) 451.4 to 168.9), 13 -OH-d₂- -T (m/z 433.4 to 150.0), 13 -OH- -T (m/z 445.4 to 162.9), 13 -OH-d₃- -T (m/z 448.4 to 166.0), and OH-d₄-PK (m/z 470.4 to 189.0). -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 of OH-d₆- -T, OH- -T, OH-d₃-SRR- -T were 2.4 minutes, while that of OH-d₄-PK was 3.9 minutes. Quantitation of 13 OH- -T was found to be linear over 3 orders of magnitude (56–56,000 fmol on column, $R^2 = 0.998$), with a lower limit of quantification (signal/noise = 10) of 56 fmol on column, and a lower limit of detection (signal/noise = 5) of 14 fmol on column.

To confirm their identities, accurate masses of OH-d₂- -T and OH-d₄-PK 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 Synergi Hydro-RP column, described above. Both TOFMS and product ion scans were analyzed using Peakview version 1.1.0.1 (AB Sciex). The d₂- -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 putative OH-d₂- -T 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 d₂- -T side chain and the expected d₂- -T cleavage pattern in negative mode, which results in the loss of 1 of the 2 deuterium atoms. d₄-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 putative OH-d₄-PK had a measured m/z of 470.3704 (0 ppm error from calculated exact mass). The measurements of d₄-PK and OH-d₄-PK are consistent with the addition of a single O atom in the d₄-PK side chain.

Statistics

To evaluate statistical differences, the data were analyzed using one-way ANOVA with Tukey Multiple Comparisons using Prism 5 statistical software (Graphpad Software, Inc). To normalize variances between groups when these were statistically different, the data was logarithmically transformed prior to statistical evaluation. Michaelis-Menten enzyme kinetics calculations were also performed using non-linear kinetics to estimate Vmax and Km, using Graphpad's non-linear kinetics calculator. The kinetic information in the table is shown as "apparent" because the concentrations in the microsomes were not measured. Statistical analysis of kinetic parameters was assessed using one-way ANOVA with Tukey post-tests. An unpaired t-test was used to compare kinetic parameters with and without competitor the vitamin (Table). Shown are means ± SEM; data were considered significantly different at $P < 0.05$.

Results

Vitamin E and Liver Slices

Incubation of liver slices with various Ts was carried out to investigate the relative hydroxylation of the forms of interest. The 2*S*- α -T could not be separately identified from the endogenous liver α -T; however, when incubated with 2*S*- α -T the liver slice α -T increased compared with the α -T concentrations in the slices incubated with vehicle (none) or with the labeled Ts (Figure 3A). To estimate 2*S*- α -T accumulated by the liver slices, the liver slice *unlabeled* α -T concentrations were estimated by averaging the endogenous α -T present in the various incubation conditions (with the exception of those with added 2*S*- α -T), this average was then subtracted from the α -T present in the 2*S*- α -T-incubated slices. Using this estimate of 2*S*- α -T, the liver slices incubated with the various vitamin E forms were found to contain similar concentrations of d₆- α -T, d₂- α -T, or 2*S*- α -T (Figure 3B).

The hydroxylation products, 13-OH-d₆- α -T, 13-OH-d₂- α -T, or 13-OH- α -T, made by the slices were measured. To estimate 13-OH-2*S*- α -T, the average unlabeled 13-OH- α -T in slices incubated with d₆- α -T, d₂- α -T, or none, was subtracted from the total 13-OH- α -T in the 2*S*- α -T-incubated slices. The 13-OH-d₂- α -T and 13-OH-2*S*- α -T concentrations were nearly double those of 13-OH-d₆- α -T incubated slices (Figure 3C). Thus, the liver slices preferentially metabolized non-2*R*- α -T, as has been observed in humans [14,17].

Insect microsomes expressing human CYP4F2 (S-CYP4F2)

To compare vitamin E and K as substrates for S-CYP4F2, optimized incubation conditions, mass spectrometry parameters, as well as confirmed identification of the hydroxylated metabolites, were used for these experiments (see methods). There were no significant differences between d₆-*RRR*- α -T or d₃-*SRR*- α -T, when used as substrates with regard to the estimated apparent Michaelis-Menten constant (*K*_m) or in the apparent maximal velocity (*V*_{max}), while the d₂- α -T substrate resulted in a significantly higher apparent *V*_{max} compared with either of the α -Ts (Figure 4A, Table). A higher apparent *V*_{max} was obtained when d₄-PK was incubated with S-CYP4F2 (*P*=0.0001), but the apparent *K*_m was not statistically different from those of the vitamin Es (Table). Overall, the S-CYP4F2 catalytic efficiency (*V*_{max}/*K*_m) for the vitamin K substrate was about 5 times greater than for the α -T or β -T substrates.

To investigate the effect of the presence of one vitamin on the other's omega-hydroxylation, we examined S-CYP4F2-mediated catabolism of the vitamins incubated together. d₄-PK (0, 10, 20 and 50 μ M) was incubated with S-CYP4F2 in the presence of d₆-*RRR*- α -T (0, 10, 20 and 50 μ M) or d₃-*SRR*- α -T (0, 10, 50, and 100 μ M). Each vitamin concentration was incubated with the entire range of the other vitamin (Figures 4B and 4C). The apparent d₄-PK *V*_{max} decreased from 198 $\pm$ 23 to 137 $\pm$ 16 (*p* = 0.0333) in the presence of α -T, but no effects on the apparent *K*_m were observed with addition of the competing α -T substrates (Table). There were no differences in the formation of OH- α -T metabolites when vitamin E was incubated with S-CYP4F2 in the presence of d₄-PK (Table).

Discussion

Our studies show contradictory evidence concerning vitamin E metabolism, 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-d₂- α -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 metabolite concentrations were both significantly greater than that of 13-OH-d₆-*RRR*- α -T. When the same comparison was made with S-CYP4F2, a greater *V*_{max} for the α -T substrate was calculated than that for the *RRR*- and *SRR*- α -T

substrates, which did not differ from each other. These findings are consistent with those reported by Sontag and Parker [18] suggesting that structural differences between α -T and β -T in the chroman ring, but not differences in side chain stereochemistry, influence α -hydroxylation of the vitamin E forms by CYP4F2. Thus, the mechanism of discrimination between α -T stereoisomers is not likely to 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 α -T stereoisomers, leading to accumulation of non-2R- α -T substrate for catabolism. Given the increased α -CEHC excretion when *all rac*- α - compared with *RRR*- α -T is administered to humans [17] or the increased α -CEHC excretion when α -tocopherol is administered [14], the liver slice experiments appear to more accurately mimic the in vivo situation.

Knowing that elevated hepatic α -T concentrations lead to reduced vitamin K status [27], we hypothesized that excess vitamin E may up-regulate catabolic pathways, especially α -hydroxylation by CYP4F2. However, we found that the in vitro apparent catalytic CYP4F2 efficiency for PK was significantly greater than for the α -T substrates (Table). We also investigated whether α -T could increase CYP4F2 activity and found that the α -T added to the vitamin K-S-CYP4F2 enzyme incubations did not stimulate vitamin K's hydroxylation, but rather it may have decreased it. Thus, these studies indicate that CYP4F2 activity is not enhanced by excess α -T. Moreover, our previous studies in rats injected with α -T showed that livers from α -T injected rats contained decreased CYP4F2 protein and mRNA concentrations relative to vehicle injected rats [28]. The net effect of these findings is that excess α -T should decrease rather than increase vitamin K metabolism.

The typical rat hepatic α -T concentrations are in the nanomole/g range, while those of phylloquinone are in the picomole/g range. However, in vitro experiments where α -T is at 1000-fold greater concentrations than PK are not possible to carry out because of the limitation in solubility for incubation with the microsomes, as well as the sensitivity of the measurements of the hydroxylated metabolites. The studies presented herein suggest that α -T is not likely to potentiate vitamin K's catabolism in vivo. These latter findings are in agreement with our findings that vitamin E did not increase the urinary excretion of vitamin K metabolites, 5C- and 7C-aglycones, while the urinary vitamin E metabolite, α -CEHC, increased 100-fold [28]. The source of the decreased vitamin K status with excess vitamin E may depend upon other mechanisms for xenobiotic disposal, such as phase III transporters [28]; we are investigating these possibilities in additional studies.

CYP4F2 is the only P450 enzyme demonstrated to be capable of vitamin E and K α -hydroxylation, which is the initial step in their metabolism and excretion [2,4]. With the aid of a synthetic standard synthesized by Suhara and Okano [29], the Rettie group has now unequivocally determined that CYP4F2 catalyzes the α -hydroxylation of the highly structurally related PK analog, menaquinone-4 (Edson et al, unpublished observations). These data coupled with the in vivo evidence that PK is α -hydroxylated, the first step in forming the tail-shortened aglycone metabolites in human urine [30], renders it highly likely that the product of CYP4F2-catalysed PK metabolism is the terminal alcohol.

CYP4F2 polymorphisms are associated with altered fatty acid metabolism [31] and, in regard to arachidonic acid hydroxylation, the V433M polymorphism is correlated with elevated blood pressure and may predispose individuals to hypertension, which may be related to diminished 20-hydroxyeicosatetraenoic acid (20-HETE) production [32]. Importantly, the V433M variant is also linked to negative health outcomes related to ischemic stroke [32,33] and myocardial infarction [34]. CYP4F2 polymorphisms also contribute to the wide variability in individual response to warfarin [8,34], which demonstrates the importance of CYP4F2 in regulating vitamin K concentrations. These

associations warrant further investigations in order to determine which CYP4F2-specific metabolite(s) are influencing disease outcomes 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 α -T and 2S- α -T are more readily α -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 whole body retention of *RRR*- α -T and is necessary for the increased excretion of both α -T and 2S- α -T. This finding is in contrast to what is observed in isolated insect microsomes expressing human CYP4F2. In the latter system, vitamin E catabolism is dependent upon differences in the chroman ring, which contribute to varying rates of metabolism, but side chain stereochemistry has no influence. Furthermore, vitamin K is apparently more readily hydroxylated by CYP4F2 than is any of the vitamin E forms, but vitamin E did not augment vitamin K's catabolism. These data indicate that the decreased vitamin K status observed in subjects taking vitamin E supplements [35,36] is not caused by stimulation of CYP4F2 activity by vitamin E; therefore, other mechanisms for vitamin E effects on vitamin K status in humans must be sought.

Acknowledgments

We are grateful to Dr. Brent Flickinger (Archer Daniels Midland) for supplying 2S- α -T; Dr. Thomas Netscher (DSM Nutritional Products) for supplying 2S,4 R,8 R- α -5,7-(C²H₃)₂ tocopheryl acetate (d₃-*SRR*- α -T); Dr. James Clark (Cognis Nutrition and Health) for supplying 2R,4 R,8 R- α -5,7-(C²H₃)₂ tocopheryl acetate (d₆- α -T); Dr. Jeffrey Atkinson, (Brock University, ON, Canada) for supplying α -3,4-(²H) tocopheryl acetate (d₂ T); Dr. Tomohiro Saito (Eisai Food and Chemical Co., LTD) for supplying α -tocotrienol.

Financial Support

We acknowledge funding from the NIH (GM06797 to AR; NIDDK and ODS, DK 067930 to MGT) and the USDA National Institute for Food and Agriculture (2009-35200-05031 to MGT). Archer Daniels Midland provided an unrestricted grant to MGT.

Abbreviations

20-HETE	20-hydroxyeicosatetraenoic acid
α-TTP	α -T transfer protein
ATP	adenosine triphosphate
CEHC	carboxy ethyl hydroxy chroman
CYP4F2	cytochrome P450 4F2
d	deuterated
NADPH	nicotinamide adenine dinucleotide phosphate
PK	phyloquinone
S-CYP4F2	CYP4F2 Supersomes
T	tocopherol

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Figure 1. The 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. Chromatograms of vitamin E and K metabolites

S-CYP4F2 were incubated with each of the vitamin substrates without (filled chromatograms) or with NADPH, which is necessary for S-CYP4F2 to generate the metabolite (see Methods). Chromatograms of the extracts show peaks identified as OH-d₆-RRR-α-T, OH-d₃-SRR-α-T, OH-d₂-γ-T, and OH-d₄-PK. (A) Addition of substrates to aqueous reaction mixtures without pre-incubation with S-CYP4F2. (B) Pre-incubation of substrates with S-CYP4F2 prior to the addition of aqueous buffer. Note that in both A and B tracings in the absence of NADPH the “non-specific” peaks are approximately the same height (relative intensity=approximately 2000 or less).

Figure 3. Vitamin E metabolism by liver slices

Liver slices were incubated for 24 hours with medium containing 10 μ M vitamin E (d_6 - α -T, 2S- α -T, or d_2 - γ -T) or the equivalent amount of vehicle alone (< 0.5% ethanol, vehicle). Data are means $\pm$ SEM, n=6 for d_6 - α -T, n=5 for 2S- α -T, and n=3 for d_2 - γ -T. **A)** Unlabeled α -T (nmol/g liver) was measured in the liver slices. **B)** Exogenous d_6 - α -T or d_2 - γ -T concentrations (nmol/g) in the liver slices were measured, while 2S- α -T uptake was approximated by subtracting the unlabeled α -T (average of the conditions shown in A, with the exception of the 2S- α -T incubation) from the α -T in the 2S- α -T supplemented slices. No statistically significant differences were found between concentrations of added T in the

three incubation conditions. **C)** 13'-OH-T (pmol/g liver) produced from the exogenous vitamin E taken up by the liver slices. 13'-OH-2*S*-T was estimated by subtracting the average OH- -T found in all conditions from the 13'-OH- -T found in the slices incubated with 2*S*- -T. (ANOVA $P=0.0006$ of logarithmically transformed data, Tukey multiple comparisons shown as columns labeled with different letters, $P<0.05$.)

Figure 4. Vitamin E and K metabolism by CYP4F2

A. CYP4F2-mediated metabolite concentrations (mean $\pm$ SEM) following 30-minute incubations of indicated concentrations of each vitamin substrate ($\text{d}_2\text{-}\gamma\text{-T}$, $\text{d}_3\text{-SRR-}\alpha\text{-T}$, $\text{d}_6\text{-RRR-}\alpha\text{-T}$, or $\text{d}_4\text{-PK}$) with 25 pmol S-CYP4F2, and estimated non-linear curve fits as described in Table 1, are shown. The Michaelis-Menten curves were generated, compared and found not to be statistically different for the $\gamma\text{-Ts}$; thus, a single $\gamma\text{-T}$ Michaelis-Menten curve (global) was generated. 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 OH-PK when PK was incubated simultaneously with

either d₃-*SRR*-T or d₆-*RRR*-T (PK+ vitamin E) and in C for OH-d₃-*SRR*-T or OH-d₆-*RRR*-T when d₃-*SRR*-T or d₆-*RRR*-T was incubated with PK. *Each vitamin E substrate was investigated separately.

Table

CYP4F2-mediated metabolism of vitamin substrates

Substrate	Apparent Km $\pm$ SE (μ M)	Apparent Vmax $\pm$ SE (pmol catabolite / min / nmol CYP4F2)	Apparent Catalytic Efficiency (Vmax/Km)
Vitamin E [*] (d ₆ -RRR- -T and d ₃ -SRR- -T)	32 $\pm$ 12	85 $\pm$ 13 ^a	2.6
d ₂ - -T	80 $\pm$ 26	215 $\pm$ 39 ^b	2.7
d ₄ -PK	14 $\pm$ 6	198 $\pm$ 23 ^b	15.8
ANOVA	NS	P=0.0001	
Interactions between vitamins			
d ₄ -PK plus Vitamin E [*] (d ₆ -RRR- -T or d ₃ -SRR- -T) KI = 30 $\pm$ 27 μ M	6 $\pm$ 4	137 $\pm$ 16	22.8
t-test, PK vs (PK+ vitamin E)	NS	P=0.033	
Vitamin E [*] plus d ₄ -PK (d ₆ -RRR- -T or d ₃ -SRR- -T) KI = 82 $\pm$ 20 μ M	32 $\pm$ 5	68 $\pm$ 4	1.9
t-test, 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 (Figure 4A) for the apparent kinetic parameters are shown for individual vitamins; interactions are shown in Figure 4B for OH- d₄-PK for PK vs (PK+ vitamin E), and Figure 4C for OH- -T for vitamin E vs (vitamin E+ PK). Michaelis-Menten model for d₄-PK concentrations was R²=0.8358 with df=15, and for d₂- -T was R²=0.9830 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 -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 R²=0.8358, with df=18. Statistical significance was assessed using ANOVA on the means $\pm$ 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 d₆-RRR- -T or d₃-SRR- -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 R²=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 R²=0.7363, with df=29. Note that the presence of the other vitamin had no statistically significant effect on the apparent Km, but vitamin E did decrease the estimated apparent Vmax of PK.