

RESEARCH ARTICLE

Vitamin E decreases extra-hepatic menaquinone-4 concentrations in rats fed menadione or phylloquinone

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Scope: 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).

Methods and results: In order to investigate these hypotheses, rats were fed phylloquinone (PK) or menadione (MN) containing diets (2 μ mol/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 h after the seventh 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 downregulation of hepatic cytochrome P450 expression and modified expression of tissue ATP-binding cassette transporters.

Conclusion: 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.

Keywords:

ABC transporters / 5C- and 7C-aglycones / α -CEHC / α -Tocopherol / Xenobiotic pathways

1 Introduction

Studies showing that high doses of vitamin E lead to impaired vitamin K-dependent coagulation processes have been

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Abbreviations: ABC transporters, ATP-binding cassette gene family of transporters; α -T, α -tocopherol; BCRP1, breast cancer resistance protein 1; CEHC, carboxy ethyl hydroxy chroman; CYPs, cytochrome P450s; GGCX, γ -glutamyl carboxylase; MDR1, multidrug resistance protein; MN, menadione; MK-4, menaquinone-4; MRP1, multidrug resistance-associated protein 1; OATP, organic anion-transporting polypeptide; PK, phylloquinone; UBIAD1, UbiA prenyltransferase domain-containing protein 1

reported since the 1940s [1–5]. 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 [6]. 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 10 years by nearly 40 000 women aged 45 years and older and demonstrated that vitamin E supplements decrease the risk of thromboembolism [7]. 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 effects, which were prevented by increased vitamin K intake [8]. 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

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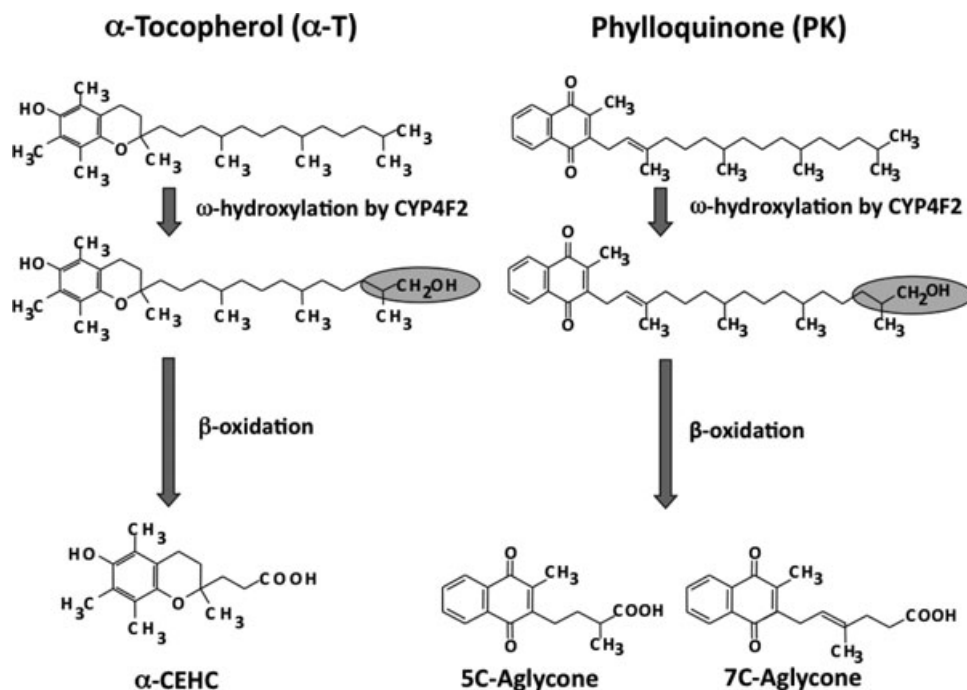


Figure 1. The metabolic pathway of vitamins E and K. The metabolic pathway of vitamins E and K involves their initial ω -hydroxylation by CYP4F2 [16, 17] followed by multiple rounds of β -oxidation to yield their respective urinary metabolites, carboxy ethyl hydroxy chroman (CEHC) for vitamin E [18] and 5C- and 7C-aglycone for vitamin K [14].

tissues. Tovar et al. [9] 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 total 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 [10]. Vitamin K refers to a family of compounds with a common 2-methyl-1,4-naphthoquinone head group. PK is the dietary form and is used to synthesize the tissue-specific form, menaquinone-4 (MK-4) [11]. In nature, the phytyl side chain of PK is cleaved to release the intermediate, menadiolone (MN), followed by geranylgeranylation, to form MK-4 [11, 12]. 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 [12]. The site of MN release is also unknown and has been theorized to occur in the intestines [11], 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 [11].

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) [8]. 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 [13]. 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 [14, 15]. As with xenobiotics, lipophilic compounds, such as vitamins E and K, are metabolized in processes involving: (i) phase I enzymes, or cytochrome P450s (CYPs), responsible for ω - and β -oxidation; (ii) phase II enzymes responsible for conjugation where increased polarity enhances water solubility; and (iii) 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 [16, 17]. Subsequently, the vitamins undergo recurrent rounds of β -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) [18] and for vitamin K the 5 and 7 carbon chain aglycone metabolites predominate [14] (Fig. 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 transporters 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 [19]. 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 [20].

Given that α -T and PK share a similar metabolic pathway and that α -T modulates xenobiotic factors [20–22], we hypothesized that α -T upregulates 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 [11]. 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.

2 Methods

2.1 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-h 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*- α -tocopheryl acetate/kg) and contained equimolar concentrations (2 μ mol/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 (both gifts from 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-h 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 \times g, 15 min) and stored at -80°C 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 -80°C until analysis. Additionally, during tissue collection, aliquots of liver were stored in RNAlater (Ambion, Austin, TX) for measurement of xenobiotic enzyme and transporter gene expression.

2.2 Vitamin E measurements

Plasma and tissue α -T concentrations were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) as previously described [23]. 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 [24]. 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; Sigma, St. Louis, MO).

2.3 Vitamin K measurements

Plasma PK and MK-4 were extracted using a modification of the method of Podda et al. [23] 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 -phyloquinone (d_4 -K1, (Buchechem BV, The Netherlands))), 4 mL of hexane was added. Samples were mixed by repeated inversion for 1 min. An aliquot of the organic phase was dried under nitrogen and resuspended in 100 μ L 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 [25]. PK and MK-4 were eluted with 4% ether in hexane, dried under nitrogen and resuspended in

100 μ L 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 μ L sample loop, and a column oven set at 30°C. Separation was achieved using an analytical HPLC column (Synergi Hydro-RP, 150 mm L \times 4.6 mm id., 4- μ m particle size, Phenomenex, Torrance, CA) fitted with a precolumn (AQ C18, 4 \times 3 mm id. SecurityGuard, Phenomenex). The mobile phase consisted of 100% methanol (flow = 1 mL/min for 13 min increased to 1.5 mL/min for 5 min and returned 1 mL/min for 2 min). Typical retention times for MK-4, PK, and d₄-K1 were 8.0, 13.3, and 13.3 min, 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 μ A, 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/h, and the cone gas (nitrogen) at 25 L/h. 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, d₄-K1 (*m/z* 454). The dwell time for each of the ions was set to 0.20 s. 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 S/N of 10/1 and 3/1, respectively.

Urinary 5C- and 7C-aglycone vitamin K catabolites were determined according to methods published previously [14, 26]. Five hundred microliters of urine sample was desalted with deionized 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 -1.2 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. Chro-

matograms 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.

2.4 qRT-PCR

Total RNA was isolated from tissue samples stored in RNAlater (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 (<http://www.ncbi.nlm.nih.gov>): (i) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5'-TAAAGGGCATCTG-3' and reverse 5'-TTACTCCTTGAGG, (ii) CYP4F1 forward 5'-GGAACCTTCGTCGCCTCCGTGG-3' and reverse 5'-CGCAACGGCAGCTGAGGCAT-3', (iii) CYP4F4 forward 5'-TGCCCTCAGCTTCGGTTGCC-3' and reverse 5'-TGCC TCCTGAGGCCAGGTCC-3', and (iv) CYP3a23/3a1 (CYP3A) forward 5'-CCTGGCAGTCGTCCTGGTGC-3' and reverse 5'-ACTGGGCCAAAATCCCGCCG-3'. The following previously published rat xenobiotic transporter primer sets were also utilized: (i) ABC, subfamily G, member 2 (Abcg2, alias BCRP1), (ii) solute carrier organic anion transporter family, member 1a4 (Slco1a4, alias OATP2), (iii) ABC, subfamily C, member 1 (Abcc1, alias MRP1), and (iv) ABC, subfamily B member 1B (Abcb1b, alias MDR1) [20]. Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA) and the 7900 HT 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 [27].

2.5 Immunoblotting

Rat microsomes were prepared from liver samples as previously described [21]. 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, Santa Cruz, CA). Secondary antibodies were either anti-goat or anti-rabbit-HRP-conjugated antibodies (Santa Cruz

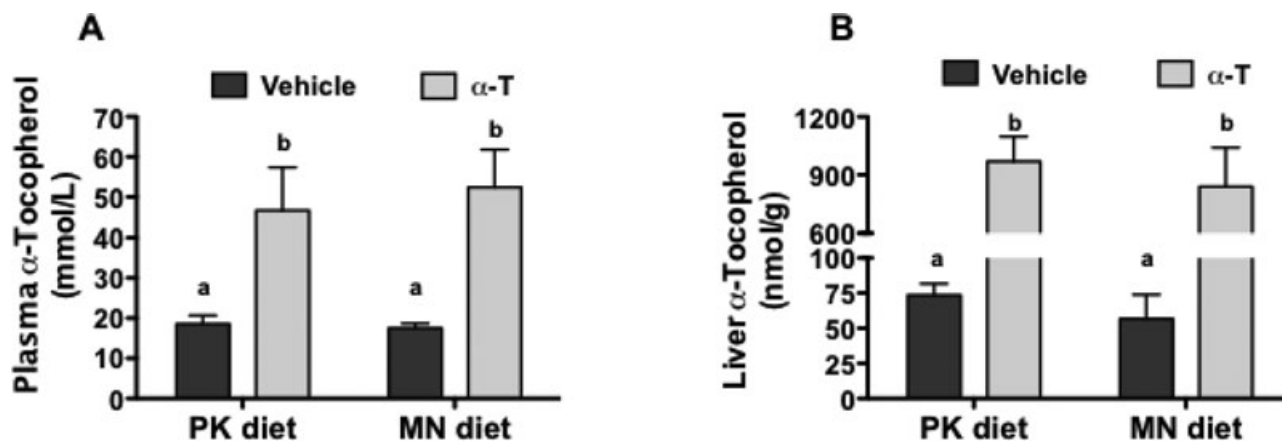


Figure 2. Subcutaneous vitamin E injections markedly increase plasma and hepatic α -tocopherol concentrations. Rats fed diets containing either PK or MN received subcutaneous injections of α -T (100 mg/kg) or vehicle for 7 days. Mean values \pm SD of (A) plasma and (B) liver α -T concentrations are shown. 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, $p < 0.001$ ($n = 5$).

Biotechnology, Inc.). The proteins were visualized with enhanced chemiluminescence (Perkin Elmer, 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, MD, <http://imagej.nih.gov/ij/>, 1997–2011).

2.6 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 \pm SD ($n = 5$ per group).

3 Results

3.1 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 h after their final injection, rats were sacrificed and blood and tissues were collected. As previously reported [22], this study showed 7 days of vitamin

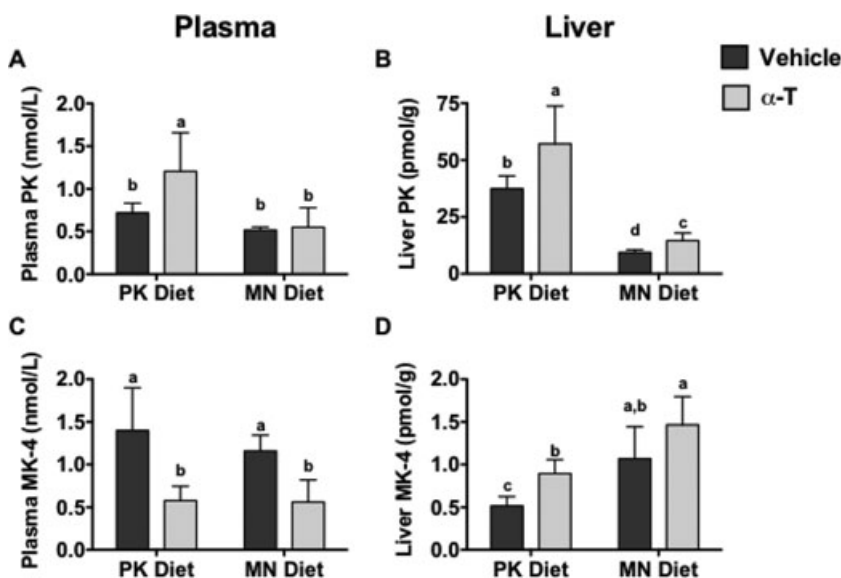


Figure 3. Subcutaneous vitamin E injections increased plasma and hepatic PK concentrations in PK and MN fed rats. The vitamin K concentrations in plasma and liver are shown from the same rats in Fig. 2. (A) PK concentrations in plasma (effect of diet, $p = 0.0020$, effect of injections, $p = 0.0408$), (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 \pm SD. Bars in each panel not bearing the same letter are significantly different, $p < 0.05$ ($n = 5$).

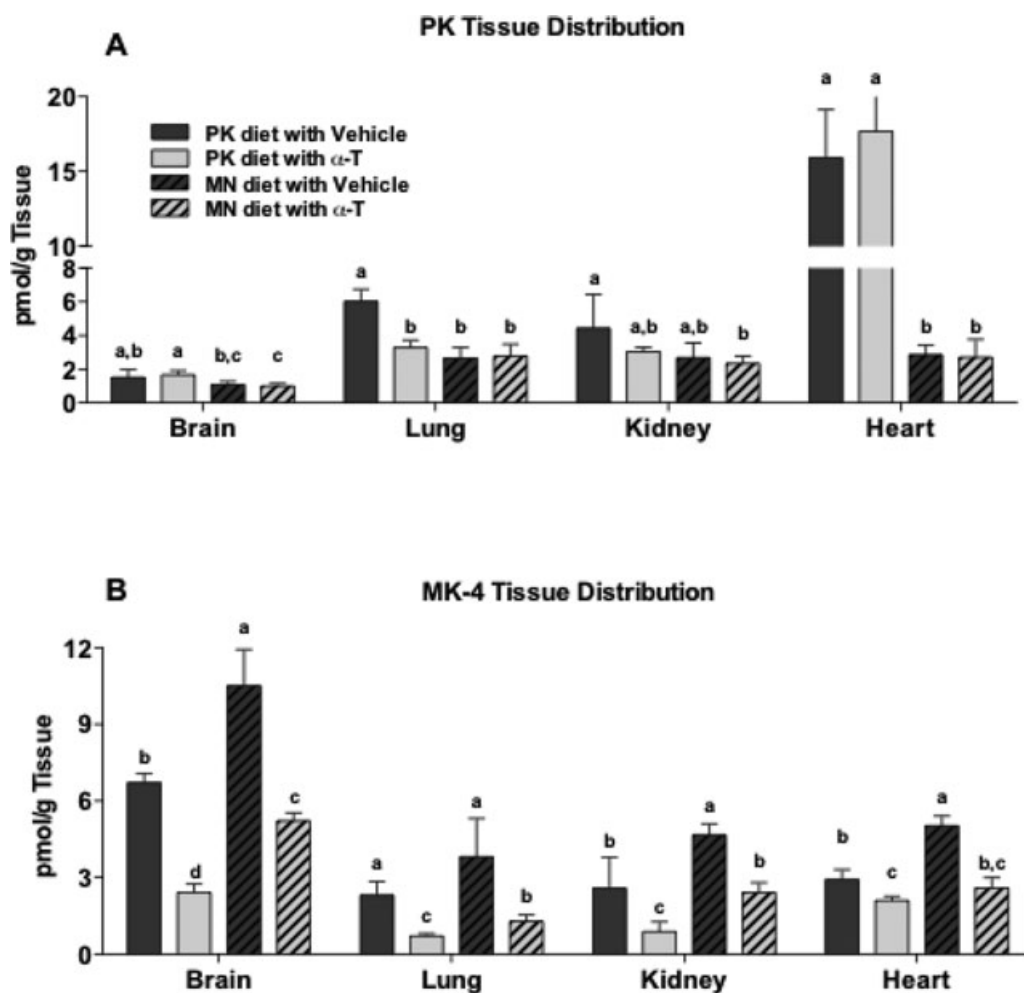


Figure 4. Subcutaneous vitamin E injections decreased extra-hepatic MK-4 concentrations. PK and MK-4 concentrations in extrahepatic tissues are shown from the same rats in Fig. 2. (A) PK concentrations were reduced in all extra-hepatic tissues of rats fed the MN diet ($p < 0.0100$); α -T injections decreased 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, $p < 0.05$ ($n = 5$).

E injections more than doubled plasma α -T concentrations and increased liver α -T concentrations more than tenfold (Fig. 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 (Fig. 3A). In contrast, α -T-injections significantly decreased plasma MK-4 concentrations, irrespective of dietary vitamin K form fed to the rats (Fig. 3C).

Hepatic PK concentrations were altered in response to both diet ($p < 0.0001$) and vitamin E injections ($p < 0.0006$) (Fig. 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 diet groups post-vitamin E injections. Both diet ($p < 0.0001$) and vitamin

E injections ($p < 0.0010$) altered the hepatic MK-4 concentrations (Fig. 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.

3.2 Extra-hepatic vitamin K concentrations

Vitamin E injections had little effect on extrahepatic tissue PK concentrations, irrespective of diet. In MN-fed rats, extrahepatic tissue PK concentrations were reduced, especially the heart (Fig. 4A). It should be noted that PK was not detected in the MN-diet (data not shown). Moreover, in MN rats, PK concentrations were unchanged by the α -T injections. Sim-

ilarly, 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) (Fig. 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 three 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.

3.3 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) (Fig. 5). Urinary α -CEHC excretion increased nearly 100-fold post-vitamin E injections from day 1 to day 8 ($p < 0.0001$, Fig. 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 = $\sim 5:1$) whereas in humans the 5C catabolite has been found to be the major form (molar ratio = $\sim 4:1$) [14].

3.4 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 [16, 17], the mRNA expression of CYP4F1 and CYP4F4 (rat homologues of human CYP4F2 [28]), and CYP3A were evaluated. All CYP mRNAs were downregulated in the liver of vitamin E injected rats compared to vehicle, irrespective of form of dietary vitamin K given (Fig. 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 (Fig. 7 A and C).

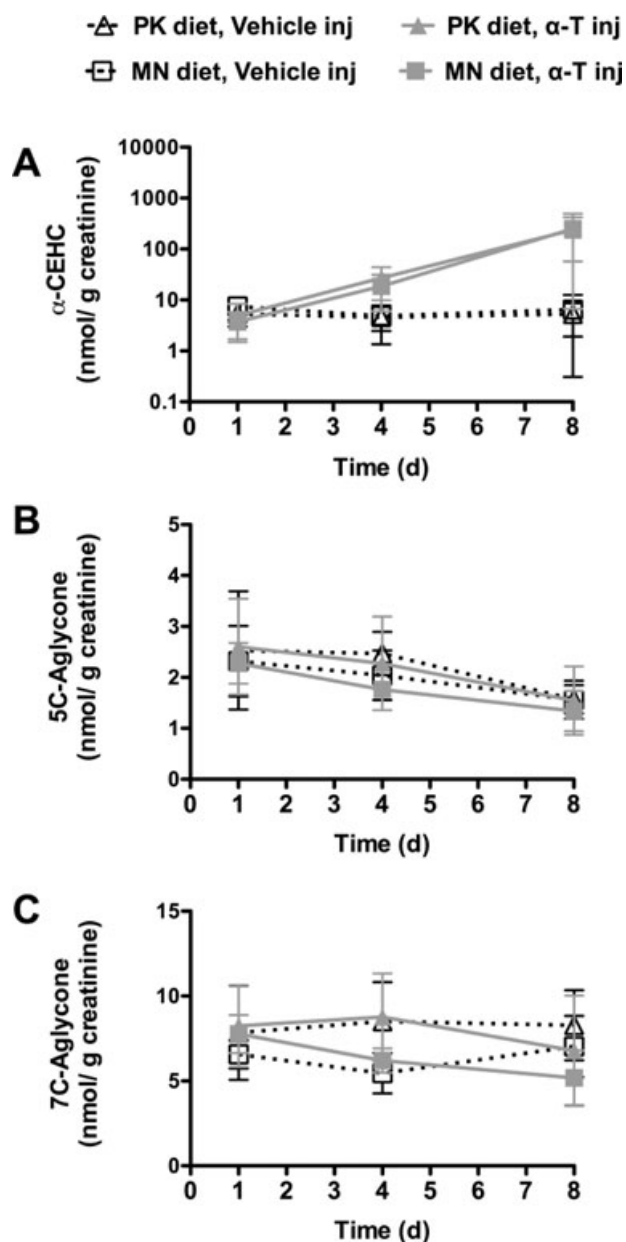


Figure 5. Vitamin E injections had no effect on vitamin K metabolite excretion. Urinary metabolite excretion is shown from the same rats in Fig. 2. α -CEHC excretion (A) increased significantly over time with vitamin E injections (effect of injection, $p < 0.0001$). Although 5C-Aglycone excretion (B) 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.

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 (Fig. 8). Specifically, OATP was found to be highly downregulated by vitamin E injections compared to vehicle (Fig. 8D), while MDR1 and BCRP1 mRNA expression was upregulated. (Fig. 8A and B). In contrast, neither dietary

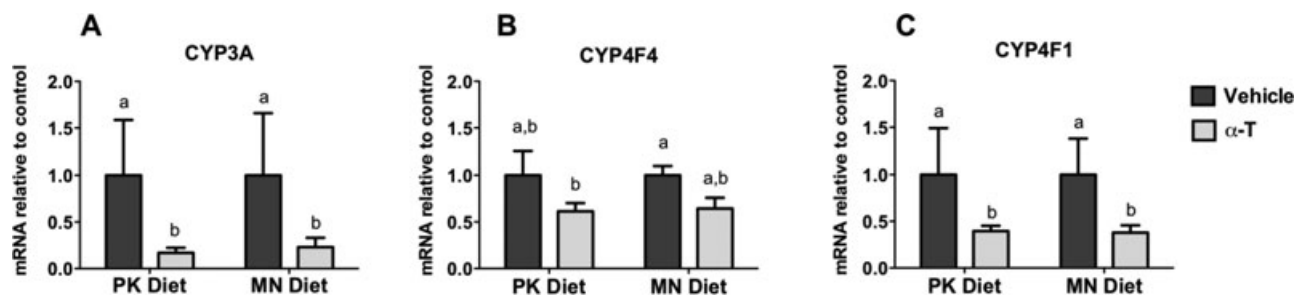


Figure 6. Vitamin E injections decreased CYP mRNA. The hepatic mRNA expression of the following CYPs: (A) CYP3A (effect of injection, $p = 0.0001$), (B) CYP4F4 (effect of injection, $p = 0.0039$), and (C) CYP4F1 (effect of injection, $p < 0.0001$) are shown from the same rats in Fig. 2. The expression of mRNA are shown relative to averaged PK-fed, vehicle-injected measurements (control). Data are mean values \pm SD. Bars in each panel not bearing the same letter for each tissue are significantly different, $p < 0.05$ ($n = 5$).

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 [29] (Fig. 7A and B). Additionally, BCRP1 protein expression was only observed in rats receiving the vitamin E injections and only in microsomes, not membranes.

4 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 MK-4 has a specific role in these tissues. Moreover, MK-4 is also the major form of vitamin K in rat [30] and human [31] 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 may be consequences for loss of vitamin K activity in other tissues. Further research is needed to assess these responses.

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. [9] 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. Our use of vitamin E injections may have caused a

more dramatic redistribution of vitamin K than was observed with dietary vitamin E. Others have 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 [32, 33]. If so, 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 (Fig. 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 [12]. 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.

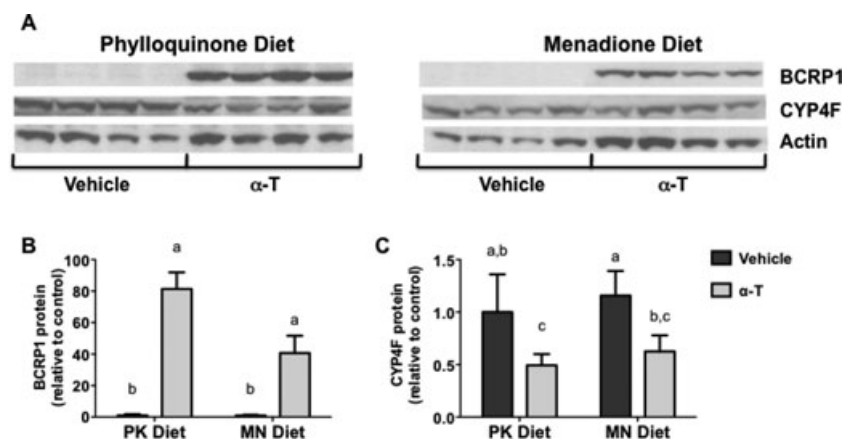


Figure 7. Protein expression of CYP4F and BCRP1 in liver microsomes are shown from the same rats in Fig. 2. (A) Western blot of BCRP1, CYP4F, and actin. Expression of (B) BCRP1 and (C) CYP4F normalized to actin are shown relative to PK-fed, vehicle-injected measurements (control). There was no effect 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 \pm SD. Bars not bearing the same letter for each tissue are significantly different, $p < 0.05$ ($n = 5$).

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 [34], 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 [20], 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 [16, 17] 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 [20], it is still unclear how vitamin E mediates its effects on expression of xenobiotic proteins. Reduced expression of CYP enzymes, however, supports evidence that increased rate of metabolism of vitamin K is not likely to 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 [20, 21]. 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

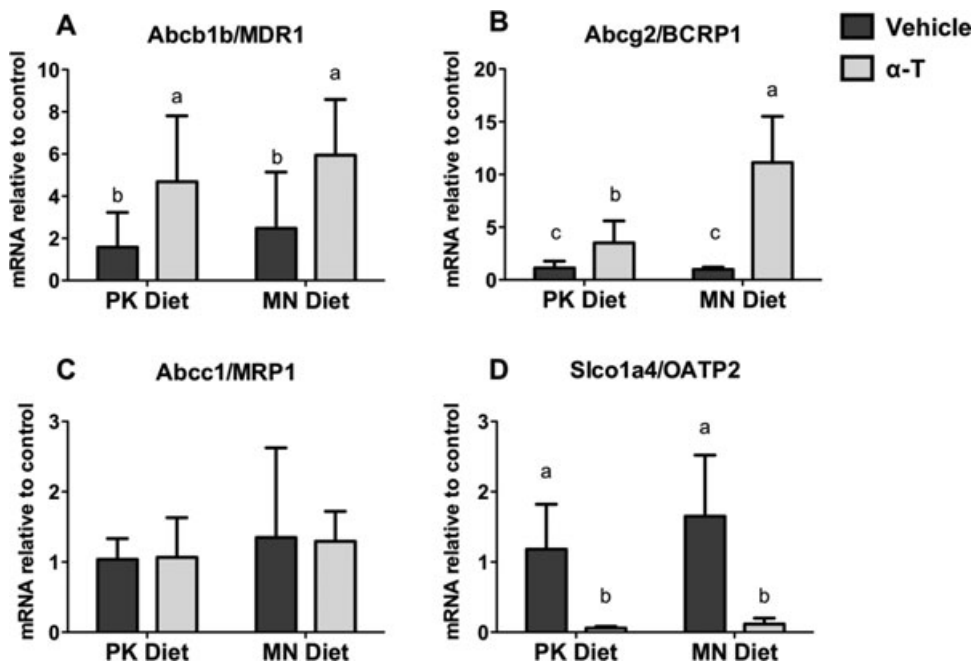


Figure 8. Vitamin E increased BCRP1 and MDR1 and decreased OATP gene expression. Hepatic mRNA expression is shown from the same rats in Fig. 2 for the following xenobiotic transporters: (A) Abcb1b/MDR1 (effect of injection, $p = 0.0208$) (B) Abcg2/BCRP1 (effect of injection, $p = 0.0005$), (C) Abcc1/MRP1, and (D) Slco1a4/OATP2 (effect of injection, $p < 0.0001$). Data are means \pm SD. Bars not bearing the same letter for each tissue are significantly different, $p < 0.05$ ($n = 5$).

membranes of many tissues and in the liver it functions to facilitate excretion of various compounds into bile [35]. Additionally, ABC transporters, such as BCRP1, are distributed intracellularly where they may accumulate and transport compounds including nutrients into vesicles [36]. 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 upregulated 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 [29], it should be noted that conflicting *in vitro* evidence suggest that posttranslational glycosylation of BCRP1 may be critical for stabilization of BCRP1 for its subsequent localization to the plasma membrane [37]. 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 increased following vitamin E injections. These data support the concept of vitamin redistribution 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 [11, 12]. 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 [19, 38, 39]. Moreover, tissue culture studies have provided evidence that MN is a substrate for the efflux transporter BCRP1 [40]. 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.

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