Author

During her sophomore year, Asal Sadatrafiei became interested in applying what she was learning in the classroom to a hands-on situation and she started thinking about getting involved in research. She approached Dr. Blumberg about his interests and was soon working on several projects in his lab. Her passion for research grew as she found value in conducting successful experiments. Now completing her second year in the University of California, San Francisco Pharmacy program, Asal says her UCI research experiences were excellent preparation for her pharmaceutical studies because the subject of her research furthered understanding of the biological mechanisms of drug metabolism and drug-nutrient interactions.

Key Terms

- Cytochrome P450
- Drug Metabolism
- Drug-Nutrient Interactions
- Gene Expression
- SXR
- Tocotrienols
- Vitamin E

Activation of the Steroid and Xenobiotic Receptor by Vitamin E Tocotrienol Isoforms Leads to Selective Target Gene Expression

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Abstract

For decades it has been known that vitamin supplements, including Vitamin E, affect drug metabolism. Vitamin E exists in eight isoforms: α-, β-, γ-, δ- tocopherol and α-, β-, γ-, δ- tocotrienol. All isoforms of Vitamin E are initially metabolized by ω-oxidation, which is catalyzed by cytochrome P450 enzymes (CYP). The CYP family of isoenzymes is one of the major groups of drug metabolizing enzymes and is regulated by the steroid and xenobiotic receptor, SXR. We show here that the four tocotrienols, but not tocopherols, specifically bind to and activate SXR. Surprisingly, tocotrienols show tissue-specific induction of SXR target genes; in primary hepatocytes, tocotrienols are able to up-regulate expression of CYP3A4, but not UDP glucuronosyltransferase 1A1 (UGT1A1) or multidrug resistance protein-1 (MDR1) genes, whereas tocotrienols induce MDR1 and UGT1A1 but not CYP3A4 expression in intestinal LS180 cells. These findings provide a molecular mechanism to explain why vitamin supplements affect the absorption and effectiveness of drugs. Knowledge of drug-nutrient interactions may help reduce the incidence of decreased drug efficacy.

Faculty Mentor

This work illustrates that vitamins may have unexpected effects on the absorption and effectiveness of drugs. Asal and her coworkers showed that tocotrienol forms of Vitamin E (commonly found in cereal grains) activate the steroid and xenobiotic receptor, SXR. This activation leads to increased expression of genes involved in drug and xenobiotic metabolism and could lead to decreased effectiveness of prescription drugs. Paradoxically, this effect is expected to be stronger with natural Vitamin E which contains both tocopherol and tocotrienol forms as compared to synthetic Vitamin E. Asal worked in my laboratory for more than two years and this experience whetted her interest in research. This research was particularly important in Asal's choice of pharmaceutical research as her future field of study.

Bruce Blumberg
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Introduction

Drug-drug interactions are a common problem in medical practice. In contrast, drug-nutrient interactions are not as widely considered when prescribing medications. Although drug-nutrient interactions are not as common as drug-drug interactions, they can have an impact on therapeutic outcome. There is evidence that vitamin supplements can affect the absorption and effectiveness of drugs (Trovato et al., 1991). Many of these effects can be attributed to the steroid and xenobiotic receptor (SXR), which regulates the induction of xenobiotic (foreign compounds and drug metabolizing enzymes) response to pharmacological levels of endogenous hormones, natural steroids, xenobiotic chemicals, and bioactive dietary compounds (Blumberg et al., 1998). SXR is a member of the nuclear receptor family of ligand-activated transcription factors. SXR is highly expressed in the liver, small intestine, and colon in humans and it is responsible for regulation of oxidation (phase I), conjugation (phase II), and transport (phase III) genes (Kliewer et al., 2002). The phase I genes refer to the cytochrome P450 monooxygenases, mainly CYP3A4, a crucial component of the body's defense mechanism against xenobiotics. The induction of the CYP3 enzymes is a source of concern for possible drug-nutrient and drug-drug interactions because the CYP3 family is responsible for the metabolism of about 60% of all prescription medications (Cholerton et al., 1992; Maurel, 1996; Rendic and Di Carlo, 1997; Michalets, 1998).

Vitamin E is fat-soluble and exists in eight different naturally-occurring isoforms, (α-, β-, γ-, and δ-) tocopherols and tocotrienols, differing in the number and position of methyl groups on the chroman ring (Saito et al., 2003). All eight Vitamin E isoforms are absorbed by the intestine, transported by chylomicrons in lymph, and then incorporated into hepatic cells (Traber et al., 1992; Kayden and Traber, 1993). Unlike other fat-soluble vitamins, Vitamin E is not accumulated in the liver to toxic levels. All forms of Vitamin E are metabolized by degradation of the side chain via initial ω-oxidation (Landes et al., 2003), catalyzed by cytochrome P450 enzymes (CYP), namely CYP3A4, the first one suggested to be involved in Vitamin E metabolism (Brigelius-Flohe, 2003), and CYP4F2. The metabolic rate of Vitamin E is highest for tocotrienols and lowest for α-tocopherol, which has been suggested to contribute to its more potent biological activity (Sontag and Parker, 2002).

Vitamin E acts as an antioxidant, protecting cells from potentially damaging by-products of metabolism such as free radicals (Karbownik et al., 2001; Yoshida et al., 2002). Free radicals can lead to DNA damage and consequent induction or promotion of carcinogenesis, resistance to chemotherapy, and cardiovascular disease. Other benefits of Vitamin E include the role of γ-tocotrienol, which stimulates sodium excretion in the prevention of hypertension (Saito et al., 2003).

The goal of this study was to determine which isoforms of Vitamin E result in the activation of SXR and induction of the xenobiotic metabolism pathway. We found that tocotrienols activate SXR, leading to upregulation of SXR target genes. The induction of SXR target genes differed between liver and intestinal cells, suggesting that SXR ligands have the ability to regulate differentially the expression of target genes. The activation of SXR by Vitamin E suggests that consumption of dietary supplements high in tocotrienols, such as palm oils (Yoshida et al., 2003) and over-the-counter vitamins, may lead to increased metabolism of other dietary compounds, xenobiotics, and prescription drugs.

Materials and Methods

Human Cell Culture and Reagents

All experiments were carried out in accordance with the Institutional Review Board at the University of California, Irvine, and were consistent with federal guidelines. Human internal epithelial cell line LS180 and hepatoma cell line HepG2 were obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. The cells were seeded into 6-well plates and grown in DMEM-10% FBS until they reached 70-80% confluence. The medium was replaced with DMEM containing 10% resin-charcoal stripped FBS 24 hr before treatment. Immediately before treatment, the medium was removed; the cells were washed once with phosphate buffered solution (PBS) and then treated with different reagents or ethanol at the appropriate time.

Human primary hepatocytes were obtained from Liver Tissue Procurement and Distribution System, Pittsburg, PA (LTPADS) as attached cells in 6-well plates. The hepatocytes were maintained in Hepatocyte Medium (Sigma, St. Louis, MO) for at least 24 hr before treatment. Rifampicin (RIF), mifepristone (RU486), and clotrimazole were used as the control (Sigma, St. Louis, MO). The Vitamin E compounds were purchased from Calbiochem (San Diego, CA).

Transient Transfection and Luciferase Assay

To test the ability of various isoforms to activate SXR, LS180 and HepG2 cells were seeded into 12-well plates

overnight and transiently cotransfected with the SXR expression plasmid, Luciferase reporter constructs, and CMX-β-galactosidase transfection control plasmids with Lipofectamine 2000 (Invitrogen Life Technology, Carlsbad, CA) in serum-free DMEM. 24-48 hr post-transfection, the cells were treated with 10 μM of the α-, β-, γ-, and δ- isoforms of the tocopherols and tocotrienols, with ethanol, or known SXR ligands RIF, RU486, and clotrimazole as positive controls. The cells were lysed 24 hr after treatment and β-galactosidase and luciferase assays were performed (Blumberg et al., 1998). Reporter gene activity was normalized to the β-galactosidase transfection controls and the results expressed as normalized RLU per OD β-galactosidase per minute to facilitate comparisons between plates. Each data point represents the average of triplicate experiments +/- standard error of the mean (SEM) and was replicated in independent experiments. The error bars represent the SEM, a statistical value defined as the standard deviation divided by the square root of the number of samples.

Competitive Ligand Binding Assay

N-terminal histidine-6-tagged human SXR ligand binding domain was expressed in *Escherichia coli* together with the SRC-1 receptor interaction domain as described (Tabb et al., 2003). Active protein was refolded from inclusion bodies solubilized in denaturation buffer (6 M guanidium-HCL, 50 mM HEPES pH 7.4, 0.2 M NaCl, 25 mM DTT, 1% w/v Triton-X100) by rapid 10-fold dilution into binding buffer (50 mM HEPES pH 7.4, 1 M sucrose, 0.2 M NaCl, 0.1 mM DTT, 0.1% w/v CHAPS) followed by dialysis overnight at 4 ºC against binding buffer. Binding assays were performed by coating 96-well Nickel Chelate FlashPlates (Perkin Elmer Life Sciences, Boston, MA) with a ten-fold molar excess of protein for one hr at 22 ºC in binding buffer (50 mM Hepes, pH 7.4, 200 mM NaCl, 1 M sucrose, 0.1% CHAPS). Unbound protein was removed from the wells by washing four times with binding buffer. ^{3}H-SR12813 (Amersham-Pharmacia BioSciences, Piscataway, NJ) was added to a final concentration of 50 nM in each well, either alone or with competitive ligands in binding buffer as indicated (Jones et al., 2000). Incubation was continued for 3 hr at room temperature. Total counts were measured using a Topcount Scintillation Counter (Packard, Meriden, CT). Counts remaining after the addition of 10 μM clotrimazole were taken as non-specific background and subtracted from all wells. All assays were performed in triplicate and reproduced in independent experiments.

RNA Isolation and Quantitative Real-time (QRT-PCR) Analysis

Total RNA was isolated from LS180 cells and primary hepatocytes using TRIzol Reagent (Invitrogen Life Technology, Carlsbad, CA) according to the manufacturer-supplied protocol (Tabb et al., 2003). For RT-PCR analysis, 1 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase according to the manufacturer-supplied protocol (Invitrogen Life Technology, Carlsbad, CA). Quantitative real time RT-PCR was performed using gene specific primers (Sigma, St. Louis, MO) and the SYBR Green PCR kit (Applied Biosystems, Foster City, CA) in a DNA Engine Opticon—Continuous Fluorescence Detection System (MJ Research, Walthon, CA). All samples were quantified using the comparative Ct method, also known as the $2^{-\Delta\Delta Ct}$ method, for relative quantification of gene expression, normalized to GAPDH (Livak and Schmittgen, 2001).

Results

Tocotrienols, but not Tocopherols, Activate SXR in Ls180 and Hepg2 Human Cells

Previous studies suggested that Vitamin E could activate SXR (Landes et al., 2003). To determine which isoforms of Vitamin E directly activate SXR, transfection assays were utilized. Since SXR functions as a steroid and xenobiotic sensor primarily in liver and intestine tissue *in vivo*, we chose human hepatic cell line HepG2 and human intestinal epithelial cell line LS180 as models. The known SXR ligands clotrimazole, RIF, and RU486 showed a 5- to 18-fold induction of SXR activity in LS180 and a 22- to 47-fold activation in HepG2 (Figure 1). At 10 μM, all four of the tocotrienols activate SXR in both cell lines, which show the same trend, although the effect was more strongly observed in HepG2 cells, which is consistent with the responses to the positive controls. All four of the tocotrienols significantly activated SXR in both the LS180 (3- to 5- fold) and HepG2 (6- to 13-fold) cells. The four tocopherols did not activate SXR in either cell line. Although SXR activation by tocotrienols is as robust as the positive control compounds, all four tocotrienols showed reproducible activity. These findings demonstrate that the tocotrienols, but not tocopherols, activate SXR.

Tocotrienols Specifically Bind SXR in Vitro

Since tocotrienols activated SXR in transient transfections (Figure 1), we then sought to determine whether tocotrienols bind to purified SXR protein *in vitro*, using a sensitive scintillation proximity ligand-binding assay (Tabb et al., 2003). This assay employed ^{3}H-SR12813 and recom-

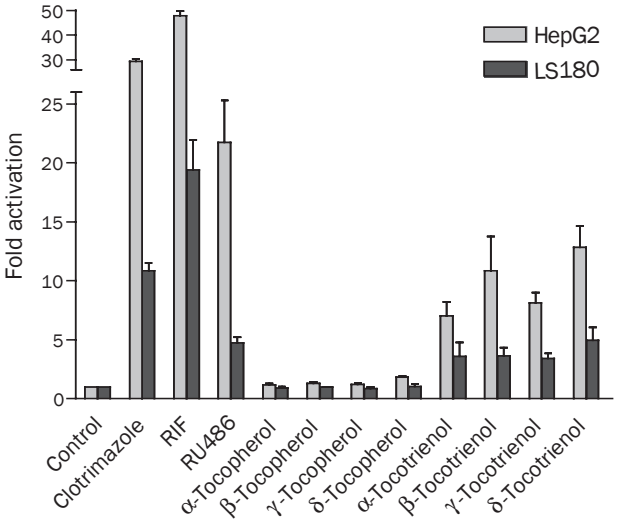

Figure 1

Activation of SXR by tocotrienols. LS180 and HepG2 cells were transiently transfected with SXR together with SXRE-luciferase reporter and CMX-β-galactosidase transfection control plasmids. After transfection, cells were treated with control medium or medium containing 10 μM clotrimazole, RIF, RU486, or the various Vitamin E compounds.

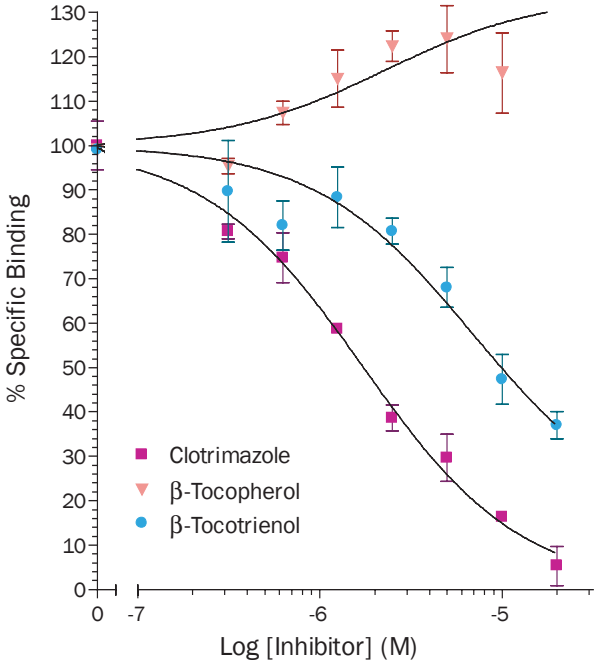

Figure 2

β-Tocotrienol, but not β-tocopherol, specifically binds to the purified SXR ligand-binding domain. His_6-SXR LBD was co-expressed with the SRC-1 receptor interaction domain and purified. The receptor complex was bound to Nickel Chelate FlashPlates and incubated with 50 nM of ^{3}H-SR12813 in the presence of the indicated concentration of β-tocotrienol, β-tocopherol or clotrimazole. Values represent the average of triplicates ± SEM and were replicated in independent experiments.

binant His6-tagged-SXR coexpressed with the SRC-1 receptor interacting domain (Dussault et al., 2001) and Nickel-Chelate FlashPlates (Perkin Elmer Life Sciences, Boston, MA). SR12813 interacts specifically with SXR with a dissociation constant of 40 nM (Jones et al., 2000). β-tocotrienol and clotrimazole (positive control) were able to displace ^{3}H-SR12813 from the SXR LBD in a dose-dependent manner, whereas the β-tocopherol did not compete for receptor binding, an outcome consistent with our transfection results (Figure 2). Tocotrienols α-, γ- and δ- were also able to specifically bind to SXR, whereas all the tocopherols failed to effectively bind SXR (data not shown). The K_i, the enzyme inhibition constant, for tocotrienols binding to SXR was determined to be ~5 μM, a value in the range of other known SXR ligands (Moore et al., 2000; Dussault, et al. 2001). The K_i, defined as the concentration of competing ligand at which half of the binding sites will be occupied, reveals the affinity of the compounds for the receptor. The affinity of the compounds is inversely related to the inhibition constant. From these results, it can be inferred that tocotrienols specifically bind to and activate SXR, whereas the tocopherols failed to activate. These results, then, suggest that tocotrienols function as genuine ligands for SXR.

Tocotrienols Selectively Increase SXR Target Gene Expression in Various Tissues

Next, we sought to determine whether tocotrienols induce the expression of known SXR target genes in cells. Three genes were selected: CYP3A4 (phase 1), UGT1A1 (phase 2), and MDR1 (phase 3), representing each of the three phases of drug metabolism. The two cell models used were LS180 cells, intestinal cells known to metabolize all forms of Vitamin E and express SXR target genes involved in drug metabolism, and primary human hepatocytes, the most appropriate model for the study of liver-specific metabolism. HepG2 cells were not suitable because they were not able to express CYP3A genes in response to SXR ligands. Cells were treated with 10 μM of solvent control, positive control (clotrimazole, RIF or RU486) or various Vitamin E compounds for 24 hr and the total RNAs isolated. QRT-PCR was performed to detect the expression levels of the known SXR target genes CYP3A4, UGT1A1 and MDR1.

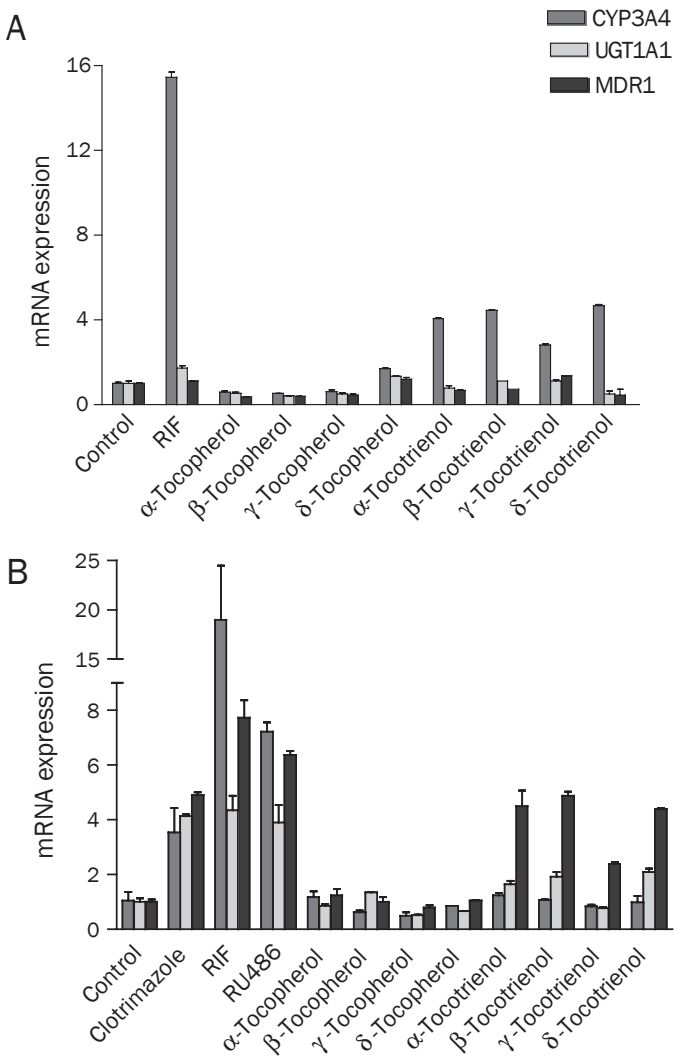

Figure 3

Tocotrienols selectively increase SXR target gene expression in various tissues. Human primary hepatocytes (3A) or LS180 intestinal epithelial cells (3B) were treated with control medium or medium containing 10 μM RIF or the various Vitamin E compounds. Total RNA was isolated and gene expression levels were detected by QRT-PCR, where all values were normalized to GAPDH control.

In accordance with the receptor activation data, the four tocotrienols, but not tocopherols, were able to induce phase 1 SXR target gene CYP3A4 mRNA expression 3- to 5-fold compared to solvent controls in primary human hepatocytes (Figure 3A). No significant increase in either the phase 2 UGT1A1 or phase 3 MDR1 gene expression was observed after treatment with tocotrienols or RIF. In contrast to the results seen in primary hepatocytes, the four tocotrienol compounds increased the expression of mRNAs encoded by the phase 2 UGT1A1 gene (1.5- to 2-fold except with γ-tocotrienol) and the phase 3 MDR1 gene (2- to 4-fold) in the intestinal LS180 cells, whereas they did not increase the expression of CYP3A4 (Figure 3B). The three known SXR ligands clotrimazole, RIF, and RU486 were able to induce all of the three tested SXR target genes CYP3A4, UGT1A1, and MDR1 in LS180 cells. These results suggest that tocotrienols may act as tissue-specific SXR ligands that can induce different SXR target gene expressions depending on the tissue.

Discussion

The present study investigated the effects of Vitamin E on SXR activation and the induction of SXR target genes involved in drug metabolism. The first goal of this project was to determine which Vitamin E isoforms directly activate SXR. The results showed that α-, β-, γ-, and δ-tocotrienol, and not the corresponding tocopherols, specifically bind and activate the nuclear receptor SXR in transfected hepatic and intestinal cell lines *in vitro*. The tocotrienols also selectively induced expression of only CYP3A4 in primary hepatocytes, whereas they induced expression of only UGT1A1 and MDR1 in LS180 cells. These findings confirm and extend previous results suggesting that Vitamin E can activate SXR (Landes et al., 2002). Therefore, tocotrienols may not only induce their own metabolism, but also alter the metabolism of steroids, drugs, xenobiotic chemicals, and bioactive dietary compounds normally metabolized by an SXR-dependent pathway.

Humans consume Vitamin E in foods such as vegetable oils, nuts, green leafy vegetables, and fortified cereals. Tocopherols are found in the germ of cereal seeds and in polyunsaturated vegetable oils. Tocotrienols are found in the aleurone and subaleurone layers of cereal seeds and in palm oils (Yoshida et al., 2003). The components of over-the-counter Vitamin E supplements vary based on the brand. Interestingly, the so-called natural Vitamin E supplements, which contain mixed tocopherols and tocotrienols, have the potential for causing interactions, whereas synthetic α-tocopherols would not be expected to cause such effects.

Our data demonstrate the activation of SXR by 10 μM concentrations of tocotrienols. Pharmacokinetic studies show that the plasma concentration of tocotrienols can reach >2000 ng/ml (>5 μM) following oral administration of a single dose of 300 mg mixed tocotrienols (Yap, 2001). This concentration can be reached in human plasma and concentrations in the liver may be even higher than those measured in plasma (Landes et al., 2003). This would be expected to result in increased activation of SXR *in vivo*, and therefore increase expression of the drug metabolizing enzymes regulated by SXR. This leads to the inference that ingestion

of foods or dietary supplements containing high levels of tocotrienols accelerated metabolism and consequently, reduced efficacy of many prescription drugs. The results of this investigation suggest that it may not be in the best interest of patients on medications necessary for disease or symptomatic treatment to supplement their diet with high doses of tocotrienol-containing Vitamin E. Our findings agree with previous research in drug-nutrient interactions that several natural foods and herbs are able to induce drug metabolism (Sorensen, 2002). Knowledge of drug-nutrient interactions can help reduce the incidence of decreased drug efficacy.

In summary, this study shows that all four tocotrienols are able to efficiently activate SXR and specially bind to SXR, whereas tocopherols do not activate or bind to SXR. Tocotrienols are also able to selectively regulate SXR target genes in different tissues: in primary hepatocytes, they induce CYP3A4 gene expression; in intestinal LS180 cells, they induce MDR1 and UGT1A1 gene expression, but have no effect on CYP3A4. Future studies will include analysis of additional SXR target genes to elucidate the selective SXR target gene expression.

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