Cytochrome P450 Regulation by α -Tocopherol in *Pxr*-Null and *PXR*-Humanized Mice

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ABSTRACT

The pregnane X receptor (PXR) has been postulated to play a role in the metabolism of α -tocopherol owing to the up-regulation of hepatic cytochrome P450 (P450) 3A in human cell lines and murine models after α -tocopherol treatment. However, in vivo studies confirming the role of PXR in α -tocopherol metabolism in humans presents significant difficulties and has not been performed. *PXR*-humanized (h*PXR*), wild-type, and *Pxr*-null mouse models were used to determine whether α -tocopherol metabolism is influenced by species-specific differences in PXR function in vivo. No significant difference in the concentration of the major α -tocopherol metabolites was observed among the h*PXR*, wild-type, and *Pxr*-null mice through mass spectrometry-based

metabolomics. Gene expression analysis revealed significantly increased expression of *Cyp3a11* as well as several other P450s only in wild-type mice, suggesting species-specificity for α -tocopherol activation of PXR. Luciferase reporter assay confirmed activation of mouse PXR by α -tocopherol. Analysis of the *Cyp2c* family of genes revealed increased expression of *Cyp2c29*, *Cyp2c37*, and *Cyp2c55* in wild-type, h*PXR*, and *Pxr*-null mice, which suggests PXR-independent induction of *Cyp2c* gene expression. This study revealed that α -tocopherol is a partial agonist of PXR and that PXR is necessary for *Cyp3a* induction by α -tocopherol. The implications of a novel role for α -tocopherol in *Cyp2c* gene regulation are also discussed.

Introduction

α -Tocopherol is not accumulated in the body like other lipid-soluble vitamins; rather, it is transported to the liver and, through a series of oxidation reactions, converted to α -carboxyethyl hydroxychroman (α -CEHC) (Scheme 1). It is then conjugated and excreted from the body. Metabolism to α -CEHC involves an initial ω -oxidation step that converts tocopherol to 13'-hydroxy- α -tocopherol. It was reported that cytochrome P450 (P450) enzymes induced after α -tocopherol dosing perform this ω -oxidation step (Birringer et al., 2001). However, contradictory studies have failed to ascertain which enzymes and regulatory mechanisms are involved. For example, CYP4F2 was increased after α -tocopherol dosing in cell culture models (Sontag and Parker, 2002), but no change in CYP4F2 was seen in rats

injected with α -tocopherol (Mustacich et al., 2006). The same rats, however, showed increases in CYP3A, CYP2B, and CYP2C protein. *Cyp3a11* (the murine homolog of human *CYP3A4*) gene expression was increased in C57BL/6 mice dosed with α -tocopherol (Mustacich et al., 2009), but the expression of other *Cyp3a* subfamily members as well as the *Cyp2b13*, *Cyp2c44*, and *Cyp2e1* genes was not changed. A further study showed an increase of hepatic CYP3A protein in C57BL/6 mice fed an α -tocopherol diet (Traber et al., 2005). These studies revealed that CYP3A enzymes appear to be involved in rodent ω -oxidation of α -tocopherol, but the activity of the other P450 enzymes is not conclusive.

Human CYP3A isoforms are involved in the metabolism of over 50% of drugs. In cellular and rodent models, α -tocopherol supplementation may interfere with drug metabolism through the induction of CYP3A4, which is regulated by the pregnane X receptor (PXR). α -Tocopherol was shown to act as a ligand for PXR in HepG2 cells (Landes et al., 2003). Thus, it could potentially induce its own metabolism by increasing the expression of CYP3A4 and ω -oxidation to 13'-hydroxy- α -tocopherol. However, another study revealed that activation of PXR in wild-type mice by pregnenolone-16 α -carbonitrile (PCN) or in *PXR*-humanized (h*PXR*) mice by rifampicin decreased vitamin E metabolism compared with *Pxr*-null mice (Cho et al., 2009). This may have been due to down-regulation of β -oxidation by PCN and rifampicin.

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ABBREVIATIONS: CAR, constitutive androstane receptor; α -CEHC, α -carboxyethylhydroxychroman; α -CMBHC, α -carboxymethylbutylhydroxychroman; P450, cytochrome P450; ESI, electrospray ionization; FBS, fetal bovine serum; h*PXR*, *PXR*-humanized; MRM, multiple reaction monitoring; MS, mass spectrometry; PCN, pregnenolone-16 α -carbonitrile; PPAR- α , peroxisome proliferator-activated receptor- α ; PXR, pregnane X receptor; OPLS-DA, orthogonal projection to latent structures discriminant analysis; UGT, uridine 5'-diphospho-glucuronosyltransferase; UPLC, ultraperformance liquid chromatography; UPLC-ESI-QTOF-MS, ultraperformance liquid chromatography electrospray ionization time-of-flight mass spectrometry.

Scheme 1. Scheme of α -tocopherol metabolism.

The contradictory observations in previous studies could primarily be due to different model systems used for comparison. Indeed, it is known that mouse PXR does not have the same response as human PXR to ligands. The DNA-binding domain for PXR is 95% homologous between humans and mice, yet they only share between 75–80% of amino acids in the ligand-binding domain (LeCluyse, 2001). Therefore, although mouse models can be ideal for biologic research, the genetic differences that exist between mice and humans can have a major impact on drug metabolism. To overcome this problem, hPXR and Pxr-null mice can be used to show the species-specific response.

The National Health and Nutrition Examination Survey (NHANES) in 1999–2000 showed that $\sim 10\%$ of U.S. adults took a vitamin E supplement (Picciano and McGuire, 2009), and as PXR is also involved in the detoxification of a large number of drugs from the body, it is essential to characterize α -tocopherol modulation of PXR and the downstream effects on the expression of drug-metabolizing enzymes in appropriate mouse models. Thus, the expression of hepatic P450 genes was analyzed in hPXR, Pxr-null, and wild-type mice after α -tocopherol dosing to determine whether α -tocopherol metabolism is influenced by species-specific differences in PXR function. Metabolomics based on ultraperformance liquid chromatography–mass spectrometry (UPLC-MS) were also used to identify and quantitate the urinary metabolites of α -tocopherol. This was to determine whether species-specific differences in PXR would affect the metabolic end-products of α -tocopherol. This study could potentially shed light on the possibility for drug-drug interactions when supplementing the diet with α -tocopherol.

Materials and Methods

Reagents. PCN, corn oil, rifampicin, and chlorpropamide were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals required for UPLC-MS were obtained from Thermo Fisher Scientific (Waltham, MA) and were of the highest purity grade.

Animals, Diets, and Experimental Design. All animal studies were conducted in accordance with Institute of Laboratory Animal Resource (ILAR) guidelines and approved by the National Cancer Institute Animal Care and Use Committee. From 3 weeks of age, male hPXR ($n = 13$), Pxr-null ($n = 13$), and wild-type ($n = 9$) mice bred at NCI-Bethesda (Bethesda, MD) were maintained under a standard 12-hour light/dark cycle with water. Mice were fed ad libitum a purified diet containing vitamin-free casein, sucrose, cornstarch, dextrose, L-cystine, cellulose, tocopherol-stripped soybean oil, mineral mix (Dyets, Inc., Bethlehem, PA), vitamin mix with no vitamin E (Dyets, Inc.), and choline bitartrate. The mineral mix consisted of calcium carbonate, potassium citrate, potassium phosphate monobasic, sodium chloride, potassium sulfate, magnesium oxide, ferric citrate, zinc carbonate, manganous carbonate, cupric carbonate, potassium iodate, sodium selenate, ammonium vanadate, and sucrose. The vitamin mix consisted of niacin, calcium pantothenate, pyridoxine HCl, thiamine HCl, riboflavin, folic acid, biotin, vitamin B₁₂, vitamin A palmitate, vitamin D₃, vitamin K₂/dextrose mix, and sucrose. For acclimatization purposes, the mice were placed in individual metabolic cages for 24 hours at 6 and 7 weeks of age. At 8 weeks of age, the mice were placed in metabolic cages for a 24-hour predose urine collection. After urine collection, half of the mice were switched to the same vitamin E-deficient base diet but supplemented with 500 mg/kg DL- α -tocopheryl acetate (Glanbia Nutrition, Carlsbad, CA); the remaining half continued on the vitamin E-deficient diet. This dosage was chosen to be equivalent to the dosage received by a 70-kg human taking vitamin E supplements. These supplements typically contain 400–600 mg of α -tocopherol. For a 70-kg human, this equates to approximately 5.7–8.6 mg α -tocopherol/kg human weight. Therefore, for a 30-g mouse who eats a 5-g vitamin E-enriched diet/day, they will ingest 2.5 mg of α -tocopherol. At 9 and 10 weeks of age, the mice were placed in metabolic cages for 24-hour urine collection and then were killed under CO₂. Livers were harvested, flash frozen in liquid N₂, and stored at -80°C .

A second set of mice were gavaged with strong PXR activators to compare the expression of PXR activated genes with those induced by α -tocopherol. From 3 weeks of age, hPXR ($n = 9$), Pxr-null ($n = 9$), and wild-type ($n = 10$) mice bred at NCI-Bethesda were maintained under a standard 12-hour light/dark cycle with water and fed ad libitum the vitamin E-deficient diet. At 6 weeks of age, wild-type ($n = 5$), Pxr-null ($n = 4$), and hPXR ($n = 4$) mice were gavaged with 100 μL of corn-oil daily for 4 days. The remaining wild-type ($n = 5$) and Pxr-null ($n = 5$) mice were gavaged with PCN (50 mg/kg per mouse dissolved in corn oil), and the hPXR ($n = 5$) mice with rifampicin (10 mg/kg per mouse dissolved in corn oil) daily for 4 days. On day 5, the mice were killed by CO₂ asphyxiation, and their livers were harvested, flash frozen in liquid N₂, and stored at -80°C .

Chemical Synthesis. To verify the identities of α -CEHC acyl glucuronide, α -CEHC ether glucuronide, and α -CEHC glycine, standards were synthesized in-house as described previously elsewhere (Johnson et al., 2012).

Urine Preparation for UPLC-ESI-QTOFMS-Based Metabolomics. Urine samples were thawed, and 50 μL was added to a microcentrifuge tube containing 50 μL of acetonitrile/water (50:50 v/v) and 5 μM chlorpropamide stored at 4°C . The samples were vortexed for 1 minute each and then centrifuged at 14,000g for 20 minutes at 4°C to remove proteins and particulates. The supernatant was transferred to an UPLC vial (Waters Corp., Milford, MA). Pooled samples were also made for quality control containing 5 μL of each sample. The samples were then randomized and analyzed by ultraperformance liquid chromatography electrospray ionization time-of-flight mass spectrometry (UPLC-ESI-QTOF-MS) as described previously (Johnson et al., 2011) using a reverse-phase 50×2.1 mm Acquity 1.7 μm BEH C18 column (Waters Corp.) and an Acquity UPLC system (Waters Corp.). Mass spectrometry was performed on a Waters QTOF-Premier-MS operating in ESI[−] and ESI⁺ mode.

The mass spectral data were centroided, integrated, and deconvoluted to generate a multivariate data matrix using MarkerLynx (Waters Corp.). Peak picking, alignment, deisotoping, and integration were performed automatically by the software and then were transformed into a multivariate matrix containing aligned peak areas with matched mass-to-charge ratios (m/z) and retention times. The data were normalized to the peak area of the internal standard chlorpropamide, which appeared at a retention time of 5.3 minutes, 275.024 [M-H][−] and 277.041 [M+H]⁺, and were imported to SIMCA-P+ software (Umetrics, Kinnelon, NJ) for multivariate data analysis. The ESI⁺ and

ESI⁺ data were Pareto-scaled and analyzed by partial least squares discriminant analysis and orthogonal partial least squares discriminant analysis (OPLS-DA). For identification of metabolites, OPLS-DA models were constructed comparing predose to postdose samples. Ions with a $p(\text{corr})$ value above 0.8 and peak area above 100 were subjected to tandem MS. Further confirmation of identity was then performed by repeating the tandem MS fragmentation using authentic standards at 100 μM in water and in urine.

Biomarkers were quantitated by multiple reaction monitoring (MRM) on an Acquity UPLC coupled to a XEVO triple-quadrupole tandem MS (Waters Corp.) to obtain the actual concentration of each metabolite normalized to the endogenous creatinine concentration. Standard calibration curves were made, and the following MRM transitions were monitored for creatinine (114.0 $\rightarrow$ 86.1 m/z ESI⁺), α -CEHC ether glucuronide (455.2 $\rightarrow$ 279.2 m/z ESI⁺), α -CMBHC ether glucuronide (497.1 $\rightarrow$ 165.1 m/z ESI⁺), and α -CEHC glycine (336.2 $\rightarrow$ 261.2 m/z ESI⁺), using authentic standards. Urine was deproteinized in 50% acetonitrile and diluted 1:2. An internal standard of chlorpropamide (277.1 $\rightarrow$ 110.9 m/z ESI⁺) was added to each sample with a final concentration 1 μM . The samples were quantitated using TargetLynx (Waters Corp.) software.

Gene Expression Analysis. Complementary DNA was synthesized from 1 μg total RNA using the Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA). For quantitative polymerase chain reaction analysis, primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA) based on GenBank sequence data and crossed exon-exon junctions. The quantitative polymerase chain reactions contained 25 ng of cDNA, a 150 nM concentration of each primer, and 5 μl of SYBR Green polymerase chain reaction Master Mix (Applied Biosystems) in a total volume of 10 μl . All reactions were performed in triplicate on an Applied Biosystems Prism 7900HT Sequence Detection System. Relative mRNA levels were calculated by the comparative threshold cycle method using β -actin as the internal control.

Transient Transfection Assay. HepG2 cells (ATCC, Manassas, VA) were plated in 12-well plates and cultured in Dulbecco's modified Eagle's medium + 10% fetal bovine serum (FBS). Cells were transfected with 10 ng of mouse PXR, 100 ng of the firefly luciferase DR3 reporter construct (kind gift of Grace Guo, University of Kansas Medical Center), and 2 ng of the control *Renilla* plasmid pRL-Sv40 with FuGENE Transfection Reagent (Promega, Madison, WI). Twenty-four hours after transfection, the media were removed and replaced with media containing 10 μM PCN (in dimethylsulfoxide) or increasing concentrations of α -tocopherol. For α -tocopherol dosing, α -tocopherol stock solution in ethanol was added dropwise to FBS and stored for 4 hours at 4°C, as previously described (Parker and Swanson, 2000). This α -tocopherol-enriched FBS was then diluted 1:10 with Dulbecco's modified Eagle's medium before addition to the cells. Twenty-four hours after treatment, the cells were lysed, and the luciferase assay was performed using the Dual Luciferase Assay Kit (Promega). Firefly luciferase values were normalized to *Renilla* values for each well and were represented as fold over the control treatment (dimethylsulfoxide).

Statistical Analysis. Concentrations of α -tocopherol metabolites, gene expression fold change, and gene relative expression were expressed as mean $\pm$ S.E.M. after Student's *t* test on unpaired samples using GraphPad Prism v5.00 (GraphPad Software, Inc., San Diego, CA) with the assumption that there was a normal population distribution. Comparisons with $P < 0.05$ were statistically significant and were noted on each graph. The Mann-Whitney *U* test was also performed because of the low sample number in each group, which confirmed that each comparison was statistically significant (unpublished data).

Results

Metabolomics Analysis. UPLC-ESI-QTOFMS analysis was performed on urine samples collected from mice before and after dosing with α -tocopherol deficient and enriched diets. The projection to latent structures discriminant analysis models constructed for each mouse strain clearly showed clustering based on the diet the mice were fed (Fig. 1, A–C). OPLS-DA models were then generated for each mouse strain to compare urine from mice fed the α -tocopherol-deficient diets with urine from 7 days of α -tocopherol supplementation or 14 days of

α -tocopherol supplementation. Ions that were highly correlated to α -tocopherol dosing were subjected to tandem MS and compared against authentic standards that were synthesized in-house. The ions were confirmed as α -CEHC ether glucuronide, α -CMBHC ether glucuronide, and α -CEHC glycine. The urinary concentrations of these metabolites were quantitated using MRM, revealing no statistically significant difference in the concentration of each urinary metabolite among the wild-type, *Pxr*-null, and *hPXR* mice (Fig. 2). There was also no statistically significant difference between day 7 and day 14 for each mouse strain in the excretion of the metabolites.

P450 Gene Expression. Gene-expression analysis was performed on a number of hepatic P450s from mice fed the α -tocopherol-deficient and α -tocopherol-enriched diets (Fig. 3A). Induction of *Cyp3a11* by α -tocopherol was confirmed in wild-type mice; however, *Cyp3a11* expression was unchanged in the *Pxr*-null and *hPXR* mice. In wild-type mice, *Cyp4f13* was induced 1.6-fold ($P = 0.001$) after α -tocopherol supplementation, but no changes were observed in *Pxr*-null or *hPXR* mice. *Cyp4f14*, *Cyp4f15*, *Cyp4f16*, and *Cyp4f18* expression was unaffected by α -tocopherol supplementation in all mouse strains studied. To determine whether α -tocopherol could up-regulate other drug metabolizing P450 enzymes, gene expression analysis was performed on mRNA encoded by *Cyp1a2*, *Cyp2e1*, *Cyp2b10*, and *Cyp4a10*. Of these genes, *Cyp1a2*, *Cyp2e1*, and *Cyp2b10* were up-regulated only in the wild-type mice, 1.5-fold ($P = 0.027$), 1.9-fold ($P = 0.027$), and 7.3-fold ($P = 0.022$), respectively. *Cyp4a10* was statistically significantly decreased in *hPXR* mice 2.4-fold ($P = 0.017$).

As members of the *CYP2C* family have been identified as PXR and CAR targets, the expression of several *CYP2C* family members was investigated (van Waterschoot et al., 2009; Konno et al., 2010). *Cyp2c29* was increased 2.4-fold ($P = 0.040$), 1.5-fold ($P = 0.002$), and 1.8-fold ($P = 0.044$) in the wild-type, *Pxr*-null, and *hPXR* mice, respectively, and in mice fed the α -tocopherol-enriched diet compared with the deficient diet. The relative expression of this gene was highest compared with other genes in the *Cyp2c* family (Fig. 3B). *Cyp2c55* was increased 2.2-fold ($P = 0.007$) and 2.9-fold ($P = 0.007$) in *Pxr*-null and *hPXR* mice, respectively; however, the relative gene expression was very low. In addition, *Cyp2c37* was increased 2.0-fold ($P = 0.005$) in the *hPXR* mice. *Cyp2c44* showed an increased trend in *Pxr*-null mice fed the α -tocopherol-enriched diet (1.4-fold, $P = 0.052$) whereas the expression of *Cyp2c39* was not affected by α -tocopherol in any of the mouse strains.

Gene-expression analysis was also performed on the same hepatic phase I drug metabolizing enzymes in mice gavaged with strong PXR activators (Fig. 4). PCN was given by oral gavage to wild-type and *Pxr*-null mice, and rifampicin to *hPXR* mice. This was done to compare the expression of PXR activated genes with those induced by α -tocopherol. PCN gavage to wild-type mice resulted in the up-regulation of the following genes: *Cyp3a11*, 20.9-fold ($P < 0.001$); *Cyp2b10*, 160-fold ($P = 0.001$); *Cyp2c29*, 9.2-fold ($P = 0.001$); and *Cyp2c55*, 303.3-fold ($P = 0.001$). After the rifampicin gavage to *hPXR* mice, *Cyp3a11* was up-regulated 4.5-fold ($P < 0.001$); *Cyp2b10*, 16.5-fold ($P = 0.005$); *Cyp2c29*, 3.7-fold ($P = 0.001$); *Cyp2c55*, 171.9-fold ($P = 0.002$); and *Cyp2c37* 1.9-fold ($P = 0.001$). However, in the *Pxr*-null mice, only *Cyp2c29* was increased after PCN gavage, 1.6-fold ($P = 0.035$).

To determine whether α -tocopherol can activate mouse PXR, *in vitro* reporter gene assays were performed. α -Tocopherol was administered at doses of 2.5, 25.0, and 50.0 μM . PXR was activated 1.7-fold ($P < 0.001$) and 1.6-fold ($P = 0.015$) at 50 μM and 2.5 μM doses of α -tocopherol, respectively (Fig. 5). As a positive control, PCN activated PXR 9.5-fold ($P = 0.005$).

Fig. 1. Projection to latent structures discriminant analysis scores plot from urine samples analyzed by UPLC-ESI-QTOFMS. (A) Wild-type mice ($R^2X = 0.403$, $R^2Y = 0.993$, $Q^2 = 0.859$). (B) *Pxr*-null mice ($R^2X = 0.218$, $R^2Y = 0.856$, $Q^2 = 0.531$). (C) *hPXR* mice ($R^2X = 0.301$, $R^2Y = 0.948$, $Q^2 = 0.731$). □, the predose α -tocopherol deficient diet; △, the day-7 α -tocopherol-deficient diet; ◇, the day 14 α -tocopherol-deficient diet. ■, the predose α -tocopherol-enriched diet. ▲, the day-7 α -tocopherol-enriched diet. ◆, the day-14 α -tocopherol-enriched diet.

UGT Gene Expression Analysis. As glucuronide conjugates of α -CEHC and α -CMBHC were identified in mouse urine after dietary supplementation with α -tocopherol, and UDP glucuronosyltransferase (UGT) genes are regulated by PXR (Buckley and Klaassen, 2009; Mackenzie et al., 2010), gene expression analysis of the major hepatic UGT isoforms was performed. There was no difference in the expression of the major *Ugt* isoforms between the mice fed the α -tocopherol-deficient and α -tocopherol-enriched diets (Fig. 6A).

However, PCN gavage to wild-type mice did induce the expression of *Ugt1a10*, 2.8-fold ($P = 0.001$); *Ugt1a1*, 3.1-fold ($P < 0.001$);

Ugt2b34, 3.2-fold ($P < 0.001$); *Ugt1a9*, 9.4-fold ($P < 0.001$); and *Ugt2b5*, 1.4-fold ($P = 0.005$) (Fig. 6B). PCN gavage to *Pxr*-null mice resulted in the up-regulation of one gene, *Ugt1a10*, 1.8-fold ($P = 0.015$). Rifampicin dosing to *hPXR* mice up-regulated *Ugt1a10*, 2.7-fold ($P = 0.009$); *Ugt1a1*, 1.5-fold ($P = 0.025$); *Ugt2b34*, 2.5-fold ($P = 0.038$); *Ugt1a9*, 8.4-fold ($P = 0.009$); and *Ugt1a7c* 2.1-fold in *hPXR* mice ($P = 0.007$). The *Ugt1a6* genes were down-regulated after PCN or rifampicin dosing as follows: *Ugt1a6b*, 1.3-fold ($P = 0.025$) in wild-type mice and 1.8-fold ($P = 0.005$) in *hPXR* mice; and *Ugt1a6a*, 2.0-fold in *hPXR* mice ($P = 0.007$).

Fig. 2. Mean concentrations of α -tocopherol metabolites identified in urine. Urinary α -tocopherol metabolite concentrations were normalized to urinary creatinine and expressed as μmol of metabolite per mmol of creatinine. No α -tocopherol metabolites were detected in the mice fed an α -tocopherol-deficient. Error bars are S.E.M. after two-tailed Student's *t* test. No statistical significance was observed among mouse strains for the excretion of each metabolite.

Fig. 3. (A) Hepatic gene expression analysis by quantitative polymerase chain reaction (qPCR) from mice fed α -tocopherol-deficient and α -tocopherol-enriched diets. (B) Relative hepatic gene expression of *Cyp2c* genes by qPCR from mice fed α -tocopherol-deficient and α -tocopherol-enriched diets. All values were normalized to β -actin and expressed as fold change. Error bars are S.E.M., statistical significance as determined by two-tailed Student's *t* test between the α -tocopherol-deficient diet and α -tocopherol-enriched diets. **P* < 0.05; ***P* < 0.01.

Discussion

MS-based metabolomics was used to reveal three urinary α -tocopherol metabolites in wild-type, *Pxr*-null, and *hPXR* mice: α -CEHC ether glucuronide, α -CMBHC ether glucuronide, and α -CEHC glycine. These metabolites were produced via an initial ω -hydroxylation step that converts α -tocopherol into 13'-hydroxy- α -tocopherol and then a series of β -oxidation steps to produce α -CMBHC and α -CEHC. Earlier studies revealed that the ω -hydroxylation step was performed by P450s, predominantly CYP3A4 in HepG2 cells, or *Cyp3a11* in mouse models (Birringer et al., 2001, 2002; Traber et al., 2005; Mustachich et al., 2009). This enzyme is regulated by PXR (Bertilsson et al., 1998). It was also hypothesized that α -tocopherol could be a ligand for PXR, thus inducing its own metabolism (i.e., autoinduction). After dosing with α -tocopherol, *Cyp3a11* was up-regulated only in wild-type mice, while dosing with known murine and human PXR-specific ligands up-regulated *Cyp3a11* in both the wild-type and *hPXR* mice. The possibility does exist that α -tocopherol activates human PXR but that this form of activated PXR is not able to transactivate the murine *Cyp3a11* promoter while a strong human PXR agonist like rifampicin is able to activate *Cyp3a11*. The activation of murine PXR by α -tocopherol was confirmed through luciferase reporter assay, although PXR activation by α -tocopherol was minimal (Fig. 5).

Previously, species-specific responses to ligand activation by human and mouse PXR were seen for a number of xenobiotics (Lehmann et al., 1998; Cheng et al., 2011). Although α -tocopherol induces *Cyp3a11* expression, no statistically significant difference in the concentration of α -tocopherol urinary metabolites was observed between wild-type and *Pxr*-null mice. Therefore, the basal protein level of CYP3A may be sufficient to metabolize α -tocopherol in

wild-type, *hPXR*, and *Pxr*-null mice. Indeed, there is no difference in basal CYP3A expression between wild-type, *hPXR*, and *Pxr*-null mice (Ma et al., 2007).

The CYP4F family was postulated to be involved in the ω -hydroxylation of α -tocopherol (Sontag and Parker, 2002, 2007), and studies have linked the regulation of this family to PPAR α (Issemann and Green, 1990; Kalsotra and Strobel, 2006). Substrates for human CYP4F enzymes are leukotriene B₄ (Chen and Hardwick, 1993), arachidonic acid, and α -tocopherol (Sontag and Parker, 2007); however, limited substrates have been found for mouse CYP4F enzymes (Kalsotra and Strobel, 2006). A recent study revealed that *Cyp4f14* is the major vitamin E ω -hydroxylase in mice (Bardowell et al., 2012). However, *Cyp4f13* was the only *Cyp4f* gene up-regulated in the present study in wild-type mice, and no *Cyp4f* genes were increased in the *Pxr*-null or *hPXR* mice after α -tocopherol dosing. In contrast to the up-regulation of *Cyp4f13* in wild-type mice after α -tocopherol dosing, the known peroxisome proliferator-activated receptor- α (PPAR α) target gene *Cyp4a10* was repressed in *hPXR* mice after α -tocopherol dosing, suggesting a complex role for α -tocopherol in PPAR α signaling.

Regulation of other cytochrome P450s that are under the control of various nuclear receptors were investigated, including constitutive androstane receptor (CAR), and the aryl hydrocarbon receptor. Expression of the aryl hydrocarbon receptor target gene *Cyp1a2* and CAR target gene *Cyp2b10* were significantly up-regulated in the wild-type mice after α -tocopherol dosing, revealing possible novel roles for α -tocopherol in the regulation of these enzymes. The *Cyp2c* family of genes was also up-regulated after α -tocopherol treatment.

The *Cyp2c* family is regulated by PXR (*Cyp2c55*) (van Waterschoot et al., 2009; Konno et al., 2010) and CAR in mice (*Cyp2c37*,

Fig. 4. Hepatic gene-expression analysis by qPCR from wild-type and *Pxr*-null mice gavaged with corn oil or PCN, and hPXR mice gavaged with corn oil or rifampicin. All values were normalized to β -actin and expressed as fold change. Error bars are S.E.M., statistical significance as determined by two-tailed Student's *t* test between corn oil gavage and PCN/rifampicin. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

Cyp2c29) (Jackson et al., 2004, 2006; van Waterschoot et al., 2009; Konno et al., 2010). However, human *CYP2C9* regulation was shown to be performed by PXR, CAR (Al-Dosari et al., 2006), vitamin D receptor (Drocourt et al., 2002), hepatocyte nuclear factor 4 α (Ibeanu

and Goldstein, 1995), hepatocyte nuclear factor 3 γ (Bort et al., 2004), glucocorticoid receptor (Gerbal-Chaloin et al., 2002), and CCAAT/enhancer-binding protein α (Jover et al., 1998; Al-Dosari et al., 2006). The activation of PXR by PCN reveals that *Cyp2c29* and *Cyp2c55* are regulated by mouse PXR whereas *Cyp2c29*, *Cyp2c55*, and *Cyp2c37* are regulated by rifampicin-activated human PXR. The results show that activation of PXR cannot only induce *Cyp3a11* but also *Cyp2b10*. *Cyp2b10* is induced by CAR, but it has been shown that PXR can regulate *Cyp2b* genes, and in turn CAR can regulate *Cyp3a* genes (Xie et al., 2000). Therefore, it is apparent that there is cross-talk between PXR and CAR-mediated gene regulation, and that regulation of the *Cyp2c* gene family by α -tocopherol may involve both PXR and CAR. Indeed, the increased expression of *Cyp2b10* in α -tocopherol-treated wild-type mice and *Cyp2c29*, *Cyp2c55*, and *Cyp2c37* in the *Pxr*-null mice dosed with α -tocopherol indicates that α -tocopherol may activate CAR.

As PXR can also up-regulate UGT (Xie et al., 2003; Buckley and Klaassen, 2009), gene-expression analysis of the major hepatic *Ugt* isoforms was performed. There was no statistically significant change in *Ugt* expression after α -tocopherol dosing (Fig. 6). The same isoforms were also analyzed from mice dosed with PCN or rifampicin, and some *Ugts* were induced, including the known PXR target gene *Ugt1a1* (Xie et al., 2003). However the induction was not as robust as was seen with the *Cyp* genes, suggesting that *Ugt* genes are not as responsive to xenobiotic induction.

Fig. 5. Activation of mouse PXR by α -tocopherol. HepG2 cells were transfected with mouse PXR, DR3 reporter luciferase, and pRL-SV40 constructs. Cells were treated with 10 μ M PCN or 2.5, 25.0, or 50.0 μ M α -tocopherol for 24 hours. The firefly luciferase value was normalized to the *Renilla* luciferase value and was expressed as fold change over dimethyl sulfoxide (DMSO), PCN/rifampicin. **P* < 0.05; ****P* < 0.001.

Fig. 6. Hepatic gene-expression analysis of UGTs by qPCR. (A) Wild-type, *Pxr*-null, and *hPXR* mice fed α -tocopherol-deficient and α -tocopherol-enriched diets. (B) Wild-type and *Pxr*-null mice gavaged with corn oil or PCN, and *hPXR* mice gavaged with corn oil or rifampicin. All values were normalized to β -actin and expressed as fold change. Error bars are S.E.M., statistical significance as determined by two-tailed Student's *t* test between corn oil gavage and PCN/rifampicin. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

Previously, *Cyp3a11* and *Cyp2b10* had been shown to be activated by PCN in wild-type mice, but a much larger increase in *Cyp2b10* was seen after induction of CAR by 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene, showing that CAR has a greater effect on *Cyp2b10* expression than PXR (Maglich et al., 2002). Others (Mustacich et al., 2009) have proposed that α -tocopherol regulates drug metabolizing enzyme-expression through CAR rather than PXR due to low PXR activation in HepG2 cells (Landes et al., 2003) and primary hepatocytes (Zhou et al., 2004). The induction of *Cyp3a11* and *Cyp2b10* by α -tocopherol is lost in *Pxr*-null mice, indicating that PXR is the primary nuclear receptor involved in α -tocopherol induction of these genes. However, the involvement of CAR in induction of *Cyp2c29* cannot be ruled out because of the induction observed in *Pxr*-null mice after α -tocopherol dosing. The absence of *Cyp3a11* induction in *hPXR* mice indicates that α -tocopherol is a mouse PXR partial agonist and the induction of the *Cyp2c* genes in all three mouse strains studied is linked to CAR or another transcription factor.

In humans, CYP2C9, CYP2C8, and CYP2C19 metabolize approximately 20% of pharmaceutical drugs. Therefore, coadministration of α -tocopherol with other xenobiotics could result in faster clearance and lower plasma levels of a therapeutic drug. There have been numerous clinical trials of vitamin E for disease amelioration, which have resulted in contradictory data. There are at least two known polymorphisms in the *CYP2C9* promoter, which allows for the possibility of reduced response to transcriptional activation by PXR that could contribute to interindividual variation (Yin et al., 2008). *CYP3A4* is also highly polymorphic with known mutations of varying prevalence in different ethnic groups in the promoter region as well as the coding region, which could affect α -tocopherol induction of *CYP3A4* as well as the metabolism of α -tocopherol (McGraw and Waller, 2012). Therefore, genetic polymorphisms and coadministration with other xenobiotics

could be routes for further investigation when investigating disparities seen in trials of the therapeutic value of vitamin E supplementation.

Authorship Contributions

Participated in research design: Johnson, Bonzo, Cheng, Krausz, Idle, Gonzalez.

Conducted experiments: Johnson, Bonzo, Cheng, Krausz.

Contributed new reagents or analytic tools: Kang, Luecke.

Performed data analysis: Johnson, Bonzo.

Wrote or contributed to the writing of the manuscript: Johnson, Bonzo, Cheng, Idle, Gonzalez.

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