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Cytochrome P450 Regulation by α-Tocopherol in *Pxr*-null and Humanized-*PXR* mice

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Running Title: The regulation of CYP gene expression by α-tocopherol

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Nonstandard abbreviations: CAR, constitutive androstane receptor; α-CEHC, α-carboxyethylhydroxychroman; α-CMBHC, α-carboxymethylbutylhydroxychroman; CYP, cytochrome P450; ESI, electrospray ionization; h*PXR*, humanized-*PXR*; MDA, multivariate data analysis; MRM, multiple reaction monitoring; MS, mass spectrometry; PCN, pregnenolone-16α-carbonitrile**;** PLS-DA, projection to latent structures discriminant analysis; PXR, pregnane X receptor; OPLS-DA, orthogonal projection to latent structures discriminant analysis; UPLC, Ultra-performance liquid chromatography; UPLC-ESI-QTOFMS, Ultra-performance liquid chromatography electrospray ionization time-of-flight mass spectrometry;

Abstract

The pregnane X receptor (PXR) has been postulated to play a role in the metabolism of α-tocopherol due to the upregulation of hepatic cytochrome P450 (CYP) 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 between 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 cytochrome 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 suggesting 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; 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 (CYP) enzymes induced after α-tocopherol dosing carry out 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 homologue 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 *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 CYP enzymes are 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 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 16α-carbonitrile (PCN) or in *PXR*-humanized (h*PXR*) mice by rifampicin decreased vitamin E metabolism compared to *Pxr*-null mice (Cho et al., 2009). This may have been due to downregulation of β-oxidation by PCN and rifampicin. 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 biological research, the genetic differences that exist between mice and humans can have a major impact on drug metabolism. To overcome this problem h*PXR* 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 ~ 10% of U.S adults took a vitamin E supplement (Picciano and McGuire, 2009), and as PXR is also involved in the detoxication 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 CYP genes were analyzed in h*PXR*, *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.. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS)-based metabolomics was 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. Pregnenolone 16α-carbonitrile (PCN), corn oil, rifampicin, and chlorpropamide were obtained from Sigma Aldrich (St. Louis, MO). All other chemicals required for UPLC-MS were obtained from 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 three weeks of age, male h*PXR* (n=13), *Pxr*-null (n=13), and wild-type (n=9) mice bred at NCI-Bethesda were maintained under a standard 12 h light/12 h dark cycle with water. Mice were fed *ad libitum* a purified diet containing vitamin free casein, sucrose, cornstarch, dyetrose, L-cystine, cellulose, tocopherol stripped soybean oil, mineral mix (Cat#210050, Dyets, Inc, Bethlehem, PA), vitamin mix with no vitamin E (Cat# 319508, 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 h at six and seven weeks of age. At eight weeks of age the mice were placed in metabolic cages for a 24 h 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 dose was chosen to be equivalent to the dose 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 a-tocopherol/kg human weight. Therefore for a 30 g mouse who eats 5 g vitamin E enriched diet/day they will ingest 2.5 mg of α-tocopherol. At nine and 10 weeks of age, the mice were placed in metabolic cages for 24 h urine collection and then euthanized under CO_2. Livers were harvested, flash frozen in liquid N_2, and stored at -80ºC.

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 three weeks of age, h*PXR* (n=9), *Pxr*-null (n=9), and wild-type (n=10) mice bred at NCI-Bethesda, were maintained under a standard 12 h light/12 h dark cycle with water and fed *ad libitum* the vitamin E deficient diet. At six weeks of age, wild-type (n=5), *Pxr*-null (n=4), and h*PXR* (n=4) mice were gavaged with 100 µl corn-oil daily for four days. The remaining wild-type (n=5) and *Pxr*-null (n=5) mice were gavaged with PCN (50 mg/kg/mouse dissolved in corn oil), and the h*PXR* (n=5) mice with rifampicin (10 mg/kg/mouse dissolved in corn oil) daily for four days. On day five, the mice were killed by CO_2 asphyxiation and their livers harvested, flash frozen in liquid N_2 and stored at -80ºC.

Chemical Synthesis. In order 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 (Johnson et al., 2012).

Urine preparation for UPLC-ESI-QTOFMS-based metabolomics. Urine samples were thawed and 50 µl added to a microcentrifuge tube containing 50 µl acetonitrile:water (50:50 v/v)

and 5 μM chlorpropamide stored at 4ºC. The samples were vortexed for 1 min each and centrifuged at 14,000 x g for 20 min at 4ºC 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 UPLC-ESI-QTOFMS as described previously (Johnson et al., 2011) using a reverse-phase 50 x 2.1 mm ACQUITY® 1.7 μm BEH C18 column (Waters Corp, Milford, MA) and an ACQUITY® UPLC system (Waters Corp, Milford, MA). 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, Milford, MA). 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 min, 275.024 $[M-H]^-$ and 277.041 $[M+H]^+$ and 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 (PLS-DA) 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(corr) value above 0.8 and peak area above 100 were subjected to tandem MS. Further confirmation of identity was then carried out 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 (QqQMS) (Waters Corp, Milford, MA), 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 → 86.1 *m/z* ESI^+), α-CEHC ether glucuronide (455.2→ 279.2 *m/z* ESI^+), α-CMBHC ether glucuronide (497.1→ 165.1 *m/z* ESI^+) and α-CEHC glycine (336.2 → 261.2 *m/z* ESI^+), using authentic standards. Urines were deproteinized in 50% acetonitrile and diluted 1:2. An internal standard of chlorpropamide (277.1→ 110.9 *m/z* ESI^+) was added to each sample with final concentration 1 μM. The samples were quantitated using TargetLynx (Waters Corp, Milford, MA) software.

Gene expression analysis. cDNA was synthesized from 1 μg total RNA using Superscript II reverse transcriptase kit (Invitrogen, Carlsbad, CA). For qPCR analysis, primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA) based on GenBank sequence data and crossed exon-exon junctions. qPCR reactions contained 25 ng of cDNA, 150 nM of each primer and 5 μl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) 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, Manasses, VA) were plated in 12-well plates and cultured in DMEM + 10% fetal bovine serum (FBS). Cells were transfected with 10 ng 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 post-transfection, media was removed and replaced with media containing 10 μM PCN (in DMSO) or increasing concentrations of α-tocopherol. For α-tocopherol dosing, α-tocopherol stock solution in ethanol was added drop-wise to FBS and stored for 4 hrs at 4°C as described (Parker and Swanson, 2000). This α-tocopherol-enriched FBS was then diluted 1:10 with DMEM prior to addition to the cells. Twenty-four hours after treatment, cells were lysed and luciferase assay was performed using the Dual Luciferase Assay Kit (Promega). Firefly luciferase values were normalized to *Renilla* values for each well and represented as fold over control treatment (DMSO).

Statistical Analysis. Concentrations of α-tocopherol metabolites, gene expression fold change and gene relative expression were expressed as mean ± standard error of the mean (SEM) after Student's t-test on unpaired samples using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA) with the assumption that there was a normal population distribution. A comparison with a *P*-value <0.05 was statistically significant and noted on each graph. Mann-Whitney U-test was also carried out due to the low sample number in each group which confirmed that each comparison was significant (data not shown).

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RESULTS

Metabolomics analysis: UPLC-ESI-QTOFMS analysis was carried out on urine samples collected from mice pre- and post-dosing with α-tocopherol deficient and enriched diets. PLS-DA models were constructed for each mouse strain that clearly showed clustering based on the diet the mice were fed (Figure 1A-C). OPLS-DA models were then generated for each mouse strain comparing urine from mice fed the α-tocopherol deficient diets to 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 significant difference in the concentration of each urinary metabolite between the wild-type, *Pxr*-null and h*PXR* mice (Figure 2). There was also no significant difference between day 7 and 14 for each mouse strain in the excretion of the metabolites.

CYP gene expression. Gene expression analysis was performed on a number of hepatic CYPs from mice fed the α-tocopherol deficient and enriched diets (Figure 3A). Induction of *Cyp3a11* by α-tocopherol was confirmed in wild-type mice; however, *Cyp3a11* expression was unchanged in the *Pxr*-null and h*PXR* mice. In wild-type mice, *Cyp4f13* was induced 1.6-fold (P=0.001), after α-tocopherol supplementation, while no changes were observed in *Pxr*-null or h*PXR* mice. *Cyp4f14*, *Cyp4f15*, *Cyp4f16* and *Cyp4f18* expression was unaffected by α-tocopherol supplementation in all mouse strains studied. In order to determine if α-tocopherol could

upregulate other drug metabolizing CYP enzymes, gene expression analysis was carried out on mRNAs encoded by *Cyp1a2, Cyp2e1, Cyp2b10* and *Cyp4a10*. Of these genes, *Cyp1a2, Cyp2e1,* and *Cyp2b10* were upregulated 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 significantly decreased in h*PXR* 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 h*PXR* mice, respectively, in mice fed the α-tocopherol enriched diet, compared to the deficient diet. The relative expression of this gene was highest compared to other genes in the *Cyp2c* family (Figure 3B). *Cyp2c55* was increased 2.2-fold (*P*=0.007) and 2.9-fold (*P*=0.007) in *Pxr*-null and h*PXR* mice; however the relative gene expression was very low. In addition, *Cyp2c37* was increased 2.0–fold (*P*=0.005) in the h*PXR* mice. *Cyp2c44* showed an increased trend in *Pxr*-null mice fed the α-tocopherol enriched diet (1.4-fold, *P*=0.052) while the expression of *Cyp2c39* was not affected by α-tocopherol in any of the mouse strains.

Gene expression analysis was also carried out on the same hepatic phase I drug metabolizing enzymes in mice gavaged with strong PXR activators (Figure 4). PCN was given by oral gavage to wild-type and *Pxr*-null mice, and rifampicin to h*PXR* mice. This was carried out in order to compare the expression of PXR activated genes with those induced by α-tocopherol. PCN gavage to wild-type mice resulted in the upregulation 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). Following rifampicin gavage to h*PXR* mice, *Cyp3a11* was upregulated 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).

In order to determine whether α-tocopherol can activate mouse PXR, *in vitro* reporter gene assays were carried out. α-Tocopherol was administered at doses of 2.5, 25, and 50 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 (Figure 5). As a positive control, PCN activated PXR 9.5-fold (*P*=0.005).

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 carried out. There was no difference in the expression of the major *Ugt* isoforms between the mice fed the α-tocopherol deficient and enriched diets (Figure 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) (Figure 6B). PCN gavage to *Pxr*-null mice resulted in the upregulation of one gene, *Ugt1a10,* 1.8-fold (*P*=0.015). Rifampicin dosing to h*PXR* mice upregulated; *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 h*PXR* mice (*P*=0.007). The *Ugt1a6* genes were downregulated after PCN or rifampicin dosing; *Ugt1a6b,* 1.3-fold (*P*=0.025)

in wild-type mice and 1.8-fold (*P*=0.005) in h*PXR* mice, *Ugt1a6a*, 2.0-fold in h*PXR* mice (*P*=0.007).

Discussion

MS-based metabolomics was used to reveal three urinary α-tocopherol metabolites in wild-type, *Pxr*-null and h*PXR* 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 carried out by CYPs, predominantly CYP3A4 in HepG2 cells, or *Cyp3a11* in mouse models (Birringer et al., 2001; Birringer et al., 2002; Traber et al., 2005; Mustacich 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 upregulated only in wild-type mice, while dosing with known murine and human PXR-specific ligands upregulated *Cyp3a11* in both the wild-type and h*PXR* 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 (Figure 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 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, h*PXR*, and *Pxr*-null mice. Indeed, there is no difference in basal CYP3A expression between wild-type, h*PXR*, and *Pxr*-null mice (Ma et al., 2007).

The CYP4F family was postulated to be involved in ω-hydroxylation of α-tocopherol (Sontag and Parker, 2002; Sontag and Parker, 2007), and studies have linked the regulation of this family to PPARα (Issemann and Green, 1990; Kalsotra and Strobel, 2006). Substrates for human CYP4F enzymes are leukotriene B_4 (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 upregulated in the present study in wild-type mice, and no *Cyp4f* genes were increased in the *Pxr*-null or h*PXR* mice after α-tocopherol dosing. In contrast to the upregulation of *Cyp4f13* in wild-type mice after α-tocopherol dosing, the known PPARα target gene *Cyp4a10* was repressed in h*PXR* mice after α-tocopherol dosing, suggesting a complex role for α-tocopherol in PPARα signalling.

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 (AhR). Expression of the AhR target gene *Cyp1a2* and CAR target gene *Cyp2b10* were significantly upregulated in the wild-type mice after α-tocopherol dosing, revealing possible novels roles for α-tocopherol in regulation of these enzymes. The *Cyp2c* family of genes was also upregulated 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, Cyp2c29*) (Jackson et al., 2004; Jackson et al., 2006; van Waterschoot et al., 2009; Konno et al., 2010). However human *CYP2C9* regulation was shown to be carried out by PXR, CAR (Al-Dosari et al., 2006), vitamin D receptor (Drocourt et al., 2002), hepatocyte nuclear factor 4α (HNF4α) (Ibeanu and Goldstein, 1995), HNF3γ (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 while *Cyp2c29*, *Cyp2c55* and *Cyp2c37* are regulated by rifampicin-activated human PXR. The results show that activation of PXR can not 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 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 upregulate UGT's (Xie et al., 2003; Buckley and Klaassen, 2009), gene expression analysis of the major hepatic *Ugt* isoforms were carried out. There was no significant change in *Ugt* expression after α-tocopherol dosing (Figure 6). The same isoforms were also analysed 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.

Previously *Cyp3a11* and *Cyp2b10* have 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 due to the induction observed in *Pxr*-null mice after α-tocopherol dosing. The absence of *Cyp3a11* induction in h*PXR* 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, co-administration 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 resulting 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 inter-individual 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 that could affect α-tocopherol induction of *CYP3A4* as well as the metabolism of α-tocopherol (McGraw and Waller, 2012). Therefore, genetic polymorphisms and co-administration 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: Kang, Luecke

Performed Data Analysis: Johnson, Bonzo

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

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Footnotes

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C.H.J. and J.A.B. contributed equally to the work.

Legend for Schemes

Scheme 1. Scheme of α-tocopherol metabolism.

Legend for Figures.

Figure 1. PLS-DA 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 ($R^2X = 0.218$, $R^2Y = 0.856$ $Q^2 = 0.531$), C:h*PXR* ($R^2X = 0.301$, $R^2Y = 0.948$, $Q^2 = 0.731$); □ predose α-tocopherol deficient diet, △ day 7 α-tocopherol deficient diet, ◇ day 14 α-tocopherol deficient diet ■ predose α-tocopherol enriched diet,▲ day 7 α-tocopherol enriched diet, ◆ day 14 α-tocopherol enriched diet.

Figure 2. Mean concentrations of α-tocopherol metabolites identified in urine. Urinary α-tocopherol metabolite concentrations were normalised to urinary creatinine and expressed as μmol of metabolite per mmol of creatinine. No α-tocopherol metabolites were detected in α-tocopherol deficient diet fed mice. Error bars are SEM after two-tailed Student's t-test. No statistical significance was observed between mouse strains for the excretion of each metabolite.

Figure 3. A: Hepatic gene expression analysis by qPCR from mice fed α-tocopherol deficient and enriched diets, B; Relative hepatic gene expression of *Cyp2c* genes by qPCR from mice fed α-tocopherol deficient and enriched diets. All values were normalized to β-actin and expressed as fold change. Error bars are SEM, significance as determined by two-tailed Student's t-test between α-tocopherol deficient diet and α-tocopherol enriched diets. *P*-value: $^{**}P<0.01$, $^{*}P<0.05$

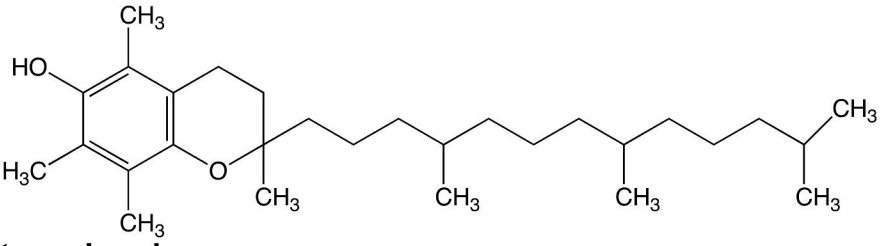
Figure 4. Hepatic gene expression analysis by qPCR from wild-type and *Pxr*-null mice gavaged with corn oil or PCN, and h*PXR* mice gavaged with corn oil or rifampicin. All values were normalized to β-actin and expressed as fold change. Error bars are SEM, significance as

determined by two-tailed Student's t-test between corn oil gavage and PCN/rifampicin. *P*-value: ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$

Figure 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 μM PCN or 2.5, 25, or 50 μM α-tocopherol for 24 hrs. The firefly luciferase value was normalized to *Renilla* luciferase value and expressed as fold change over DMO. PCN/rifampicin. *P*-value:***$P<0.001$, *$P<0.05$

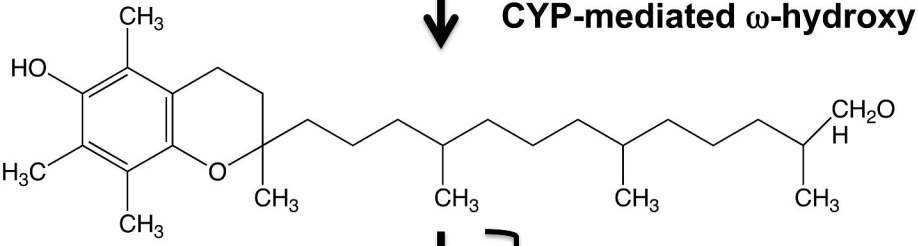
Figure 6. Hepatic gene expression analysis of UGT's by qPCR. A: Wild-type, *Pxr*-null, and h*PXR* mice fed α-tocopherol deficient and enriched diets. B: Wild-type and *Pxr*-null mice gavaged with corn oil or PCN, and h*PXR* mice gavaged with corn oil or rifampicin. All values were normalized to β-actin and expressed as fold change. Error bars are SEM, significance as determined by two-tailed Student's t-test between corn oil gavage and PCN/rifampicin. *P*-value: ****$P<0.0001$, ***$P<0.001$, **$P<0.01$, *$P<0.05$

Scheme 1



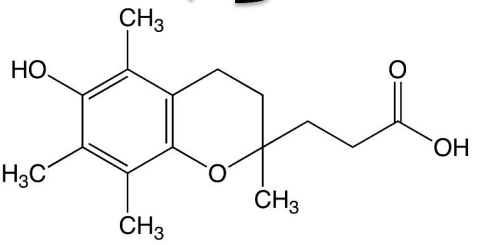
α-tocopherol

CYP-mediated ω-hydroxylation



13'-hydroxy-α-tocopherol

β-hydroxylation steps



α-carboxyethylhydroxychroman

Figure 1

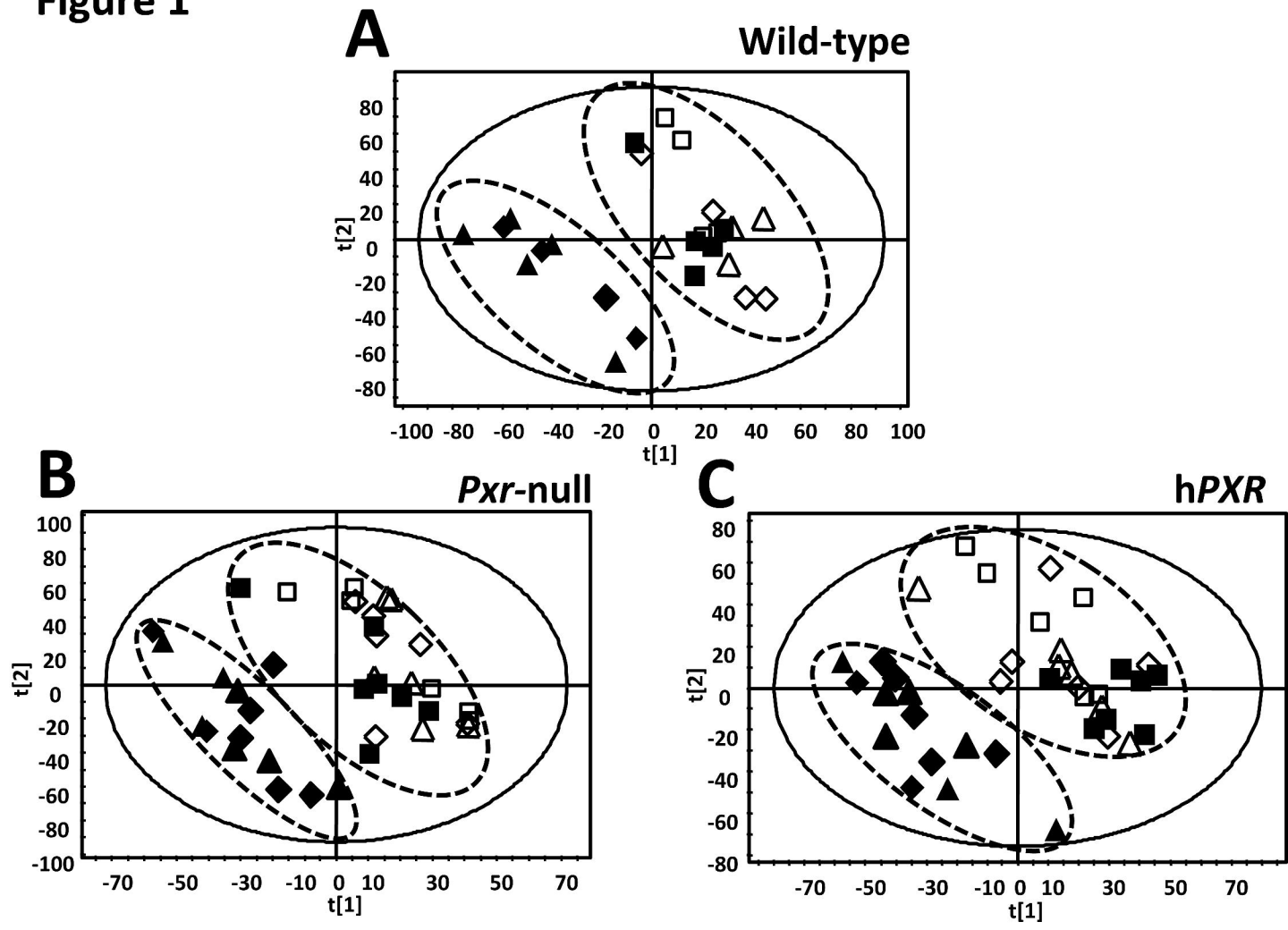


Figure 2

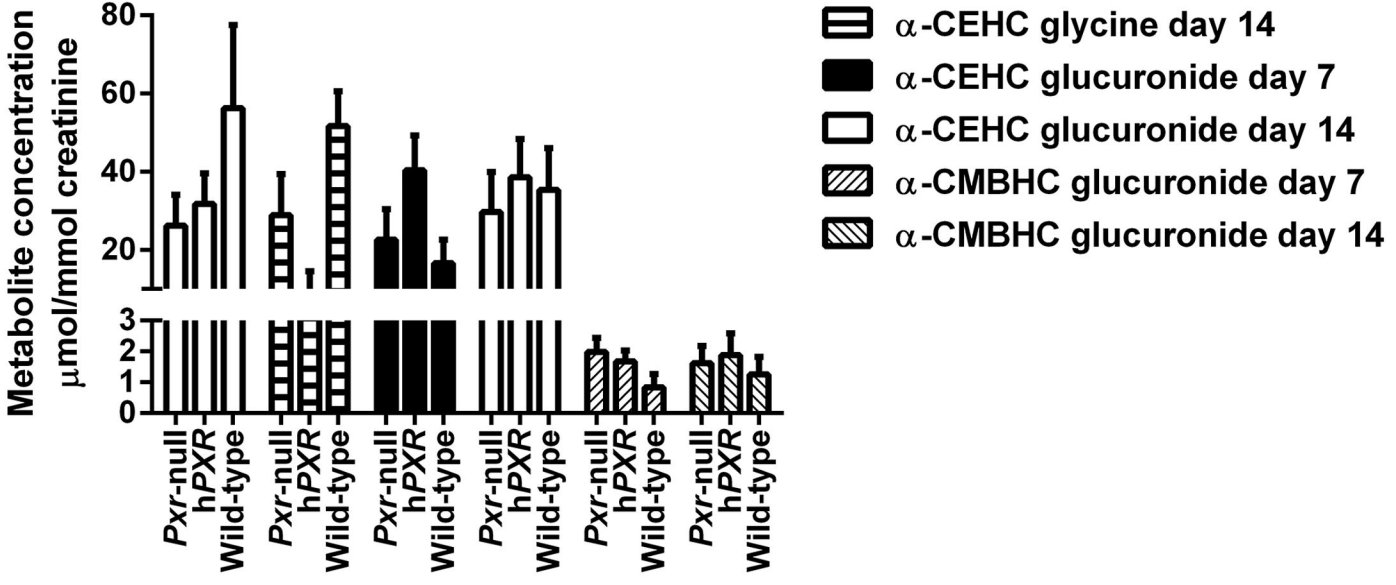

Metabolite concentration
µmol/mmol creatinine
80
60
40
20
3
2
1
0
Pxr-null
hPXR
Wild-type
α-CEHC glycine day 7
α-CEHC glycine day 14
α-CEHC glucuronide day 7
α-CEHC glucuronide day 14
α-CMBHC glucuronide day 7
α-CMBHC glucuronide day 14

Figure 3

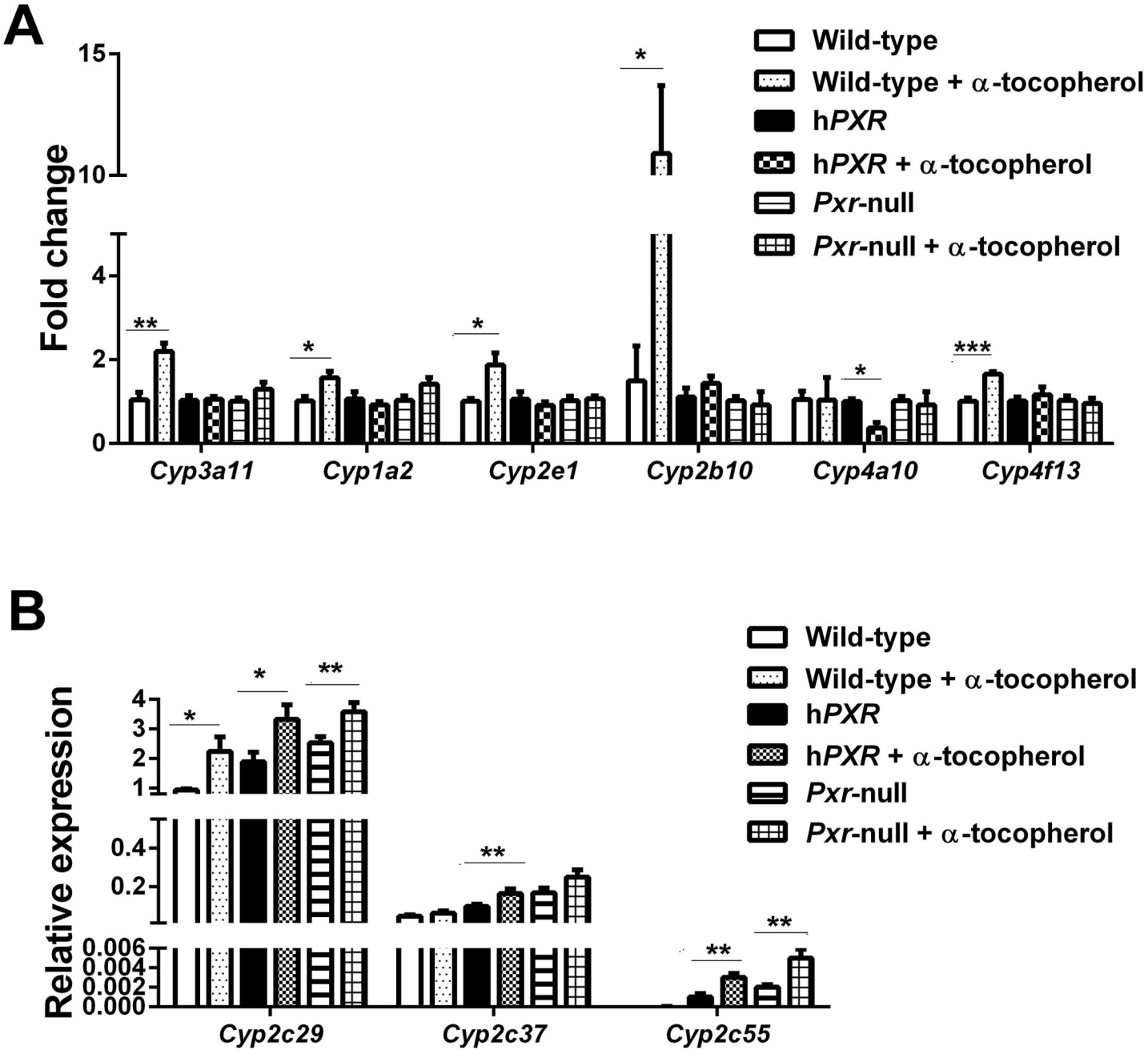

A
Fold change
15
10
4
2
0
Wild-type
Wild-type + α-tocopherol
hPXR
hPXR + α-tocopherol
Pxr-null
Pxr-null + α-tocopherol
**
*
*
*
*
*
***
Cyp3a11
Cyp1a2
Cyp2e1
Cyp2b10
Cyp4a10
Cyp4f13
B
Relative expression
4
3
2
1
0.4
0.2
0.006
0.004
0.002
0.000
Wild-type
Wild-type + α-tocopherol
hPXR
hPXR + α-tocopherol
Pxr-null
Pxr-null + α-tocopherol
*
*
**
**
**
**
Cyp2c29
Cyp2c37
Cyp2c55

Figure 4

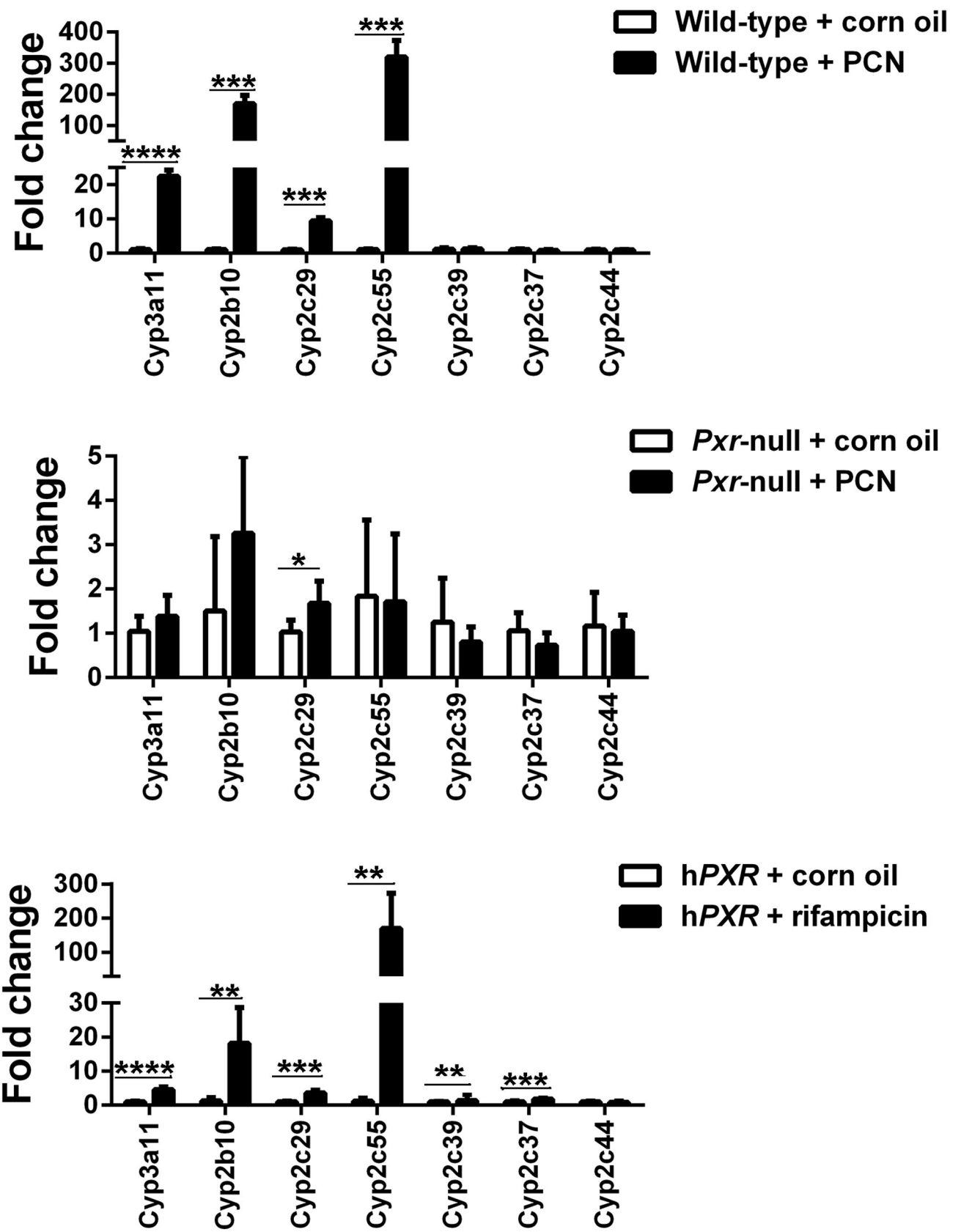

Wild-type + corn oil
Wild-type + PCN
Fold change
400
300
200
100
20
10
0
****
***
***
***
Cyp3a11
Cyp2b10
Cyp2c29
Cyp2c55
Cyp2c39
Cyp2c37
Cyp2c44
Pxr-null + corn oil
Pxr-null + PCN
Fold change
5
4
3
2
1
0
*
Cyp3a11
Cyp2b10
Cyp2c29
Cyp2c55
Cyp2c39
Cyp2c37
Cyp2c44
hPXR + corn oil
hPXR + rifampicin
Fold change
300
200
100
30
20
10
0
****
**
***
**
**
***
Cyp3a11
Cyp2b10
Cyp2c29
Cyp2c55
Cyp2c39
Cyp2c37
Cyp2c44

Figure 5

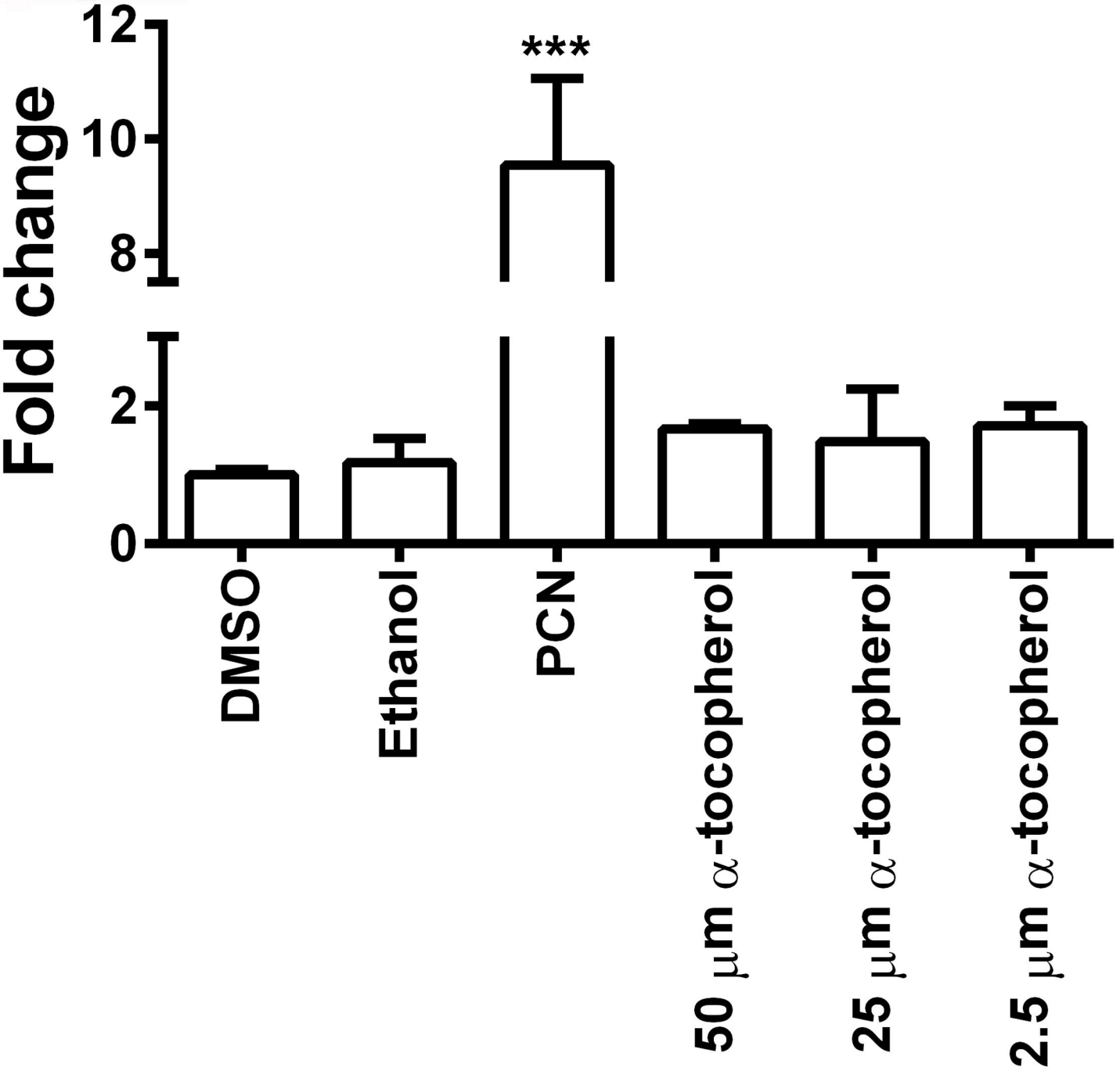
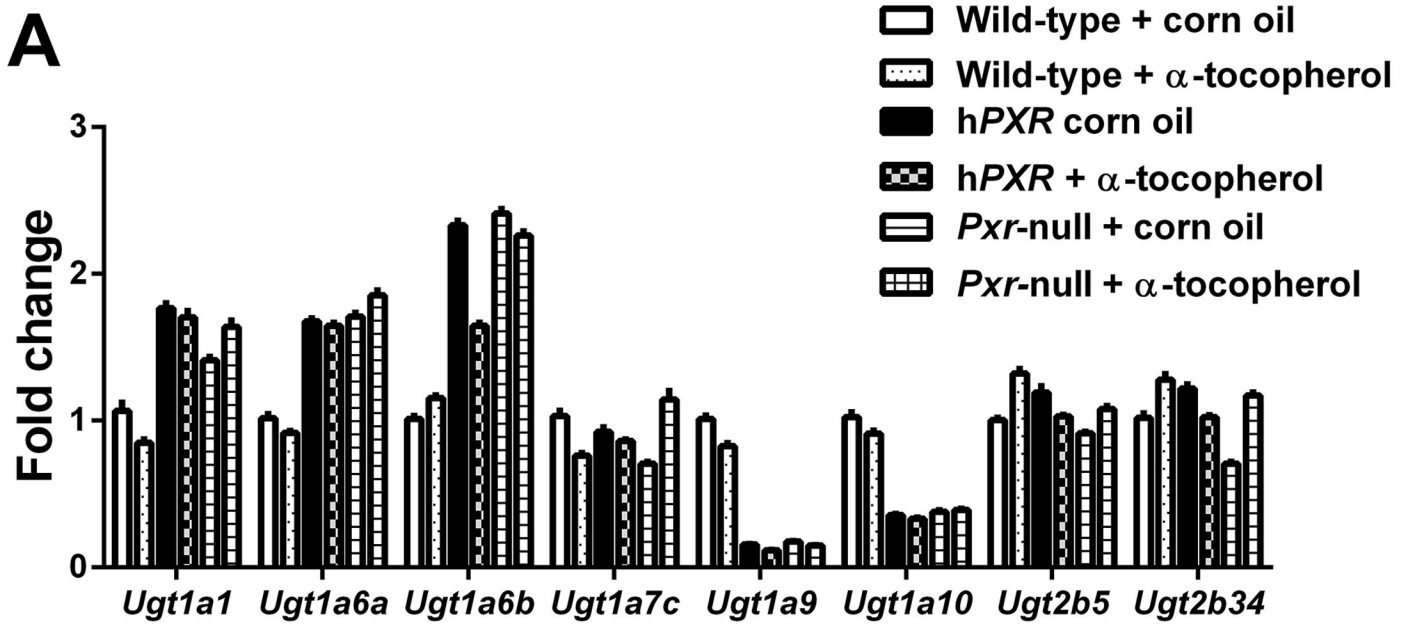
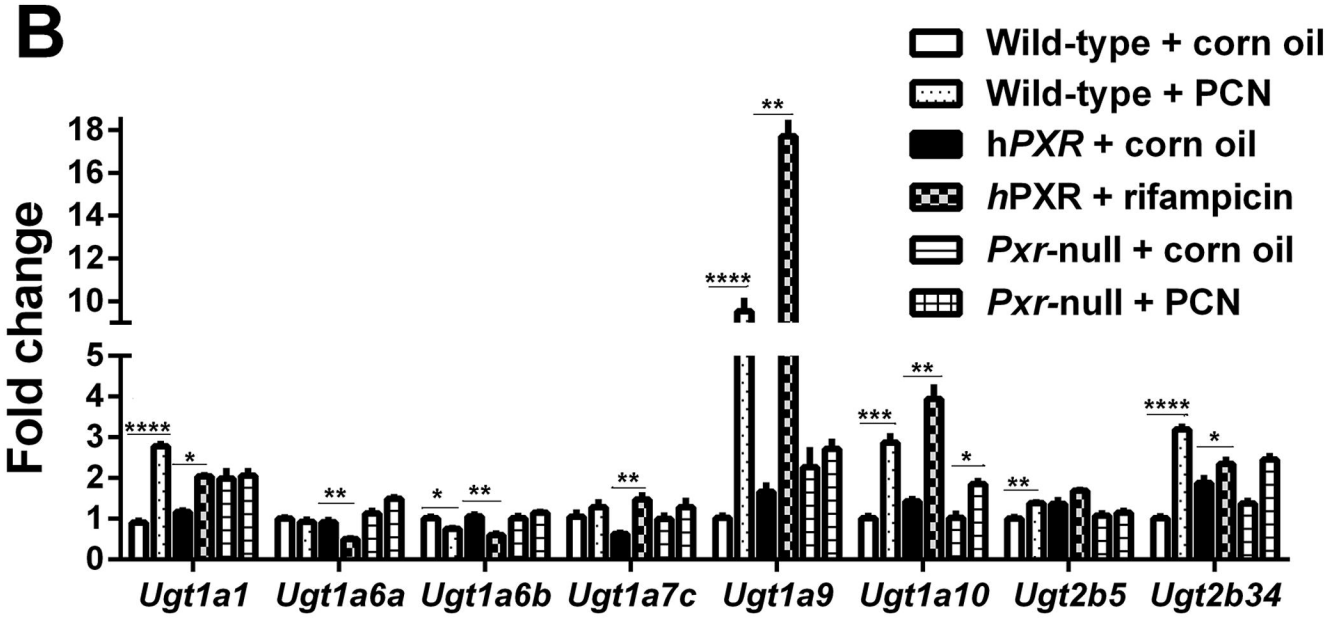

Fold change
12
10
8
2
0
***
DMSO
Ethanol
PCN
50 μm α-tocopherol
25 μm α-tocopherol
2.5 μm α-tocopherol

Figure 6


A
Wild-type + corn oil
Wild-type + α-tocopherol
hPXR corn oil
hPXR + α-tocopherol
Pxr-null + corn oil
Pxr-null + α-tocopherol
Fold change
0
1
2
3
Ugt1a1
Ugt1a6a
Ugt1a6b
Ugt1a7c
Ugt1a9
Ugt1a10
Ugt2b5
Ugt2b34


B
Wild-type + corn oil
Wild-type + PCN
hPXR + corn oil
hPXR + rifampicin
Pxr-null + corn oil
Pxr-null + PCN
Fold change
0
1
2
3
4
5
10
12
14
16
18
****
*
**
*
**
**
****
**
***
**
*
**
****
*
Ugt1a1
Ugt1a6a
Ugt1a6b
Ugt1a7c
Ugt1a9
Ugt1a10
Ugt2b5
Ugt2b34