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CYP4V2 in Bietti’s Crystalline Dystrophy: Ocular Localization, Metabolism of ω-3 Polyunsaturated Fatty Acids and Functional Deficit of the p.H331P Variant

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Number of Text Pages: 34
Number of Tables: 0
Number of Figures: 6
Number of References: 43
Number of Word in Abstract: 250
Number of Words in Introduction: 579
Number of Words in Discussion: 1309

Abbreviations:
P450, cytochrome P450; BCD, Bietti’s crystalline corneoretinal dystrophy; CYP4V2, cytochrome P450 4V2; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; ROS, rod outer segment; DLPC, 1,2-dilauroyl-sn-glycero-3-phosphocholine; BSTFA, N,O-bis(trimethylsilyl)trifluoroacetate; DTT, dithiothreitol; PBS, phosphate buffered saline; RPE, retinal pigmented epithelium; PCR, polycyclic reaction; GC-CI/MS, gas chromatography-chemical ionization/mass spectrometry; GC-FID, gas chromatography-flame ionization detection.
Abstract

Bietti’s crystalline corneoretinal dystrophy (BCD) is a recessive degenerative eye disease caused by germ-line mutations in the CYP4V2 gene. More than 80% of mutant alleles consist of three mutations; two splice site alterations and one missense mutation, viz, c.992C>A translating to p.H331P. In the present study, we analyzed the expression of CYP4 family members in human tissues and conducted functional studies with the wild-type and p.H331P enzymes to better understand the link between CYP4V2 activity and BCD. Expression analysis of 17 CYP1-4 genes showed CYP4V2 to be a major P450 in ARPE-19 cells, a human cell line spontaneously generated from normal human retinal pigment epithelium, and the only detectable CYP4 transcript. Immunohistochemical analysis demonstrated that CYP4V2 protein was present in epithelial cells of the retina and cornea, and that the enzyme was localized to endoplasmic reticulum. Recombinant reconstituted CYP4V2 protein metabolized eicosapentaenoic acid and docosahexaenoic acid, the latter an important constituent of the retina, to their respective ω-hydroxylated products at rates similar to purified CYP4F2, an established hepatic PUFA hydroxylase. The disease-associated p.H331P variant was undetectable by Western blot in HepG2 cells stably transduced with lentiviral expression vectors. Finally, over expression of functional CYP4V2 in HepG2 cells alters lipid homeostasis. Therefore, we have demonstrated that CYP4V2 protein is expressed at high levels in ocular target tissues of BCD, that the enzyme is metabolically active towards PUFAs and that the functional deficit in BCD patients carrying the H331P variant is most likely a consequence of the instability of the mutant protein.
Introduction

Bietti’s crystalline corneoretinal dystrophy (BCD) is an autosomal recessive degenerative retinopathy clinically characterized by progressive decline in central vision, night blindness and constriction of the visual field. The average age of manifestation in affected family members is around 30 years (Hu 1983). Morphologically, BCD is defined by yellow-white crystalline lipid deposits in the retina and the cornea (Bietti 1937), ultimately leading to degeneration of the retina and sclerosis of the choroidal vessels. BCD is rare in Caucasians but relatively common in Asian populations. After the genetic defect was initially linked to chromosome 4q35 (Jiao et al. 2000), Li et al. identified bi-allelic mutations in the ‘orphan’ P450 enzyme, CYP4V2, in 23 out of 25 index patients from families with BCD. Numerous research groups (Gekka et al. 2005; Lin et al. 2005; Shan et al. 2005; Wada et al. 2005; Yokoi et al. 2010) have confirmed the original genetic findings (Kelly et al. 2011). Importantly, a recent study demonstrated that more than 95% of all analyzed BCD patients have germ-line mutations in the CYP4V2 gene (Xiao et al. 2011). Although more than 34 distinct mutations have been identified, variation in exons 6 to 9 account for >80% of all mutations with at least three founder mutations existing; c.802-8_810del17insGC, c.992A>C, and c.1091-2A>G that account for 62.7%, 7.4%, and 6.4%, respectively of all mutated alleles (Xiao, 2011).

Although complex lipid deposits are also found in the circulating lymphocytes and skin fibroblasts of BCD patients (Wilson 1989; Kaiser-Kupfer et al. 1994) and expression of CYP4V2 mRNA has been detected in most human tissues (Li et al. 2004), the clinical disease phenotype seems to be restricted to the eye. The composition of these crystalline lipids has not been elucidated, but early biochemical tracer studies were indicative of a cellular defect in the
anabolism of ω-3 polyunsaturated fatty acids (PUFAs) (Lee et al. 2001). More recently, analysis of total fatty acids in the plasma of BCD patients relative to control subjects were suggestive of a defect in the synthesis of oleic acid (Lai et al. 2010).

CYP4V2 is generally referred to as an ‘orphan’ P450 because its substrate specificity is just beginning to be defined (Kelly et al. 2011). Typically, CYP4 enzymes are microsomal fatty acid ω-hydroxylases that function together with mitochondrial and peroxisomal α/β-oxidation enzymes to degrade cellular lipids. Although CYP4V2 is the most distantly related of all the human CYP4 enzymes, with a sequence identity of only ~35% (Rettie 2008), we found recently that the recombinantly expressed enzyme possesses typical CYP4 ω-hydroxylase activity towards medium-chain saturated fatty acids (Nakano et al. 2009). Therefore, it is tempting to speculate that genetic defects in the catalytic function of CYP4V2 prevent local ocular degradation of lipids that subsequently accumulate in BCD.

For the foregoing scenario to be viable, we postulated that CYP4V2 should be expressed locally in BCD target tissues, and be capable of metabolizing key ocular lipids. No quantitative data on CYP4V2 expression are available (Li et al. 2004), and no studies on protein expression in ocular tissues have been reported. Therefore, we analyzed the expression of CYP4V2 mRNA and protein in BCD target tissues and evaluated the functional activity of wild-type CYP4V2 and the most frequent coding-region BCD-associated mutation, p.H331P, with particular emphasis on the ability to process docosahexaenoic acid (DHA), which is found at high concentrations in the eye. We also stably expressed the wild-type and the mutant CYP4V2 enzymes in HepG2 cells to evaluate enzyme translation/stability and effect on endogenous lipid profiles.
Materials and Methods

Chemicals

DHA (C22:6n-3), EPA (C20:5n-3) and AA (C20:4n-6) were purchased from Cayman Chemicals (Ann Arbor, Michigan). Fast-Red was from Pierce (Rockford, IL). Myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1-cis-9) and 2,2-d2- stearic acid (C18:0-d2) were purchased from Sigma-Aldrich (St. Louis, MO). 22-Hydroxydocosanoic acid and 20-hydroxyeicosanoic acid were purchased from Larodan Fine Chemicals (Malmo, Sweden). Halt Inhibitor Cocktail was obtained from Thermo Scientific. Polyethyleneimine transfection reagent was purchased from Sigma-Aldrich (Deisenhofen, Germany) and an HIV1 helper plasmid pCD/NL-BH kindly obtained from Jakob Reiser (New Orleans). Geneticin® was purchased from Gibco (Karlsruhe, Germany). Laemmli buffer was from Bio-Rad Laboratories (Hercules, CA) and Odyssey Blocking buffer was from LI-COR Biosciences (Lincoln, NE).

Enzyme, antibodies and cell sources

CYP4 Supersomes® were from BD Bioscience (San Jose, CA), and ARPE-19 cells were from ATCC (Manassas, VA). PCR Master Mix, MultiScribe™ Reverse Transcriptase, random hexamers and Taqman expression system assay probes were from Applied Biosystems (Carlsbad, CA). Recombinant NADPH:P450 oxidoreductase and cytochrome b5 expressed in E. coli were prepared as described previously (Cheesman et al. 2003). The anti-calreticulin antibody, anti-mouse IgG antibody conjugated with Cy5® and mouse monoclonal anti-β-actin were from Abcam (Cambridge, MA). The anti-mouse antibody conjugated with IR680 dye was purchased from Rockland Immunochemicals (Gilbertsville, PA). The human tissue panel (FDA992) was purchased from US Biomax, Inc (Rockville, MD). Human retina sections were
purchased from Fred Hutchison Cancer Research Center (Seattle, WA). Human kidney and small intestine microsomes were a gift from Dr. Ken Thummel, Department of Pharmaceutics, University of Washington (Seattle, WA). Human liver samples were obtained from the Liver Bank in the School of Pharmacy, University of Washington. HepG2 cells and Sf9 cells were purchased from ATCC (Manassas, VA)

**RT-PCR analysis**

Total RNA from ARPE-19 cells was isolated by using TRI Reagent® as described in the manufacturer’s protocol (Applied Biosystems, Foster City, CA). The quality and quantity of the total RNA was determined spectrophotometrically. cDNA was synthesized from 1 μg of the total RNA using MultiScribe™ Reverse Transcriptase with random hexamers in a total volume of 10μL. Real time RT-PCR reaction mixtures contained 0.1 μg of cDNA, PCR Master Mix and Taqman gene expression probes (Applied Biosystems) for the following genes: human *GusB*, *CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP2A6*, *CYP2B6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP2E1*, *CYP2J2*, *CYP3A4*, *CYP4A11*, *CYP4B1*, *CYP4F3*, *CYP4F12* and *CYP4V2*. Amplification reactions were done on an Applied Biosystems 7900 HT-SDS instrument for 40 cycles of 95°C/15 sec, 60°C/60 sec and Ct values determined using SDS V 2.3 software. The expression levels (ΔΔCt) were determined in relation to the house keeping gene *GusB* with CYP4V2 as a calibrator.

**CYP4V2 expression, purification and quality control**

Cloning and expression of CYP4V2 in insect cells were described previously (Nakano et al. 2009). The wild-type CYP4V2 protein was purified by Ni-NTA and hydroxyapatite (HA) columns, as described elsewhere (Cheesman et al. 2003) with minor modifications. The final product was dialyzed twice against 100mM potassium phosphate at pH7.4, 20% glycerol and 0.1
8 mM EDTA and stored at -80°C. Purity was assessed by SDS-PAGE (9%) with Coomassie blue staining. Reduced carbon monoxide (CO)-bound spectra and absolute spectra were recorded on a Cary 300 UV/VIS Spectrometer (Nakano et al. 2009). The molecular weight of purified CYP4V2 was determined by LC-MS as previously described (Zheng et al. 2003).

**CYP4F2 expression and purification**

The wild-type *CYP4F2* gene was a kind gift from Drs. David E. Stec (University of Mississippi Medical Center, Jackson, MS) and Mark J. Rieder (University of Washington, Seattle, WA). To obtain the full length cDNA with the hexahistidine tag, *CYP4F2* was amplified by PCR with the specific primers, 5’-GCG CGA ATT CAT GTC CCA GCT GAG CCT GTC CTG GC-3’ and 5’-GCG CGT CGA CTC AAT GAT GAT GAT GAT GAT GAT GAT GAG TCT AAT GGT GAC TCA GGG GCT CCA CCC-3’. Preparation of a recombinant baculovirus containing *CYP4F2* with a hexahistidine tag was generated using the Bac-to-Bac® Baculovirus expression system (Invitrogen, Carlsbad, CA) as described elsewhere (Nakano et al. 2009). CYP4F2 expression and purification were performed as described above for CYP4V2 with minor modifications.

**Antibody production and assessment of cross-reactivity**

Anti-CYP4V2 antibody production was carried out by R&R Research (Stanwood, WA) using purified CYP4V2 as the antigen. Briefly, two rabbits were immunized with the purified CYP4V2 protein supplemented with complete adjuvant along with 3 boosts (1 mg total). Twelve weeks after initial immunization, the animals were sacrificed and sera isolated. Polyclonal IgG was isolated from the crude sera of pre- and post-immunized animals following fractionation with a saturated ammonium sulfate solution. The cross reactivity of the anti-CYP4V2 IgG towards other CYP4 enzymes (Supersomes® and purified CYP4B1 expressed in E.coli) was determined.
from Western blots of 0.2 pmol of purified CYP4V2, 1 pmol of Supersomes® (CYP4A11, CYP4F2, CYP4F3A, CYP4F3B, CYP4F12) and 1 pmol of purified rabbit CYP4B1.

**Immunocytochemical staining**

A detailed procedure can be found at [http://www.abcam.com](http://www.abcam.com). Briefly, after fixing and blocking, ARPE-19 cells were incubated first with anti-CYP4V2 or pre-immune rabbit IgG and then with an anti-calreticulin antibody. The secondary antibody incubation was performed with a mixture of anti-rabbit IgG antibody conjugated with fluorescein (Pierce, Rockford, IL) and anti-mouse IgG antibody conjugated with Cy5®. The processed samples were analyzed using a Zeiss LSM 510 META instrument.

**Immunohistochemical staining**

Tissue sections were first deparaffinized, rehydrated and treated with sodium citrate buffer (10mM sodium citrate, 0.05% Tween-20, pH 6.0). Sections were then blocked with secondary antibody host serum, and treated with anti-CYP4V2 antibody or preimmune IgG followed by a secondary anti-rabbit antibody conjugated with alkaline phosphatase. The sections were visualized using Fast-Red and counterstained with hematoxylin.

**DHA and EPA metabolic incubations**

Purified CYP4V2 or CYP4F2 (200 pmol) was mixed with purified NADPH-cytochrome P450 reductase and cytochrome b₅ at a molar ratio of 1:2:1 or 1:3:4, respectively (Powell et al. 1998; Nakano et al. 2009). DLPC (8µg) and the substrates, dissolved in ethanol, were added and incubated on ice for 5 min. The mixture was made up to a total volume of 500 µL with 100 mM potassium phosphate buffer (pH 7.4) and pre-incubated for 2 min at 37°C in a water bath. Metabolic reactions were initiated by the addition of 1 mM NADPH and allowed to proceed for
30 minutes. Reactions were quenched with 500 µL of cold 10% hydrochloric acid. Samples were spiked with the internal standards, 2.5 µg of 22-hydroxydocosanoic acid and 5 µg of 20-hydroxyeicosanoic acid, and extracted twice with chloroform containing 0.01% butylated hydroxytoluene. Pooled organic extracts were dried under a N₂ stream, reconstituted with ethyl acetate and methylated with diazomethane. The methylated samples were dried under N₂, reconstituted with a small volume of ethyl acetate, BSTFA [N,O-bis(trimethylsilyl)trifluoroacetate] added, and the samples heated at 60°C for 45 min prior to analysis by GC-MS. Commercially available CYP4F3B Supersomes® was employed as a bio-reactor to generate chromatographic standards for initial identification of the ω-1 hydroxylated metabolites of DHA and EPA (Fer et al. 2008).

**GC-CI/MS and GC-FID analysis of EPA and DHA metabolism**

Derivatized extracts were analyzed on a Shimadzu QP2010 Gas Chromatograph quadrupole mass spectrometer fitted with a 12 m-fused silica capillary column (SPB-1), and operating in the chemical ionization (CI) mode. Derivatized analytes were injected at a temperature of 80°C. After 1 min, the oven temperature was raised at 70°C/min to 200°C, held for 1 min, then raised at 15°C/min to 300°C. Under these conditions, the derivatized ω and ω-1 hydroxylated metabolites of DHA (EPA) eluted at 8.8 (7.9) and 9.2 (8.3) min, respectively. To quantitate the formation of ω-hydroxylated metabolites of DHA and EPA, derivatized analytes were re-injected on a gas chromatograph with flame ionization detection (GC-FID) equipped as described above for the GC-MS analysis. Due to a lack of the authentic chemical standards, we constructed standard curves for quantitation with 22-hydroxydocosanoic acid and 20-hydroxyeicosanoic acid because these compounds possess the same carbon-chain lengths as ω-hydroxy DHA and ω-hydroxy...
EPA, and therefore exhibit equivalent signal intensities on GC-FID. The ω-1 hydroxy EPA and DHA metabolites were not quantitated by GC-FID due to low signal intensities.

**HepG2 cells stably expressing CYP4V2 and p.H331P.**

The *CYP4V2 WT* and *CYP4V2 H331P* genes were amplified from pFastBac plasmids (Nakano et al., 2009) using the primers 5’-

ACGCCCTCGAGGCCACCATGGCGGGGCTCTGGCTGG-3’ (forward) and 5’-

GATCAAGTTGAAGAGGAGAAATGCAGATGAACGCTAAGGATCCGGC-3’ (reverse). The PCR amplified products were cloned into the puc2CL6IN lentiviral vector using XhoI and BamHI in the multiple cloning sites. Next, HEK293T cells were transfected using a polyethyleneimine transfection reagent with 6 µg each of an HIV1 helper plasmid pCD/NL-BH, expression construct for HIV1 gag/pol/rev (Mochizuki 1998), the envelope vector (pczVSV-G) (Petschmann 1999), and the vector plasmids puc2CL6IN, puc2CL6-4V2wtIN or puc2CL6-4V2H331PIN, respectively. Viral supernatants were harvested 48 hours after transfection, filtered through a 0.45 µm filter and used to transduce HepG2 cells. After 24 h, the transduced cells were selected with 1 mg/ml Genticin® for 7-10 days. The established cell lines were passaged at least 4 times before analysis.

**Western blot analysis**

CYP4V2 samples (10 µg of microsomal protein from Sf9 cells or 50 µg of total membranes from HepG2 cells) were mixed with Laemmli buffer containing 56 mg/mL DTT and boiled. Samples were separated on 9% SDS-PAGE gels, transferred to nitrocellulose membranes, and the membranes blocked with Odyssey Blocking buffer containing 3% goat serum. Membranes were incubated with anti-CYP4V2 and/or mouse monoclonal anti-β-actin and washed with PBS containing 0.3% Tween-20 and incubated with anti-rabbit antibody conjugated with IR800 dye.
and/or anti-mouse antibody conjugated with IR680 dye. After washing with TPBS, the CYP4V2 and β-actin bands were visualized and quantitated using the Odyssey system (LI-COR Biosciences).

**Lipid extraction and lipid profiling by GC-EI/MS**

Harvested HepG2 cells were sonicated briefly. Cell lysate (500 µg) containing 1 µg of 2,2-d2-stearic acid (internal standard) was made up to a total volume of 500 µL with 100 mM potassium phosphate buffer (pH=7.4). Total lipid was extracted using methanol and methyl-t-butyl ether as described previously (Matyash et al. 2008). The extracted fraction was reconstituted with a small amount of ethyl acetate and transesterified with methanolic sulfuric acid (10%, v/v, Sigma-Aldrich). Transesterified lipids were analyzed on a Shimadzu QP2010 gas chromatograph quadrupole mass spectrometer fitted with a 60 m-fused silica capillary column (DB-1). The analytes were injected at a temperature of 100°C. After 2 min, the oven temperature was raised at 30°C/min to 130°C, 10°C/min to 180°C, held for 2 min, 4°C/min to 210°C, 10°C/min to 235°C, held for 3 min, 4°C/min to 255°C, 10°C/min to 310°C, then held for 4 min. Under these conditions, the derivatized saturated and unsaturated fatty acids were eluted as follow: d2-C18:0; 21.30 min, C14:0; 13.86 min, C16:0; 17.77 min, C16:1; 17.36 min, C18:0; 21.30 min, C18:1 cis-9; 20.91 min, C18:1 cis-11; 21.01 min, C20:4n-6; 23.66 min, C20:5n-3; 23.57 min and C22:6n-3; 27.65 min (Supplemental Table 1). Quantitation was achieved by selected ion monitoring of the characteristic [M-31]+ and [M-43]+ fragment ions found in EI spectra of fatty acid methyl esters. Standard curves (r²>0.98) for each fatty acid were generated with the authentic chemical standards. Differences between two means within a group were tested with Student’s t-test (two-tail, two-sample unequal variance by student t-test using GraphPad Prism® (GraphPad Software, Inc., La Jolla, CA).
Results

Gene expression of cytochrome P450s in ARPE-19 cells

ARPE-19 cells are a human retinal pigment epithelial cell line that arose spontaneously from tissue of a healthy individual (Dunn et al. 1996). Although ARPE-19 cells are a well established model system for studying the function of retinal pigmented epithelium, their expression of cytochrome P450 enzymes had not been previously determined. To better characterize these cells for in vitro studies, we extracted total RNA from ARPE-19 cells and quantitated gene expression of CYP4V2 in relation to the known ocular P450, CYP1B1, to well established CYP4A and CYP4F fatty acid hydroxylases, and several other key human P450 enzymes. RT-PCR analysis of total RNA from ARPE-19 cells revealed Ct values for CYP4V2 of 29.3± 0.08, compared to Ct values of 26.6±0.24 for the house-keeping gene, GusB, demonstrating robust CYP4V2 expression in this cell line (Figure 1). In addition, we detected high expression of CYP1B1, which was transcribed at a similar level (88% of CYP4V2 mRNA expression) in these cells. CYP2E1, CYP2J2 and CYP3A4 were transcribed only at low levels (5% of CYP4V2 mRNA expression) and transcripts for the other CYP4 genes, CYP4A11, CYP4B1, CYP4F2, CYP4F3 and CYP4F12 were not detectable (Figure 1).

CYP4V2 protein expression in ARPE-19 cells

To determine whether CYP4V2 protein along with its mRNA was abundant in ARPE-19 cells, we generated a highly specific polyclonal antibody against purified human CYP4V2 (see below). As proof of the specificity of the antibody, the cross reactivity of the CYP4V2 antibody towards other human CYP family 4 enzymes was determined by Western blotting. We used rabbit CYP4B1 prepared in our laboratory (87% sequence identity to human CYP4B1) because
the human enzyme is not commercially available. As expected from the low sequence identity of CYP4V2 to other CYP family 4 members (Rettie 2008), the rabbit CYP4V2 antibody did not recognize 1 pmol each of CYP4A11, CYP4F2, CYP4F3A/B, CYP4F12 or rabbit CYP4B1 under conditions where 0.2 pmol CYP4V2 provided a strong signal (Figure 2a).

Next, we performed quantitative Western blotting on lysates of ARPE-19 cells. As shown in Figure 2b, CYP4V2 was readily detectable at levels that averaged 6 pmol/mg protein. Immunocytochemical analysis further revealed that CYP4V2 co-localized with calreticulin (Supplemental Figure 1), a well-characterized marker of endoplasmic reticulum. Therefore, CYP4V2 is expressed in ARPE-19 cells at a significant level and correctly localized to microsomal membranes.

Immunohistochemical analysis of human ocular tissues

As BCD patients exhibit crystal deposits mainly in the retina and the cornea, we utilized the anti-CYP4V2 antibody to analyze the expression of CYP4V2 protein in these human ocular tissues from healthy individuals. As shown in Figure 3, RPE cells stained strongly positive and corneal epithelium moderately positive for CYP4V2 expression, thereby demonstrating expression of CYP4V2 protein in the disease-targeted ocular tissues of BCD, and thus confirming the mRNA expression data.

Tissue distribution of CYP4V2 protein

Li et al. (2004) had demonstrated the almost ubiquitous mRNA expression of CYP4V2 in human organs. Taking advantage of the newly generated anti-CYP4V2 antibody, our immunohistochemical analysis demonstrated that human liver, reproductive organs and
endocrine organs all stained strongly positive for the CYP4V2 protein. We found strong expression in tissues and cells with a major role in fatty acid and steroid metabolism such as hepatocytes in liver, islet cells in pancreas, endothelium in hypophysis, prostate, adrenal gland, testis, breast and parathyroid, and glandular epithelium in uterus (Supplemental Table 2). To specifically analyze CYP4V2 expression in drug metabolizing tissues, microsomes were prepared from 4 individual kidneys, 4 small intestines and 9 livers, and examined by Western blot. As shown by Western blotting, (Supplemental Figure 3), CYP4V2 protein was readily detectable in human liver, while only a negligible amount or no expression was found in human kidney or small intestine, respectively

**Characterization of purified CYP4V2**

To further characterize the biochemical activity of CYP4V2, His-tagged CYP4V2 was over-expressed in insect cells and isolated by nickel affinity and hydroxyapatite chromatography. SDS-PAGE analysis revealed a single major band of ~60 kDa apparent molecular weight (Figure 4a), which was confirmed by LC-MS as 62,163 Da (data not shown). These experimentally determined values closely matched the calculated molecular weight of CYP4V2, with its heme prosthetic group covalently bound (62,160 Da) (Cheesman et al. 2003). CYP4V2 was isolated mainly in its low spin form, as indicated by the prominent Soret band in the absolute oxidized spectrum at 419 nm and the minor α and β bands at 571 and 535 nm, respectively (Figure 4b). CYP4V2 displays a typical reduced-carbon monoxide P450 spectrum with a Soret maximum near 450 nm (Figure 4c). No P420 was observed in the final enzyme preparation which had a specific content of 9.2 nmol/mg protein, indicating that >50% was holo-enzyme.
CYP4V2-dependent ω-3 PUFA metabolism

The main physiological function of normal RPE cells is to import PUFAs from the bloodstream and recycle them to maintain lipid homeostasis in photoreceptors (Bazan 2006). Therefore, we investigated the ability of purified CYP4V2 protein to metabolize the common ω-3 PUFA’s, EPA and DHA, relative to commercially available CYP4F3B Supersomes® and purified CYP4F2. Firstly, we used CYP4F3B Supersomes® as a bioreactor (Harmon et al. 2006; Fer et al. 2008), to generate metabolite standards of ω-1 hydroxy EPA and ω-1 hydroxy DHA (Figure 5a and 5d; peaks 1 and 3), and ω-hydroxy EPA and ω-hydroxy DHA (Figure 5a and 5d; peaks 2 and 4). Further evidence for the assignment of peaks 1-4 as 19-hydroxy-EPA, 20-hydroxy-EPA, 21-hydroxy-DHA and 22-hydroxy DHA, respectively, was obtained from GC-CI/MS analysis of the BSTFA-derivatized alcohols. These spectra yielded prominent fragment ions at m/z 389 [M-15]+ and m/z 315 [M-89]+ for peaks 1 and 2, and at m/z 415 [M-15]+ and m/z 341 [M-89]+ for peaks 3 and 4 (data not shown). Purified, reconstituted CYP4V2 also formed each of these metabolites in a NADPH-dependent manner (compare Figures 5b and 5c with Figures 5e and 5f), and with a higher selectivity for ω:ω-1 hydroxylation compared to CYP4F3B. Secondly, we quantitated rates of formation of 22-hydroxy DHA and 20-hydroxy EPA formation by purified, reconstituted CYP4V2 (and CYP4F2 – not shown) and found them to be 0.30±0.03 nmol•min⁻¹•nmol⁻¹ (0.23±0.04 nmol•min⁻¹•nmol⁻¹) and 0.19±0.02 nmol•min⁻¹•nmol⁻¹ (0.23±0.01 nmol•min⁻¹•nmol⁻¹), respectively. Finally, we attempted to quantitate the efficiency (V_max/K_M) of PUFA ω-hydroxylation by CYP4V2, but solubility problems and substrate inhibition at concentrations above 80 μM DHA/EPA complicated the analysis. Nonetheless, under comparable incubation conditions for the respective purified, reconstituted enzymes,
CYP4V2 metabolized DHA slightly more rapidly than did CYP4F2, and metabolized EPA at an equivalent rate.

**Characterization of the CYP4V2 p.H331P variant: expression and functional activity**

Finally, to evaluate the expression and functional consequences of the p.H331P mutation relative to wild-type CYP4V2, we expressed both enzymes in HepG2 cells using lentiviral expression vectors. mRNA analysis revealed comparable levels of expression for both the wild-type (4V2WT) and mutant (4V2H331P) transcripts (Figure 6a). However, steady-state protein expression was much higher for wild-type CYP4V2 compared to the mutant (Figure 6b). Finally, lipid analysis revealed significantly lower levels of EPA and DHA (and arachidonic acid) in HepG2 cells expressing wild-type CYP4V2 compared to those expressing the mutant enzyme (Figure 6c). These lipid profiling data are consistent with reduced metabolism of PUFAs by the p.H331P variant.
Discussion

Genetic defects in the cytochrome P450-dependent metabolism of endogenous substrates are recognized to underlie numerous diseases states. For example, steroid metabolism defects due to mutations in CYP7B1, CYP11A1 and CYP17A1 cause spastic paraplegia type 5, lipid adrenal hyperplasia and mineralocorticoid excess syndromes, respectively (Nebert 2002; Russell et al. 2009). In terms of ocular disease, mutations in CYP1B1 are a well-established cause of primary congenital glaucoma (Sarfarazi et al. 1995; Stoilov et al. 1997), although other loci, notably LTBP2, appear to be involved in development of the disease (Ali et al. 2009). In contrast, mutations in CYP4V2 are singularly associated with BCD, a situation that provides experimental opportunities for deciphering the endogenous substrate(s) for the enzyme and perhaps also for shedding light on the pathophysiology of BCD.

If CYP4V2 plays a significant metabolic role in BCD, it seems important that the enzyme be localized to target tissue(s) of BCD. CYP2A6 and CYP2C8 mRNA have been identified previously from tissue homogenates of human retina/choroid by RT-PCR (Zhang et al. 2008), and expression of CYP4V2 mRNA in the retina and in RPE cells has been reported, but no quantitative data are available (Li et al. 2004). In the present study, we found CYP4V2 and CYP1B1 to by far the most highly expressed P450 mRNAs in ARPE-19 cells. In fact, of 15 other P450s that were analyzed, only mRNA for CYP2E1, CYP2J2 and CYP3A4 were quantifiable, and these levels reached <10% that of CYP4V2. CYP4A and CYP4F are expressed at significant levels in human liver and, especially the kidney where they are believed to have important roles in 20-HETE formation (Lin et al. 1994; Zou et al. 1994; Carroll et al. 1997). In marked contrast, the mRNA data from ARPE-19 cells suggest that these more commonly studied
CYP4 enzymes may be functionally silent in these naturally transformed human RPE cells. Additionally, to facilitate analysis of CYP4V2 protein expression, we expressed and purified the recombinant enzyme to generate a highly selective polyclonal antibody for Western blotting and immunohistochemical analysis of target tissues in BCD. CYP4V2 protein was readily detected in human retinal epithelium and the endoplasmic reticulum of ARPE-19 cells, and to a lesser extent in corneal epithelium. Therefore, CYP4V2 is clearly present in ocular cells that are affected in BCD, and may be the dominant functional CYP4 enzyme at these sites. These studies establish ARPE-19 cells as an important tool for evaluating BCD-related biochemical changes in a disease-relevant cell line following knock-down of the endogenous enzyme.

We demonstrated previously that baculovirus-mediated expression of CYP4V2 in insect cells yielded a metabolically competent ω-hydroxylase of several saturated fatty acids that was sensitive to inhibition by HET0016 at nanomolar concentrations (Nakano et al. 2009). Therefore, despite its low sequence identity with other human CYP4 enzymes, CYP4V2 exhibits their prototypic functional characteristics. In the present study we purified CYP4V2 to near homogeneity and found that the recombinant enzyme was isolated from this expression system in the low-spin (ligand-free) state. Therefore, to further probe the substrate specificity of the enzyme, we turned our attention to fatty acids that are present at high concentrations in the retina.

The physiology of the retina has been studied extensively; photoreceptor cells, rods and cones, are especially well characterized. The rod outer segment (ROS) contains rhodopsin-enriched disk membranes, and the lipids of these disk membranes are predominantly
phosphatidyl choline and phosphatidyl ethanolamine. The majority of these lipids are composed of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and DHA (C22:6n-3), whereas EPA (C20:5n-3) is a minor retinal lipid (Anderson 1970). In fact, DHA in the rod outer segment (ROS) of rats represents ~50% of the total fatty acids esterified to phosphatidylethanolamine and phosphatidylserine (Fliesler 1983). The ROS together with RPE cells, possess an efficient lipid recycling system in order to regenerate disk membranes and maintain a high PUFA content (Bazan 2006). Notably, ten percent of the ROS is renewed daily in primates (Young 1967; Young 1971), and the rates of synthesis and disposal of disk membranes are about the same under normal conditions in mice (Young 1971; LaVail 1973). Because DHA homeostasis is so tightly regulated in RPE cells, we compared the substrate specificity of CYP4V2 towards the ω-3 PUFAs, EPA and DHA.

ω-3 PUFAs would be expected to undergo rate-limiting CYP4-dependant ω-oxidation followed by β-oxidation in peroxisomes and mitochondria (Hardwick 2008). CYP4F family enzymes, principally CYP4F2 and CYP4F3B, have been reported to be the main PUFA hydroxylases in human liver (Fer et al. 2008). The current study extends the repertoire of PUFA hydroxylases to CYP4V2, which we found to preferentially catalyze ω- and ω-1 hydroxylation of both EPA and DHA. Purified CYP4V2 and CYP4F2 metabolized both PUFAs at similar rates, but since CYP4F enzymes were not expressed at a significant level in ARPE-19 cells (or corneal CRL11515 cells, data not shown), CYP4V2 is likely to dominate CYP4-dependent PUFA catalysis in BCD target tissues in the eye. Moreover, CYP4V2 is expressed at readily detectable levels in human liver and so, in some subjects, the enzyme may also be a contributor to hepatic metabolism of ω-3 PUFAs. However, further studies are needed to test this hypothesis.
BCD is an autosomal recessive disease and many discrete mutations of CYP4V2 have been identified, the most frequent of which is alternative splicing at the splice-acceptor site for exon 7 (Kaiser-Kupfer et al. 1994; Lee et al. 2001; Li et al. 2004; Lee et al. 2005; Lin et al. 2005; Shan et al. 2005). As a result, 17 base pairs at the 3’ end of intron 6 and on the 5’ end of exon 7 are missing. Consequently, exon 7 is deleted and presumably the resulting truncated enzyme is non-functional. We focused here on the p.H331P mutation, the second most frequent mutation, in order to demonstrate unequivocally that a BCD-associated mutation abrogates enzyme activity, because of the relative ease of recombinant expression and subsequent analysis of this coding-region mutant. We initially expected some loss of function due to the criticality of His331’s location within the most conserved region of the I-helix that constitutes part of the active site (Supplemental Figure 4), as this could be expected to affect ligand binding or apo-protein formation. Interestingly, upon expression in HepG2 cells the p.H331P mutant protein did not accumulate, despite reasonable levels of mRNA expression. Therefore, the mechanism for a loss of function in mammalian cells due to expression of the p.H331P variant is post-transcriptional, and likely involves either increased degradation secondary to reduced protein stability or impaired translation.

Finally, comparative lipid profiling studies in HepG2 cells stably transfected with wild-type CYP4V2 or the p.H331P variant revealed significant decreases in the total levels of several PUFAs in the former cell line (Figure 6c). These studies complement the finding that DHA and EPA are substrates for reconstituted CYP4V2. However, it should be noted that CYP4V2 is not selective for DHA compared to EPA metabolism, assessed either from the catalytic studies with
purified protein or the comparative lipidomic analyses. A further caveat is that the latter experiments were conducted in HepG2 cells, not retinal or corneal cell lines, and so the relevance of altered PUFA metabolism to the disease mechanism of BCD remains to be established.

In summary, we examined CYP4V2 expression and organ distribution with an emphasis on ocular tissues. We also evaluated the enzyme’s substrate specificity for PUFAs and characterized the functional deficit of the p.H331P mutant found in BCD. We demonstrate that; (i) CYP4V2 is a major form of P450 expressed in retinal cells, (ii) the enzyme possesses ω-hydroxylase activity towards ω-3 PUFAs, (iii) the most common non-synonymous SNP found in BCD encodes a non-functional protein, and (iv) over expression of CYP4V2 in HepG2 cells alters lipid homeostasis. Because CYP4V2 appears be the only CYP4 present at significant levels in retinal cells, it may be a prominent contributor to local metabolism of PUFAs in retinal cells.
Acknowledgements

We thank Dr. Jamil Haque for recombinant expression of CYP4F2 in insect cells and Ms. Chelsea Stewart for expression and purification of the P450 co-enzymes. Also we thank Mr. Eric D. Kantor and Dr. Gail D. Anderson for assistance with GC-FID analysis. Ms. Kelly Hudkins for scoring of CYP4V2 histological tissue sections.
AUTHORSHIP CONTRIBUTIONS

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Contributed new reagents or analytic tools: Nakano, Hanenberg and Weik.

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REFERENCES


Footnotes

This work was supported by the Drug Metabolism Transport and Pharmacogenetics Research Fund in the School of Pharmacy, University of Washington and by NIH grant [GM49054].
Figure Legends

FIGURE 1. Real time RT-PCR analysis of P450 expression in ARPE-19 cells. CYP4V2 was the dominantly expressed P450 of 17 genes examined. The relative expression was calculated by the ΔΔCt method using CYP4V2 as a calibrator. The bars indicate the standard deviation of triplicate experiments.

FIGURE 2. Cross reactivity of anti-CYP4V2 IgG with other CYP4 enzymes and CYP4V2 protein expression in ARPE-19 cell lysate. (a) The anti-CYP4V2 antibody specifically reacted with CYP4V2. Lanes 1,5,9: 0.2 pmol CYP4V2, lane 2: 1 pmol rabbit CYP4B1, lane 3: 1 pmol CYP4A11, lane 4: 1 pmol CYP4F2, lane 6: 1 pmol CYP4F3A, lane 7: 1 pmol CYP4F3B, lane 8: 1 pmol CYP4F12. (b) Lanes 1-3: 330, 160 and 82 fmol of purified CYP4V2, respectively, lane 4 and 5: 10 and 30 μg of ARPE-19 cell lysate, respectively. CYP4V2 was expressed at a concentration of ~6 pmol/mg cell lysate. Band intensities were analyzed with an Odyssey system.

FIGURE 3. CYP4V2 expression in the normal human retina and cornea. Immunohistochemical staining using the anti-CYP4V2 IgG showed strong positive staining of RPE cells in retina tissue and weak staining of ganglion cells and internal/external nuclear layers in the retina and corneal epithelial cells. (a) Retina treated with preimmune IgG. (b) Retina treated with anti-CYP4V2 IgG. (c) Cornea treated with preimmune IgG. (d) Cornea treated with anti-CYP4V2 IgG. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform
layer; ONL, outer nuclear layer; PhL, photoreceptor layer; RPE, retinal pigmented epithelium; Ch, choroid; AE; anterior epithelium; SP; substantia propria.

FIGURE 4. Characterization of purified His6-CYP4V2 by SDS-PAGE and UV spectrometry. (a) Purified CYP4V2 migrated with an apparent molecular weight of ~60 kDa. (b) The absolute UV spectrum of CYP4V2 exhibited a Soret band at 419 nm and α and β bands at 571 and 535 nm. (c) The reduced CO-bound difference spectrum of purified CYP4V2 demonstrates absence of P420.

FIGURE 5. EPA and DHA metabolism catalyzed by purified CYP4V2 reconstituted with P450 reductase and cytochrome b5. (a) EPA incubated with CYP4F3B Supersomes® plus NADPH. (b) EPA incubated with reconstituted CYP4V2 minus NADPH. (c) EPA incubated with reconstituted CYP4V2 plus NADPH. (d) DHA incubated with CYP4F3B Supersomes® plus NADPH. (e) DHA incubated with reconstituted CYP4V2 minus NADPH. (f) DHA incubated with reconstituted CYP4V2 plus NADPH. Peaks 1, 2, 3 and 4 were assigned as 19-hydroxy-EPA, 20-hydroxy-EPA, 21-hydroxy-DHA and 22-hydroxy DHA, respectively.

FIGURE 6. Expression and functional consequences of the p.H331P variant of CYP4V2 stably transduced in HepG2. (a) Real time RT-PCR analysis of CYP4V2 wild-type and p.H331P mRNA in HepG2 cells. (b) Western blot analysis of CYP4V2 wild-type and p.H133P protein in HepG2 cells. β-Actin was detected as a loading control. (c) DHA, EPA and arachidonic acid accumulation in p.H331P transfected cells compared to cells expressing the wild-type enzyme.
Data are the mean and the bars indicate the standard deviation of triplicate experiments. *; p values < 0.02.
Figure 1

Relative expression

CYP1A1  CYP1A2  CYP1B1  CYP2A6  CYP2B6  CYP2C8  CYP2C9  CYP2D6  CYP2E1  CYP2J2  CYP3A4  CYP4A11  CYP4F2  CYP4F3  CYP4F12  CYP4V2
Figure 4

(a) 

(b) Absorbance vs Wavelength (nm)

(c) Absorbance vs Wavelength (nm)