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Cytochrome P450-mediated 17β-estradiol metabolism in zebrafish (*Danio rerio*)

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Abstract

Cytochrome P4501 (CYP1) and CYP3A proteins are primarily responsible for the metabolism of 17β-estradiol (E_2) in mammals. We have cloned and heterologously expressed CYP1A, CYP1B1, CYP1C1, CYP1C2, CYP1D1, and CYP3A65 from zebrafish (*Danio rerio*) to determine the CYP-mediated metabolism of E_2 in a non-mammalian species. Constructs of each CYP cDNA were created using a leader sequence from the bacterial *ompA* gene to allow appropriate expression in *Escherichia coli* without 5′ modification of the gene. Membrane vesicles were purified, and functional CYP protein was verified using carbon monoxide difference spectra and fluorescent catalytic assays with the substrates 7-ethoxyresorufin and 7-benzyloxy-4-(trifluoromethyl)-coumarin. Rates of *in vitro* E_2 metabolism into 4-hydroxyE_2 (4-OHE_2), 2-hydroxyE_2 (2-OHE_2), and 16α-hydroxyE_1 (16α-OHE_1) metabolites were determined by gas chromatography/mass spectrometry. The 2-OHE_2 metabolite was produced by all CYPs tested, while 4-OHE_2 was only detected following incubation with CYP1A, CYP1B1, CYP1C1, and CYP1C2. The 16α-OHE_1 metabolite was only produced by CYP1A. The highest rates of E_2 metabolism were from CYP1A and CYP1C1, followed by CYP1C2. CYP1B1, CYP1D1, and CYP3A65 had low rates of E_2 metabolism. E_2 metabolism by zebrafish CYP1A, CYP1C1, and CYP1C2 produced similar ratios of 4-OHE_2 to 2-OHE_2 as previous studies with mammalian CYP1As. CYP1B1 formed the highest ratio of 4-OHE_2 to 2-OHE_2 metabolites. Contrary to mammals, these results suggest that fish CYP1A and CYP1C proteins are primarily responsible for E_2 metabolism, with only minor contributions from CYP3A65 and CYP1B1. Similar to mammals, 2-OHE_2 is the predominant metabolite from CYP-mediated E_2 metabolism in fish, suggesting that all vertebrate species produce the same major E_2 metabolite.

Introduction

The major estrogen in vertebrates is 17β-estradiol (E_2). The first step in the metabolism of E_2 is NADPH-dependant oxidative metabolism catalyzed by hepatic cytochrome P450

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Declaration of interest

The authors declare that there is no conflict of interest that can be perceived as prejudicing the impartiality of the research reported.

(CYP) enzymes (Zhu & Conney 1998). Mammalian CYP enzymes are critical for E_2 metabolism, including those belonging to the CYP1A, CYP1B, and CYP3A subfamilies; yet, there are distinct differences in the CYP1 and CYP3 families across vertebrates. The CYP3A subfamily includes multiple genes in mammals; humans have four CYP3As (Qiu et al. 2008). Most fish contain only one or two CYP3As; zebrafish (*Danio rerio*) have a single CYP3A gene, CYP3A65 (Qiu et al. 2008). The CYP1 family contains more subfamilies in non-mammalian vertebrates. There are two CYP1A genes (CYP1A1 and CYP1A2) in mammals, and a single CYP1A gene in fish, with the exception of some polyploid species such as the salmonids (Goldstone et al. 2007). CYP1B1 is the single CYP1B gene found in all vertebrate lineages. CYP1Cs are found in all non-mammalian vertebrate lineages (Goldstone et al. 2007). Fish have CYP1C1 and CYP1C2 paralogs arising from a gene duplication event (Godard et al. 2005, Goldstone et al. 2007). The CYP1D subfamily, a non-mammalian subfamily which has been recently identified, contains a single CYP1D1 gene (Goldstone et al. 2009). The liver is the major site of E_2 metabolism; all CYP1s and CYP3As are known to be expressed in the liver of mammals (Bieche et al. 2007) and fish (Jonsson et al. 2007, Goldstone et al. 2009).

In mammals, the major product of E_2 oxidation is the 2-hydroxyE_2 (2-OHE_2) metabolite, with limited production of 4-hydroxyE_2 (4-OHE_2) and small levels of metabolites hydroxylated at either the 6β, 16β, or 16α positions (Lee et al. 2001). The regioselective hydroxylation of E_2 by purified CYPs has been well studied in mammals (Lee et al. 2003). CYP1A1, CYP1A2, and CYP3A4 show the highest rates of metabolism in humans, mostly as a result of high 2-hydroxylation activity (Lee et al. 2003). Consequently, these three CYPs have very low ratios of 4-OHE_2 to 2-OHE_2 formation (Lee et al. 2003). On the other hand, E_2 metabolism by mammalian CYP1B1 produces similar amounts of 4-OHE_2 to CYP1As and CYP3As, but very little 2-OHE_2, such that three times more 4-OHE_2 is formed in relation to 2-OHE_2 (Lee et al. 2003).

The two catecholestrogens, 2-OHE_2 and 4-OHE_2, are further metabolized by catechol-*O*-methyltransferase (COMT) into the major urinary estrogen metabolites: 2-methoxyE_2 (2-$MeOE_2$) and 4-methoxyE_2 (4-$MeOE_2$) respectively (Creveling 2003). However, the catecholestrogens can also be oxidized to form reactive semiquinones and quinines, which can form adducts with purine bases (Dawling et al. 2001, Belous et al. 2007). COMT has a higher affinity for 2-OHE_2 over 4-OHE_2, resulting in an increased genotoxicity of the 4-OHE_2 metabolite (Tsuchiya et al. 2005). High concentrations of 4-OHE_2 metabolites have been associated with human breast cancers (Rogan et al. 2003), and elevated CYP1B1 mRNA transcripts have been identified in many cancerous tissues including breast, testis, and ovary (McKay et al. 1995, Murray et al. 1997) suggesting that elevated levels of CYP1B1-mediated E_2 metabolism can lead to carcinogenesis. 2-OHE_2 metabolites appear to be tumor inhibitors (Tsuchiya et al. 2005).

In fish liver microsomes, the presence of E_2 2-hydroxylase activity was identified in channel catfish (Butala et al. 2004), English sole (Stein et al. 1991), scup, and winter flounder (Snowberger & Stegeman 1987), and recently, 4-OH activity was detected in channel catfish (Butala et al. 2004). The only reported E_2 metabolism by purified fish CYPs has been from Japanese eel showing 2-OH activity by CYP1A9 but not by CYP1C1; however, neither CYP

produced 4-OHE$_2$ metabolites (Uno et al. 2008). Since E$_2$ metabolism predominantly occurs in the liver, and the dominant metabolic products are 2-OHE$_2$ with smaller amounts of 4-OHE$_2$ for both mammals and fish, similar CYP families could be responsible for E$_2$ metabolism in mammalian and non-mammalian vertebrates.

In the present study, we describe our findings on the rates of formation of E$_2$ metabolites by bacterially expressed zebrafish CYPs. Zebrafish CYP1A, CYP1B1, CYP1C1, CYP1C2, CYP1D1, and CYP3A65 were each cloned and expressed in bacteria to produce catalytically active proteins used for *in vitro* assessment of E$_2$ metabolism. As fish have similar CYP content in the CYP1 and CYP3 families as other non-mammalian vertebrates, our data will serve as a model for non-mammalian vertebrate CYP-mediated E$_2$ metabolism.

Materials and Methods

Cloning of zebrafish CYPs

RNA was extracted with TRIzol reagent (Invitrogen) from pools of liver or gill tissue taken from female wild type zebrafish (*D. rerio*; n=8). cDNA was synthesized using cloned AMV RT (Invitrogen) and an oligo (dT)$_{20}$ primer. Zebrafish CYP1A, CYP1C1, CYP1C2, CYP1D1, and CYP3A65 were amplified from liver cDNA, and CYP1B1 was amplified from gill cDNA using Platinum Taq Polymerase (Invitrogen) following the manufacturer's protocol and using gene-specific primers and annealing temperatures as listed in Table 1. PCR products were gel purified, cloned into pGEM-T Easy vectors (Promega), and transformed into competent *Escherichia coli* JM109 cells (Promega). Plasmids were purified by QIAprep Spin Miniprep Kits (Qiagen) and sequenced by MobixLab (McMaster University, Hamilton, ON, USA).

To facilitate proper membrane targeting of expressed CYPs in bacteria, an *ompA*(+2) sequence consisting of 69 nucleotides was attached in frame with the CYP start site by PCR using the methods of Pritchard et al. (2006). The source for the *ompA*(+2) was purified genomic JM109 DNA for CYP1A, CYP1B1, CYP1C1, and CYP3A65. The *ompA*-CYP1A and *ompA*-CYP1C1 constructs were used as *ompA* templates for attachment to CYP1C2 and CYP1D1 respectively. The *ompA*(+2) sequence was attached using two separate PCRs using a high fidelity polymerase, Accuprime *Pfx* (Invitrogen). The first PCR used a forward primer against the *ompA* segment and a linker primer that primed against the first 21 bases of the CYP and the last 21 bases of the *ompA* segment (Table 1). This generated a ~90 nucleotide fragment containing the first 21 bases of the appropriate CYP gene, and was gel purified and used in a second PCR with the same forward primer and a CYP-specific reverse primer against the 3′ end (Table 1) of the zebrafish CYP cloned gene. The forward and reverse primers contained NdeI and XbaI restriction sites respectively. PCR fragments containing the complete CYP gene and the *ompA*(+2) sequence were digested, gel purified, ligated into the pCW vector, and co-transfected with the pACYC vector containing human NADPH–CYP reductase (CPR) into JM109 cells. Final sequences were confirmed by sequencing prior to expression.

Expression and purification of zebrafish CYPs

Overnight cultures of *E. coli* were diluted 1:100 into TB with ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml) and shaken at 30 °C and 200 r.p.m. Isopropyl β-D-1-thiogalacto-pyranoside (1 mM; Fisher Scientific, Pittsburgh, PA, USA) was added when cultures reached an OD_{600} of between 0·7 and 1·0. Expression was optimized with the addition of 0–1 mM δ-aminolevulinic acid (Ala; MP Biomedicals, Solon, OH, USA) based on maximizing the catalytic activity of purified membranes (see Catalytic assays). Ala was added to a final concentration of 0·1 mM for all CYP constructs except CYP1A and CYP1B1, where Ala was added at 0·5 and 1·0 mM respectively. Expression was allowed to proceed for another 20–24 h. Cells were harvested, and bacterial membranes were purified using previously published methods (Pritchard et al. 2006). Total protein content was measured using a bicinchoninic acid assay kit (Pierce protein research products) according to the manufacturer's protocols (Smith et al. 1985).

Total P450 analysis and cytochrome c *activity*

Total P450 content was measured by diluting membranes into P450 spectrum buffer (Pritchard et al. 1997) and measuring the carbon monoxide (CO) difference spectra using the peak absorbance difference between 450 and 490 nm and an extinction coefficient of 92/mM/cm (Omura & Sato 1964). Cytochrome *c* reductase activity of membranes was determined by measuring the absorbance change at 450 nm of a reaction mixture containing 1 mg/ml of cytochrome *c* and 0·4 mM NADH in 0·2 M potassium phosphate buffer (pH 7·7) at 37 °C using an extinction coefficient of 21·1/mM per cm (Massey 1959).

Catalytic assays

Ethoxyresorufin-*O*-deethylase activity was measured at 30 °C using methods from Hahn et al. (1993). The rate of conversion of 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC; BD Biosciences, San Jose, CA, USA) to 7-hydroxy-4-(trifluoromethyl)coumarin by purified membranes was measured in the presence of 1 mM BFC and 1·33 mM NADPH in 0·5 M potassium phosphate buffer (pH 7·4) at 30 °C, as optimized from Crespi & Stresser (2000). Catalytic assays containing cytochrome b_5 (Calbiochem, EMD Chemicals Inc., Gibbstown, NJ, USA) had 50 pmol added. All catalytic assays were normalized for total P450 in the reaction mixture. Optimization of CYP expression was based on maximizing 7-ethoxyresorufin (7-ER; CYP1A, 1B1, 1C1, 1C2, 1D1) or BFC (CYP3A65) metabolism with assays normalized for total protein.

Zebrafish liver microsomal preparation

Zebrafish (*D. rerio*) were purchased from DAP International (Etobicoke, ON, Canada). Fish were kept in a semi-recirculating system at 28–30 °C and fed three times per day, alternating between tropical flake food and fresh brine shrimp (*Artemia sp.*). Adult zebrafish were removed from our regular breeding stock, and livers were collected and pooled (male n=57; female n=38) and flash frozen in liquid nitrogen. Fish were ~1 year of age at collection, and were still actively breeding. Microsomal fractions were collected according to Stegeman et al. (1979). All samples were kept at −80 °C until use. Total protein concentrations were

determined, and the remaining microsomal pools were used to determine hepatic E_2 metabolism.

E_2 metabolism

E_2 (Sigma–Aldrich) was incubated with 0·5 mg membrane or microsomal protein at 28 °C for 2 h according to Spink et al. (1990) with and without cytochrome b_5 (100 pmol). The incubation time was chosen based on data collected during the optimization of the assay for studies with fish (Butala et al. 2004). Reactions were terminated with 20 mM ascorbic acid (Sigma–Aldrich); the deuterated estradiol internal standard was added, and metabolites were extracted according to Butala et al. (2004). The samples were derivatized with *N,O*-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (Sigma–Aldrich), evaporated under N_2 and resuspended in iso-octane (Sigma–Aldrich) prior to analysis. Samples were analyzed for the presence of estrone (E_1), estriol, E_2, 4-OHE_2, 2-OHE_2, 4-$MeOE_2$, 2-$MeOE_2$, 16α-hydroxyE_1 (16α-OHE_1), and the deuterated standard by GC/MS according to Butala et al. (2004). All samples were run in duplicate.

Materials

NADPH, leupeptin, aprotonin, phenylmethanesulphonylfluoride, and cytochrome *c* were purchased from Sigma–Aldrich. Ampicillin and chloramphenicol were purchased from Fisher Scientific. The deuterated estradiol internal standard was prepared as described in Dehennin et al. (1980) and kindly provided by Dr John Rimoldi (University of Mississippi). The pCW vector (Muchmore et al. 1989) was a kind gift from Dr FW Dahlquist (University of California Santa Barbara, Santa Barbara, CA, USA), and the pACYC expression vector containing human CPR was a kind gift from Dr T Friedberg (University of Dundee, Dundee, UK).

Results

Expression of zebrafish CYPs

The CO difference spectra were analyzed for each expressed CYP (Fig. 1), and peaks were seen at 420 and 450 nm, indicating the presence of significant amounts of both inactive and active P450. Total P450 content was between 0·139 and 0·655 nmol/mg protein (Table 2). Cytochrome *c* activity was measured to determine the levels of NADPH–CPR in each sample. There were similar levels of reductase activity in each sample, except for cells expressing human CPR only which showed approximately two- to three-fold higher activity than those co-expressed with a CYP gene (Table 2).

The catalytic activity of each recombinant protein was assessed using 7-ER and BFC as substrates to verify that the proteins were functionally active. All CYP1s showed catalytic activity for 7-ER and BFC except for CYP1D1 and CYP3A65, which showed no metabolism of BFC and 7-ER respectively (Fig. 2). CYP1D1 showed detectable but low catalytic activity for a broad range of other substrates tested (unpublished data). The addition of cytochrome b_5 significantly increased the ability of CYP1A, CYP1B1, CYP1C1, and CYP1C2 to metabolize 7-ER. However, cytochrome b_5 did not affect the metabolism of 7-ER by CYP1D1 or the metabolism of BFC by CYP3A65 (data not shown).

E_2 metabolism

E_2 metabolism data are expressed as mean±S.E.M. of reactions done in duplicate. There was no 2-MeOE_2 or 4-MeOE_2 detected in any of the samples, and no E_2 metabolites were detected in reactions with the control membranes (expressing CPR only). Since *E. coli* do not express COMT, the enzyme responsible for the methylation of hydroxylated E_2 metabolites, the measurement of 2-MeOE_2 and 4-MeOE_2 was additional negative controls for our assay. 16α-OHE_1 was only detected in reactions with CYP1A at a rate of 6·8±1·6 pmol/nmol P450 per min.

CYP1A and CYP1C1 showed the highest rates of E_2 metabolism and a strong preference for 2-OHE_2 over 4-OHE_2 (Table 3). CYP1C2 metabolized moderately less 2-OHE_2, and 4-OHE_2, than CYP1A and CYP1C1, but metabolism at the 2 position was highest (Table 3). All CYP1A, CYP1C1, and CYP1C2 had percent ratios of 4-OHE_2/2-OHE_2 between 3 and 8. CYP1B1, CYP1D1, and CYP3A65 had the lowest rates of E_2 metabolism in general (<40 pmol/nmol P450 per min), and 2-OHE_2 formation predominated. CYP1B1 had the highest proportion of 4-OHE_2 production (4-OHE_2/2-OHE_2=32%) of all the CYPs tested, but still formed twice as much 2-OHE_2 than 4-OHE_2. There was no 4-OHE_2 produced by CYP1D1 or CYP3A65 without cytochrome b_5.

Addition of cytochrome b_5 approximately doubled the rate of formation of 2-OHE_2 by CYP1A and CYP1C1, but reduced the amount of 4-OHE_2 produced (Table 3). Cytochrome b_5 decreased the rate of E_2 metabolism by CYP1B1 and CYP1C2, while metabolism by CYP3A65 was not affected. Small amounts of estrone were produced, at rates below 3·0 pmol/nmol P450/min, by CYP1A, CYP1B1, CYP1C1, and CYP1C2 with cytochrome b_5, but CYP3A65 with cytochrome b_5 produced estrone at a rate of 8·2±5·3 pmol/nmol P450/min. Estriol was detected only in samples with CYP1C1 or CYP1C2 that included cytochrome b_5. Estriol was produced at a rate of 223±19 pmol/nmol P450/min by CYP1C1 and 7±0·24 pmol/nmol P450/min by CYP1C2.

Zebrafish liver microsomes produced low, but detectable amounts of 2-OHE_2 (Table 4) and estrone. Only one replicate reaction of each produced 2-OHE_2, while both replicates produced estrone at 0·62±0·03 pmol/mg/min from female liver and 0·23±0·02 pmol/mg/min from male liver.

Discussion

E_2 metabolism in mammals is primarily catalyzed by CYP1 and CYP3A proteins (Lee et al. 2003), important CYP enzymes that are highly expressed in mammalian liver, the primary site of E_2 metabolism (Lee et al. 2001). Because E_2 metabolism also occurs predominantly in the liver of fish (Butala et al. 2004), we chose to express zebrafish CYP1s and CYP3A65 to investigate their capacity for E_2 metabolism *in vitro.*

Bacterial expression of recombinant eukaryotic CYP proteins is possible, but often involves the modification of the 5′ end to allow appropriate targeting to membranes (Gillam et al. 1995, Waterman et al. 1995). Instead of 5′ modification, we used an *ompA* strategy that involves the attachment of a leader sequence to the desired CYP gene, to target the native

CYP to the bacterial outer membrane (Pritchard et al. 1997). The leader sequence is cleaved off after targeting, allowing expression of unmodified, native CYP protein in bacteria. The expression conditions were optimized for each CYP by expressing the proteins in cultures with a range of Ala concentrations (0·1–1 mM). CYP1B1 was the only CYP to show a large increase in activity with an increase in Ala to 1 mM (data not shown); this was consistent with previous bacterial expressed human CYP1B1 (Jansson et al. 2000). All other CYPs required less, but some, Ala for optimal function. CO difference spectra confirmed that a peak around 450 nm was present indicating that active CYP heme-protein was present in the preparations (See Fig. 1 for a representative CO difference spectra). Another major peak at 420 nm represented other heme-containing proteins or inactive CYP at levels which are quite similar to other CYPs overexpressed in the same system (Pritchard et al. 2006).

In addition to CO difference spectra, functional protein was confirmed by fluorescent catalytic assays using 7-ER and BFC. 7-ER is a widely used specific substrate for CYP1 activity in mammals (CYP1A1, 1A2, 1B1; Shimada et al. 1998) and fish (CYP1A, 1B1; Hegelund et al. 2004). BFC is selective for CYP3A activity in mammals (Crespi & Stresser 2000), and was considered as a suitable substrate for fish CYP3A proteins based on studies with fish hepatic microsomes (Hegelund et al. 2004) and expressed fish CYP3A genes (Kashiwada et al. 2005). All CYP1s metabolized 7-ER to some degree. CYP1A catalyzed 7-ER metabolism at a rate 30 times greater than that of CYP1B1 (Fig. 2), a ratio comparable to that reported for human CYP1A1 and CYP1B1 (Murray et al. 2001). Unlike the CYP1As and CYP1Bs, there is no previous catalytic data available for CYP1Cs or CYP1Ds, but clearly the expressed CYP1C1, CYP1C2, and CYP1D1 are functional proteins as each were able to metabolize at least 7-ER (Fig. 2). While CYP1D1 had low activity for 7-ER and did not metabolize BFC, the function of the protein is completely unknown and it is likely that the substrates used are not optimal or specific for CYP1D1 activity. CYP3A65 did not metabolize 7-ER but did metabolize BFC (Fig. 2), similar to that reported for expressed medaka CYP3A proteins (Kashiwada et al. 2005). However, unlike medaka (Kashiwada et al. 2005), zebrafish CYP3A65 metabolism of BFC was not affected by cytochrome b_5. Collectively, the presence of P450 peaks in the CO difference spectrum, and the ability of these proteins to metabolize 7-ER and/or BFC confirm that the expressed proteins are functional. Overall, three points favor the differences seen between these expressed CYP proteins for E_2 metabolism as representative of true *in vivo* functional differences: 1) the expressed proteins are functional, 2) the CYP3A65, CYP1A, and CYP1B1 expressed proteins function as expected, and 3) there were similar activity levels of CPR present for each expressed protein.

CYP1A and CYP1C1 demonstrated the highest overall activity for E_2 compared with the other fish CYP1s. They were also the only two zebrafish CYPs to show increased metabolism of E_2 when supplemented with cytochrome b_5. The rate of overall E_2 metabolism of CYP1B1 compared with CYP1A was similar to that of the metabolism of human CYP1B1 to CYP1A1 (Lee et al. 2003). Surprisingly, both CYP1C1 and CYP1C2 metabolize E_2 to a higher degree than CYP1B1 and CYP3A65. Phylogenetically, vertebrate CYP1Cs are clustered in a clade with the CYP1Bs, which is distinct from the CYP1A/CYP1D clade (Goldstone et al. 2009). That mammalian CYP1B1 is capable of E_2 metabolism but in fish the CYP1Cs have much higher, and likely, more biologically relevant

E_2 metabolism than CYP1B1, suggests that E_2 metabolism was present in the ancestor to the CYP1B and CYP1C clade, and that the fish CYP1Cs have retained this ancestral function while fish CYP1B1 has acquired novel function. This might suggest that both the tunicate CYP1Es and CYP1Fs, homologs to the vertebrate CYP1B/1C and CYP1A clade respectively (Goldstone et al. 2007), may be functionally capable of E_2 metabolism.

Mammalian CYP genes show distinct regioselectivity in E_2 metabolism. Like mammals, fish CYPs produced 2-OHE_2, 4-OHE_2, and 16α-OHE_1 metabolites. In mammals, CYP1A1 was the only CYP1 able to hydroxylate at the 16α position, and does so in very low concentrations (Lee et al. 2003) similar to what was seen by zebrafish CYP1A. 2-OHE_2 was the predominant metabolite formed in both zebrafish (this study) and mammalian studies (Lee et al. 2003), and the 4-OHE_2 metabolite was produced to a much lower extent in both groups. In addition, Japanese eel CYP1A9 (one of two CYP1As in this species) also predominantly formed 2-OHE_2 (Uno et al. 2008), though at a lower rate than seen here, possibly due to an 8 h reaction time in that study.

Zebrafish CYP1Cs and CYP1A have a similar 4-OHE_2 to 2-OHE_2 ratio compared with mammalian CYP1As (Lee et al. 2003). Zebrafish CYP1B1 was similar to mammalian CYP1B1 in its regioselectivity, as it metabolized the largest proportion of 4-OHE_2 relative to 2-OHE_2 formation compared with any other tested zebrafish CYP. However, unlike human CYP1B1 (Lee et al. 2003), zebrafish CYP1B1 still metabolized more 2-OHE_2 than 4-OHE_2, and overall, the rates of metabolism were much less than the mammalian ortholog. Distinct from the other CYP1s, CYP1D1 did not produce any 4-OHE_2 metabolites. The lower ratio of 4-OHE_2 to 2-OHE_2 metabolism seen here from zebrafish CYP1B1 compared with human CYP1B1, and the preference of all other CYPs tested for 2-OH regioselectivity, agrees with the lower *in vivo* proportion of 4-OHE_2 seen in channel catfish (Butala et al. 2004) compared with human (Lee et al. 2001) and rat (Dannan et al. 1986; Table 4). Our zebrafish microsomal protein had only low activity for E_2, and we did not detect any 4-OHE_2 metabolites. We also detected the production of estrone, which along with the parent compound, E_2, are the primary estrogens in fish that are either glucuronidated or sulfated for elimination (Stein et al. 1991). Owing to the small size of zebrafish, a large sample size was required to obtain sufficient microsomal material for the present study; in fact, our microsomal pools required ~40–60 animals each and precluded a large number of replicates. To obtain sufficient numbers of animals, we collected livers from 1-year-old animals that were slated for removal from our breeding stock, and were already in reproductive decline. This likely led to an under-representation of the activity of zebrafish microsomal E_2 activity, and may account for the lower levels of E_2 metabolism that were seen from our zebrafish compared with other fish (Table 4). Additionally, there may be hormonal differences between species that could be responsible for the lower E_2 metabolism by zebrafish. The relatively low amounts of 4-OHE_2 detected in our samples suggest that though fish are susceptible to genotoxic damage by increased levels of E_2 (Teles et al. 2006), they may be less sensitive than mammals.

Unlike mammalian liver where CYP1A2 is the dominant CYP1, fish lack the CYP1A2 gene and CYP1A is the dominant hepatic CYP1 (Jonsson et al. 2007). All CYP1C1, CYP1C2, and CYP1B1 are constitutively expressed at similar low levels in the liver (Jonsson et al.

2007). CYP1A, CYP1B1, CYP1C1, and CYP1C2 are inducible through the AHR pathway (Jonsson et al. 2007), and their role in E_2 metabolism suggests a potential mechanism for AHR control of E_2 concentrations. Our data suggests that CYP1A would be responsible for the majority of CYP1-mediated hepatic E_2 metabolism in fish. First, CYP1A has higher *in vitro* activity for E_2 metabolism compared with all other CYP1s and CYP3A65 (Table 3). Secondly, liver expression of CYP1A is significantly higher than all other CYP1s (Jonsson et al. 2007). Thirdly, CYP1A has a much higher induction via AHR agonists (Jonsson et al. 2007) suggesting that even when induced, CYP1A-mediated E_2 metabolism is likely to predominate *in vivo.* The relatively high production of estrone compared with 2-OHE_2 using zebrafish (this study), channel catfish (Butala et al. 2004), English sole (Stein et al. 1991), winter flounder, and scup (Snowberger & Stegeman 1987) microsomes suggests that CYP3A65, a very highly expressed hepatic CYP with the highest estrone production of our expressed proteins, may also be important for *in vivo* E_2 metabolism in spite of its lower overall enzymatic activity *in vitro.*

CYP1D1 is constitutively expressed in the liver but, unlike other CYP1s, is not inducible through the AhR pathway (Goldstone et al. 2009). CYP1D1 expression was significantly less than CYP1A in liver, gut, gill, and kidney but higher in brain of zebrafish (Goldstone et al. 2009). Coupled with the lack of AHR-mediated induction of CYP1D1 (Goldstone et al. 2009), compared with all other CYP1 genes (Jonsson et al. 2007), this data suggest that CYP1D1 does not share a primary functional role with CYP1A1 in xenobiotic metabolism. Basal expression of CYP1A was threefold higher than CYP1D1 in liver (Goldstone et al. 2009); the low overall activity of CYP1D1 *in vitro* for E_2 metabolism, and the much lower expression in liver compared with CYP1A, strongly suggests that CYP1D1 is unlikely to play an important *in vivo* role in E_2 metabolism. It is possible that the low activities seen from CYP1D1 were due to the presence of predominantly non-functional protein in the preparation, because we did not see high catalytic capacity in the fluorescent assays. However, all CYPs were similarly prepared, a P450 peak was present for this preparation and functional reductase activity was similar to the other CYP preparations. As we do not know the function of this protein, it is more likely, given the different metabolic capacity documented in this study, the distinct expression profile and lack of induction of CYP1D1 by typical AhR ligands in other studies (Goldstone et al. 2009), that CYP1D1 has a function that is different and distinct from the other CYP1 genes, including E_2 metabolism.

Mammalian species have more CYP3A genes than fish, as a result of more recent local gene duplications (Thomas 2007, Qiu et al. 2008). Zebrafish CYP3A65 (Tseng et al. 2005) and human CYP3A4, CYP3A5, and CYP3A7 (Maruyama et al. 2007) show similar induction patterns in the liver. In humans, CYP3A4 efficiently metabolizes E_2 similar to the CYP1As (Lee et al. 2003), while zebrafish CYP3A65 poorly metabolizes E_2 at rates that are similar to human CYP3A5 and CYP3A7 (Lee et al. 2003). Zebrafish CYP3A65 only produced 4-OHE_2 metabolites, when supplemented with cytochrome b_5, while mammalian CYP3As do produce 4-OHE_2 regardless of whether cytochrome b_5 is present (Lee et al. 2003). Although the mammalian CYP3A subfamily is phylogenetically clustered together with other vertebrate CYP3As, the CYP3A genes from the Actinopterygii class of ray-finned fishes, of which zebrafish are a member, cluster with fish CYP3B, CYP3C, and CYP3D genes (Qiu et al. 2008). As such, fish CYP3As share less of a common evolutionary history with

mammalian CYP3As than they do with fish CYP3Bs, CYP3Cs, and CYP3Ds. In the case of fish and mammalian CYP3As, common nomenclature may not be reflective of common function. Fish CYP3As have some similar functions compared with mammalian CYP3As; zebrafish CYP3A65 can metabolize E_2, and both zebrafish (this study) and medaka (Kashiwada et al. 2005) CYP3A proteins can metabolize mammalian CYP3A fluorescent substrates such as BFC; however, there are clear differences in the total activity of fish CYP3As as compared with mammalian CYP3As.

Ours is the first study involving the cloning, heterologous expression, and metabolic analysis of purified membrane preparations of both CYP1 and CYP3A isoforms from a non-mammalian vertebrate species. Purified membrane preparations resulted in concentrated levels of catalytically active CYP proteins. Using these preparations, we found that CYP1A and CYP1C1 have the highest rate of E_2 metabolism, followed by more modest E_2 metabolism by CYP1C2. The dominant metabolite formed was 2-OHE_2, similar to studies seen in other fish (Uno et al. 2008) and humans (Lee et al. 2003). Similar to human E_2 metabolism by CYP1B1 (Lee et al. 2003), the ratio of 4-OHE_2 to 2-OHE_2 was highest in zebrafish CYP1B1, yet the overall rates of metabolism by zebrafish CYP1B1 were lower than expected. CYP3A65 had low E_2 metabolism comparable with human CYP3A5 and CYP3A7 (Lee et al. 2003), formed no 4-OHE_2, and may not contribute greatly to E_2 metabolism *in vivo.* CYP1D1, which had a different developmental and tissue expression pattern from CYP1A and was not inducible by AhR agonists in zebrafish (Goldstone et al. 2009), had functional differences compared with other CYP1s. CYP1D1 has been suggested to have an endogenous function (Goldstone et al. 2009); our study suggests that E_2 metabolism is not a likely function of this protein. While overall E_2 metabolism in fish and other vertebrates is similar to mammalian E_2 metabolism, in non-mammalian vertebrates, the primary E_2 metabolizers *in vivo* are likely CYP1A and CYP1C enzymes. The contributions by CYP1B1 and CYP3A are likely less than in mammalian species.

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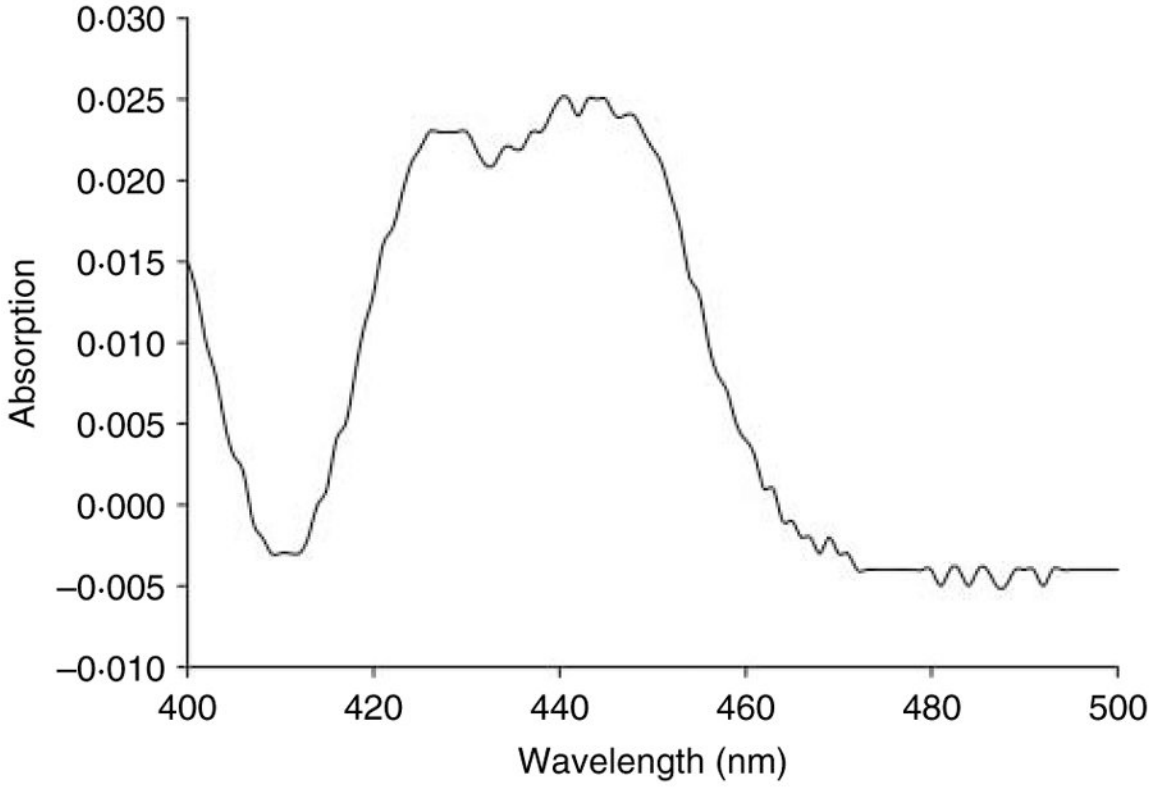

Figure 1.

Representative CO difference spectrum of recombinant zebrafish CYP purified membrane vesicles. Example shown is for membrane vesicles containing CYP1C2.

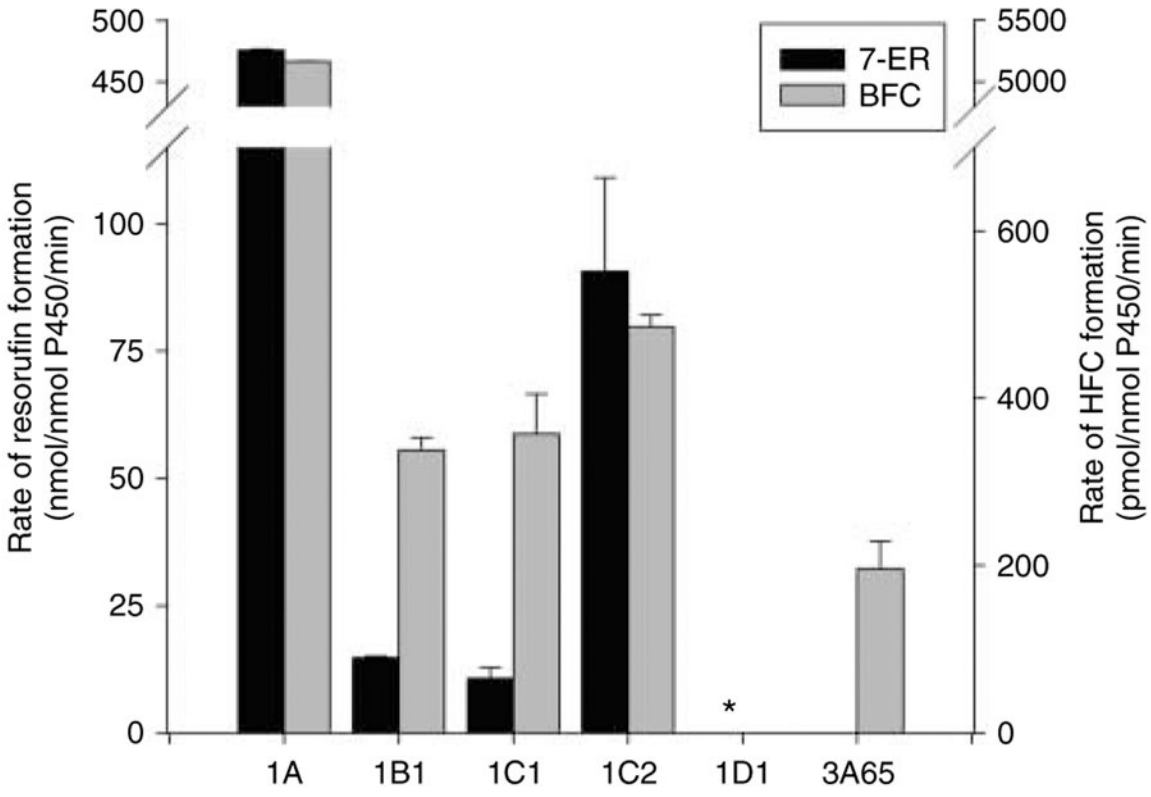

Figure 2.

Catalytic activity of recombinant zebrafish CYPs co-expressed with human NADPH–CYP reductase. Data are shown for the metabolism of 7-ethoxyresorufin (7-ER) and 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC), typical CYP1 and CYP3A substrates in mammals (Lake et al. 2009) and fish (Kashiwada et al. 2005). 7-ER and BFC are metabolized to resorufin and HFC respectively. Results are mean±S.E.M., for duplicate reactions. Catalytic activity was below the limits of detection for CYP3A65 (7-ER) and CYP1D1 (BFC). Values represent Mean±S.E.M. of reactions done in duplicate. *Resorufin was detected in CYP1D1 at a rate of 24±5 pmol/nmol P450/min.

Table 1

Primers and annealing temperatures used for (A) cloning of zebrafish cytochrome P450 (CYPs) and (B) fusion of the *ompA*(+2) segment to the zebrafish CYPs. All primers are listed in the 5′–3′ direction

A	**Forward primer**	**Reverse primer**	**Annealing temperature (°C)**	**GenBank accession number**
Construct				
CYP1A	TGGAGCTAATTGGCACTGGT	CCTGGATTTCAGGAGCTCAA	53	NM_131879
CYP1B1	CCACCCGAACTCTGAAACTC	GTTTTCTTAGCCGCCTTCATTT	53	NM_001013267
CYP1C1	GGAGGCTGAGTTTGGACTGA	CCCATTCGACTGGATGTTTT	53	NM_001020610
CYP1C2	TGAGCCATCCTCCGGTAA	GCAGTGGGTTAGACAGCACA	53	XM_686678
CYP1D1	GCGATTTGCCAACACTGATA	TGCCAACATTAGCTTGATGC	54	NM_001007310
CYP3A65	GGAGCTTCATCATCTTCAGCA	CCTCCTCCTCCTCCTCAGAC	53	NM_001020797

B	**Reverse primer**	**Linker primer**	**Annealing temperature (°C)**[b]
Construct[a]			
*omp*A-CYP1A	TCTAGAGCGGCCGCGAATTCACTA[c]	TGGAAGAATAGCCAGAGCCATXXX[d]	50, 55
*omp*A-CYP1B1	TCTAGAGCGGCCGCGAATTCACTA	AGCCAGCAGGACATCCATCATXXX	50, 55
*omp*A-CYP1C1	TCTAGAGCGGCCGCGAATTCACTA	CTGTCCGCTCCATTCCCGCATXXX	50, 55
*omp*A-CYP1C2	TCTAGAGCAGTGGGTTAGACAGCACA	CTCTGAATCCGACTGCGCCATXXX	50, 55
*omp*A-CYP1D1	TCTAGATGGAACACTGAGAGAATGATGG	GGAGATATTCTCAAGATTCATXXX	55, 53
*omp*A-CYP3A65	TCTAGAGCGGCCGCGAATTCACTA	TGTTTCTGCCGAGAAGAACATXXX	50, 55

[a] The forward primer used for *ompA*(+2)-CYP fusions was always 5′-GGAATTCCATATGAAAAAGACAGCTATCGCG-3′ with an NdeI restriction site underlined.

[b] The first temperature was used to anneal the forward and linker primer to the *ompA*(+2) template during step 1 of the PCR. The second annealing temperature is used during the second step of PCR to attach the *ompA*(+2) segment to the full-length CYP using the forward and reverse primers. See Materials and Methods section for details.

[c] The XbaI restriction site is underlined.

[d] XXX represents 5′-CGGGACGGCCTGCGCTACGGTAGCGA-3′, which primes against the 3′ end of the *ompA*(+2) segment.

Table 2

Total protein, P450 content, and cytochrome *c* activity in membrane vesicles purified from bacteria co-expressing zebrafish cytochrome P450 (CYP) genes and human NADPH–CYP reductase (CPR), or expressing CPR alone. Cytochrome *c* activity expressed as mean activity ±S.E.M. of reactions done in triplicate

	Total protein (mg/ml)	Total P450 content (nmol/mg)	Cytochrome *c* activity (nmol/mg per min)
Construct			
CPR[a]	11·65	–[b]	29·68±3·2
CYP1A	16·42	0·655	16·77±2·1
CYP1B1	19·20	0·278	16·27±0·68
CYP1C1	22·25	0·395	13·93±0·37
CYP1C2	20·50	0·473	17·52±1·0
CYP1D1	22·77	0·139	15·50±0·11
CYP3A65	17·04	0·282	18·74±0·43

[a]Human NADPH–cytochrome P450 reductase.

[b]– Total P450 content not determined.

Table 3

Rate of formation of 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2) by heterologously expressed zebrafish with and without cytochrome b_5. Data from human and eel are from published studies for comparison with the zebrafish data

	2-OHE_2 (pmol/nmol P450/min)	4-OHE_2 (pmol/nmol P450/min)	Ratio of 4-OHE_2/ 2-OHE_2 (%)
CYP			
Zebrafish[a]			
CYP1A	425·2±85·0	21·8±5·4	5
CYP1A+b_5	892·6±81·1	ND[b]	NA[c]
CYP1B1	27·1±10·7	8·8±5·7	32
CYP1B1+b_5	18·0±0·1	4·7±0·1	26
CYP1C1	510·0±1·1	40·9±3·9	8
CYP1C1+b_5	1083±33	35·2±0·7	3
CYP1C2	155·4±1·5	4·2±0·2	3
CYP1C2+b_5	57·0±1·4	1·8±0·3	3
CYP1D1	31·4±1·9	ND	NA
CYP3A65	25·6±5·3	ND	NA
CYP3A65+b_5	23·0	3·2	14
Human[d]			
CYP1A1	2523±208	163±22	7
CYP1A2	4065±156	343±24	9
CYP1B1	108±3	371±16	344
CYP3A4	355±41	78±11	22
CYP3A4+b_5	3093±91	497±27	16
CYP3A5	125±22	67±5	53
CYP3A7+b_5	146±17	55±3	58
Japanese eel[e]			
CYP1A9	177	ND	NA
CYP1C1	ND	ND	NA

[a] Data from this study, using 50 μM E_2 at 28 °C for 2 h; values represent mean±S.E.M. of duplicate reactions.

[b] ND – metabolite not detected.

[c] NA – not applicable; one or both metabolites not detected.

[d] Human data from Lee et al. (2003), using 20 μM E_2 at 37 °C for 20 min; values represent mean±S.D. of triplicate reactions.

[e] Japanese eel data calculated based on Uno et al. (2008), using 100 μM E_2, at 37 °C assuming constant rate over 8-h reaction.

Table 4

Rate of formation of 2-hydroxyestradiol (2-OHE_2) and 4-hydroxyestradiol (4-OHE_2) by liver microsomes from zebrafish. Data from other fish and mammalian species are from published studies for comparison to the zebrafish data

	Sex	*N*	2-OHE_2 (pmol/mg/min)	4-OHE_2 (pmol/mg/min)	Ratio of 4-OHE_2/2-OHE_2 (%)
Species					
Zebrafish[a]	Male	1	0·068	ND[b]	NA[c]
	Female	1	0·269	ND	NA
Channel catfish[d]	Male	3	20·3±1·1	0·748±0·032	3·7
Scup[e]	Male	5	362±141	–[f]	NA
	Female	6	157±37	–	NA
Winter flounder[e]	Male	13	296±79	–	NA
	Female	13	89±39	–	NA
English sole[g]	Juvenile	5	260±17	–	NA
Human[h]	Male	21	46·6±25·7	7·1±3·4	15
	Female	12	47·7±28·2	8·2±4·4	17
Rat[i]	Male	3–4	1710±180	390±50	23
	Female	3–4	240±20	70±20	29

[a] Data from this study, using 50 μM E_2 at 28 °C.

[b] ND – metbolite not detected.

[c] NA – not applicable; one or both metabolites not detected.

[d] Channel catfish data from Butala et al. (2004), using 50 μM E_2 at 30 °C; values represent mean±S.E.M.

[e] Data from Snowberger & Stegeman (1987), using 25 μM E_2 at 25 °C for flounder, and 30 °C for scup; values represent mean±S.D.

[f] – not investigated in this study.

[g] English sole data from Stein et al. (1991), using 100 μM E_2 at 25 °C; values represent mean±S.E.M.

[h] Human data from Lee et al. (2001), using 20 μM E_2 at 37 °C; values represent mean±S.D.

$\acute{I}$ Rat data from Dannan et al. (1986), using 200 μM E_2 at 37 °C; values represent mean±S.E.M.