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Structural features of cytochrome P450 1A associated with the absence of EROD activity in liver of the loricariid catfish *Pterygoplichthys* sp.

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ABSTRACT

The Amazon catfish genus *Pterygoplichthys* (Loricariidae, Siluriformes) is closely related to the loricariid genus *Hypostomus*, in which at least two species lack detectable ethoxyresorufin-*O*-deethylase (EROD) activity, typically catalyzed by cytochrome P450 1 (CYP1) enzymes. *Pterygoplichthys sp.* liver microsomes also lacked EROD, as well as activity with other substituted resorufins, but aryl hydrocarbon receptor agonists induced hepatic CYP1A mRNA and protein suggesting structural/functional differences in *Pterygoplichthys* CYP1s from those in other vertebrates. Comparing the sequences of CYP1As of *Pterygoplichthys sp.* and of two phylogenetically related siluriform species that do catalyze EROD (*Ancistrus sp.*, Loricariidae and *Corydoras sp.*, Callichthyidae) showed that these three proteins share amino acids at 17 positions that are not shared by any fish in a set of 24 other species. *Pterygoplichthys* and *Ancistrus* (the loricariids) have an additional 22 amino acid substitutions in common that are not shared by *Corydoras* or by other fish species. *Pterygoplichthys* has six exclusive amino acid substitutions. Molecular docking and dynamics simulations indicate that *Pterygoplichthys* CYP1A has a weak affinity for ER, which binds infrequently in a productive orientation, and in a less stable conformation than in CYP1As of species that catalyze EROD. ER also binds with the carbonyl moiety proximal to the heme iron. *Pterygoplichthys* CYP1A has amino acid substitutions that reduce the frequency of correctly oriented ER in the AS preventing the detection of EROD activity. The results indicate that loricariid CYP1As may have a peculiar substrate selectivity that differs from CYP1As of most vertebrate.

1. Introduction

Cytochrome P450 family 1 (*CYP1*) genes code for biotransformation enzymes in all vertebrate groups (Goldstone and Stegeman, 2006; Nelson, 2003). CYP1As in fish and CYP1A1 in mammals are strongly induced by and metabolize aryl hydrocarbon receptor (AHR) agonists (e.g. planar halogenated and aromatic hydrocarbons). These enzymes also are implicated in the toxic effects of many of such compounds (Rifkind, 2006). Ethoxyresorufin-*O*-deethylase activity (EROD) is widely used as a marker of CYP1A activity and induction (Parente et al., 2008; Stegeman and Livingstone, 1998; Whyte et al., 2000). Other CYP1 proteins (CYP1A2 and CYP1B1 in mammals and CYP1B1, CYP1C1, CYP1C2, and CYP1D1 in fish) also can catalyze EROD (Hirakawa et al., 2007; Shimada et al., 2007; Uno et al., 2010), but function of expressed proteins and immunoassays indicate that CYP1A is the most active catalyst for this activity in fish liver (Kloepper-Sams et al., 1987; Scornaienchi et al., 2010).

Recently, we reported that we could not detect EROD activity in microsomal preparations of liver and other organs from two fish species in the genus *Hypostomus* family Loricariidae (Siluriformes), either from control fish or fish treated with potent AHR agonists (Parente et al., 2009). The lack of EROD activity suggests that CYP1s, and perhaps especially the CYP1As in the loricariids may differ in structure and function from those of other vertebrates. The CYP1A amino acid sequence is remarkably conserved among vertebrates, with a common three-dimensional structure and similar substrate

Abbreviation: AHR, Aryl hydrocarbon receptor; BNF, ß-naphthoflavone; CYP1A, Cytochrome P450 1A; DMBA, 7,12-dimethylbenz[α]anthracene; DMSO, Dimethyl sulfoxide; ER, Ethoxyresorufin; EROD, Ethoxyresorufin-O-deethylase; i.p., intraperitoneally; ITS, internal transcribed spacer; MD, Molecular dynamics; NADPH, Reduced Nicotinamide adenine dinucleotide phosphate; OD, Optical density; PCB126, 3,3',4,4',5-pentachlorinated biphenyl; PCR, Polymerase Chain Reaction; PDB, Protein data bank; PMDB, Protein Model Data Bank; qPCR, Quantitative real time PCR; RACE reactions, Rapid amplification of cDNA ends; RMS, Root mean square; RMSD, Root-mean-squared deviation; RMSF, Root-mean-squared fluctuation; SRS, Substrate recognition site; TRIS, tris(hydroxymethyl)aminomethane; VTFM, Variable target function method.

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affinities and functions (Goldstone and Stegeman, 2006). However, single non-synonymous amino acid substitutions are able to dramatically change CYP1A1 and CYP1A2 specificity in site-directed mutagenesis studies with heterologously-expressed mammalian protein, which may alter xenobiotic detoxification, activation of pro-mutagens, and cancer susceptibility (Lewis et al., 2007; Liu et al., 2004; Taly et al., 2007; Urban et al., 2001).

In this study, we examined two other members of the Loricariidae family, *Pterygoplichthys sp.* and *Ancistrus sp.*, and also *Corydoras sp.* (Callichthyidae), a siluriform from a different family, to assess whether other Siluriformes also lack EROD activity and to provide a structural understanding for this unusual phenotype. Similar to *Hypostomus*, *Pterygoplichthys* lacks microsomal EROD activity, as well as activity with other substituted resorufins (methoxy-, pentoxy- and benzyloxyresorufin). The genus *Pterygoplichthys* is more closely related to *Hypostomus* than is *Ancistrus* while *Corydoras* more distantly related (Supplemental Fig. 1). *CYP1As* were cloned from complementary DNA (cDNA) and sequenced, and CYP1A transcript, protein, and enzyme activities were assessed in liver of fish exposed to the AHR agonists ß-naphthoflavone (BNF) or 3,3′,4,4′,5-pentachlorinated biphenyl (PCB126).

To further understand the nature of this rare condition, we sought a structural basis for the lack of ER metabolism by generating homology models for the *Pterygoplichthys* CYP1A and performing docking studies followed by molecular dynamics simulations for ER binding *in silico*. All of these procedures were extended to the CYP1A of the phylogenetically related species (*Ancistrus sp.*) that is able to catalyze EROD, and to CYP1A of zebrafish (*Danio rerio;* Cypriniformes; Cyprinidae), which is not closely related to the Siluriformes. The results suggest unusual active site features that could produce the anomalous substrate specificities of CYP1A of some loricariid species.

2. Material and methods

2.1. Fish handling and exposure

Species in three genera of Siluriformes fishes were used in this study; two from the Loricariidae family (*Pterygoplichthys* and *Ancistrus*) and one from the Callichthyidae (*Corydoras*). All fishes were purchased from a local supplier in Falmouth, MA USA. *Pterygoplichthys* identity was further characterized by the PCR amplification and sequencing of internal transcribed spacer (ITS) and partial tRNA-Pro/D-loop/tRNA-Phe conserved fragments from genomic DNA (Montoya-Burgos, 2003). Sequences (Supplemental File 2) were blasted against GenBank showing that the species used in this study was most closely related to *Pterygoplichthys scrophus* (synonyms, *Liposarcus scrophus* and *Glyptoperichthys scrophus*).

Fishes were acclimated in aquaria in the laboratory for one week in re-circulating filtered water at 28 °C. Fish were intraperitoneally (i.p.) injected with 50 mg/kg of β-naphthoflavone (BNF) or 50 mg/kg of 3,4,3′,4′,5′ pentachlorobiphenyl (PCB126) in DMSO, or to DMSO (Parente et al., 2009). A second batch of *Pterygoplichthys* and zebrafish (*Danio rerio*) was exposed to 100 μM PCB126 added in DMSO in static water exposures for 24 h, followed by 48 h in re-circulating charcoal filtered water (Jonsson et al., 2009; Jönsson et al., 2007). Fish were sampled three days after initiation of exposure. A total of 31 *Pterygoplichthys*, 7 *Ancistrus*, 11 *Corydoras* and 30 zebrafish were used in this study. The number of fish in each experimental group is described in the legend of Fig. 1.

2.2. EROD determination and CYP1A protein detection

Hepatic microsomes were prepared as described elsewhere (Stegeman et al., 1997) for spectrofluorimetric EROD determination (Stegeman et al., 1997) and CYP1A detection by immunoblotting (Goldstone et al., 2009). For *Corydoras* and zebrafish, livers from all fish of each group were pooled for microsome preparation due to the small fish size. For the other two species, microsomes were prepared from individual livers. EROD reactions in microsomes were started by the addition of NADPH to reaction mixture and fluorescence increment was determined using a Cytofluor at 30 °C. EROD values were normalized to total microsomal protein content (Lowry et al., 1951) and expressed as pmol of resorufin minute^{-1} milligram of protein^{-1}. CYP1A protein was detected in liver microsomes by western blotting using a monoclonal anti-scup CYP1A antibody (MAb1-12-3) (Stegeman, 1989). Secondary antibodies were coupled with a fluorophore (IRDye 800, LI-COR Biosciences, San Diego, CA) and signal was captured in the Odyssey Infrared fluorescent dual laser scanner (LI-COR) using laser excitation at 800 nm (sample detection) and 700 nm (BioRad Precision Plus™ blue prestained protein standard detection).

NADPH consumption by liver microsomes was measured using a Shimadzu dual beam spectrophotometer. Liver microsomes were diluted in buffer to 0.3 ml in 50 mM TRIS, 100 mM NaCl, pH 7.8 buffer with 0.106 μmol NADPH and divided between sample and reference cuvettes (0.26 μmol/ml final). Baseline was determined and then 50 μl of buffer was added to the reference cuvette and 50 μl buffer + 8 μM ER (2 μM final) was added to the sample cuvette. The time course of loss of absorbance at 340 nm was recorded for 4 min, then any change in absorbance at 572 nm was recorded for 3 min. Calculations were made using appropriate extinction coefficients from linear portions of absorbance vs time plots.

2.3. CYP1A transcript quantification and cloning

Samples of individual livers were collected for RNA extraction using STAT-60 (Invitrogen) followed by DNAse treatment. RNA quantity and quality were determined spectrophotometrically (Nanodrop ND1000, NanoDrop Technologies, Wilmington, DE). cDNA was prepared following the manufacturer's instructions for the Omniscript Reverse Transcriptase (Qiagen, Inc., Valencia, CA) with anchored oligo(dT) primers (MWG Biotech. Inc., High Point, NC) and RNasin RNase inhibitor (Promega Corp.

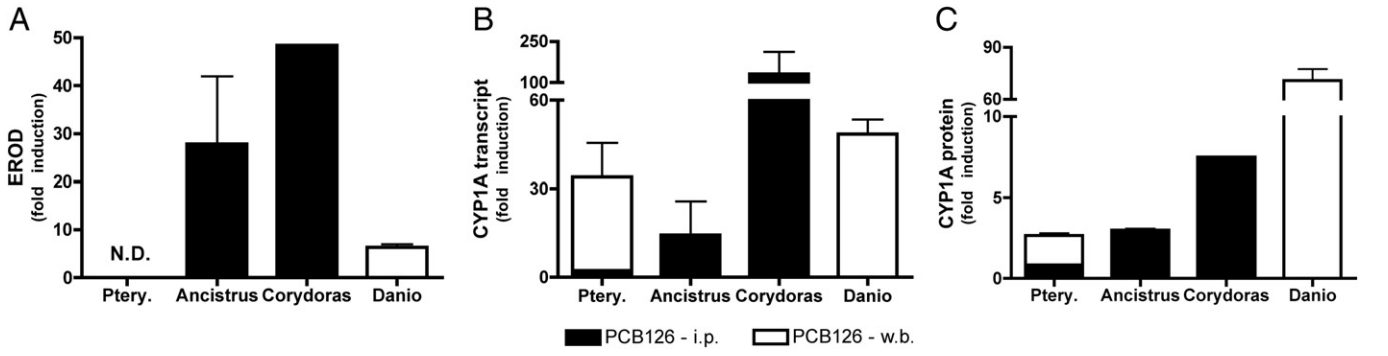

Fig. 1. CYP1A activity — EROD (A), *CYP1A* transcript (B) and CYP1A protein (C) fold inductions by intraperitoneal exposure (black bars) and exposure through the water (white bars) to PCB126 in *Pterygoplichthys* (Ptery, n = 2 and 8), *Ancistrus* (n = 2), *Coridoras* (n = 4) and *Danio rerio* (Danio, n = 3 pools of 5 fish each). Bars represent means ± standard deviation. N.D. is not detected. EROD detection limit is 2.5 pmol of resorufin.