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Zebrafish Have an Ethanol-Inducible Hepatic 4-Nitrophenol Hydroxylase that is Not CYP2E1-like

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Abstract

Zebrafish are an attractive model organism for toxicology; however, an important consideration in translating between species is xenobiotic metabolism/bioactivation. CYP2E1 metabolizes small hydrophobic molecules, e.g. ethanol, cigarette smoke, and diesel exhaust components. CYP2E1 is thought to only be conserved in mammals, but recent reports identified homologous zebrafish cytochrome P450s. Herein, *ex vivo* biochemical measurements show that unlike mammals, zebrafish possess a low-affinity 4-nitrophenol hydroxylase ($K_m \sim 0.6$ mM) in hepatic microsomes and mitochondria that is inducible only 1.5- to 2-fold by ethanol and is insensitive to 4-methylpyrazole inhibition. In closing, we suggest creating improved models to study CYP2E1 in zebrafish.

Graphical Abstract

1. Introduction

Zebrafish are an increasingly popular model organism for toxicology and pharmacology (Bailey et al., 2013; Bailey et al., 2015; Dai et al., 2014; Garcia et al., 2016; MacRae and Peterson, 2015). Compared to rodent models, zebrafish are considerably more cost-efficient and have powerful genetic tools available to study basic biological and pathological processes. Other advantages of zebrafish include a small size, short reproductive cycle, high fecundity, external development, transparent embryos, and adaptability to diverse applications (Bailey et al., 2013; Dai et al., 2014). Furthermore, many aspects of genetics and biology are known to be conserved between zebrafish and humans (Ota and Kawahara,

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2014); however, better understanding of the comparative biology of humans and zebrafish must be established (MacRae and Peterson, 2015).

Critical aspects in comparing the biology of each system for the study toxicology or pharmacology are the absorption, distribution, metabolism, elimination, and transport (ADMET) properties. The cytochrome P450 family of enzymes (CYP for specific isoforms) plays important roles in xenobiotic metabolism of many pollutants and drugs. The full suite of cytochrome P450s in zebrafish have been recently reviewed (Saad et al., 2016); compared to humans, zebrafish have many homologous xenobiotic metabolizing enzymes. For example, the 1A and 1B family of PAH-metabolizing enzymes are well-conserved in zebrafish. Similarly, human CYP3A4, which metabolizes about half of current drugs on the market (Zhou, 2008), has homologous CYP isoforms in zebrafish, CYP3C1-4. However, there has not been a clearly identified zebrafish homolog for human CYP2E1.

CYP2E1 metabolizes low molecular weight hydrophobic compounds including drugs, pollutants, and endogenous compounds (Miller, 2008). It is best known and most widely studied in its role in ethanol metabolism, where it is alternatively referred to as the microsomal ethanol oxidizing system (MEOS) (Novak and Woodcroft, 2000). However, it also bioactivates small hydrophobic environmental pollutants (e.g. benzene, 1,3-butadiene, styrene, trichloroethylene, and aniline) (Miller, 2008) and drives hepatotoxicity of the drugs acetaminophen and isoniazid through the production of reactive metabolites. Further, CYP2E1 produces reactive oxygen species directly through uncoupling of its catalytic cycle, with toxicological implications even in the absence of substrate (Cederbaum, 2006). Together, these features make CYP2E1 a critical player in the toxicological evaluation of many compounds.

Because CYP2E1 plays a critical role in toxicity, all studies involving a CYP2E1-activated compound in any model organism should consider its bioactivation. Recent studies have proposed the use of zebrafish to study alcoholic liver disease (Howarth et al., 2011; Lassen et al., 2005; Tsedensodnom et al., 2013). One study reported that zebrafish possess P450 enzymes that are homologous to mammalian CYP2E1, Cyp2y3, and Cyp2p6 (Tsedensodnom et al., 2013). The assertion of homology was based on a sequence alignment giving 43% sequence identity with human CYP2E1, cross-reactivity with antibodies raised against human CYP2E1, and a time-dependent increase in the cross-reactive protein after ethanol exposure. However, a direct comparison of enzyme activities is needed to show functional homology.

Herein, we compare CYP2E1-like activity in the zebrafish to that of mammalian CYP2E1 through *ex vivo* biochemical analysis. First, adult zebrafish were exposed to either tank water (control, n=20) or 0.5% ethanol (109mM, n=20; concentration chosen as described below) for 24 hours. Following the exposure, the zebrafish were sacrificed, and livers, hearts, and brains were dissected. Each tissue was pooled from the animals in each group and homogenized, and the microsomal and mitochondrial fractions were obtained for biochemical analysis. We assessed 4-nitrophenol metabolism by microsomes and mitochondria from each tissue fraction, first testing for CYP2E1 activity at a high (1 mM) or low (100uM) concentration. After only observing measurable activity in liver fractions, we

performed steady-state kinetics on the liver mitochondrial and microsomal fractions to obtain a kinetic profile. These experiments were performed with and without the specific CYP2E1 inhibitor 4-methylpyrazole. Finally, we compared these activities to the well-conserved mammalian CYP2E1 in rat liver microsomes.

2. Materials and Methods

2.1 Reagents

All chemicals were HPLC grade or higher. The following were purchased from Fisher Scientific (Waltham, MA): 4-nitrophenol, 4-nitrocatechol, 4-nitroanisole, 4-methylpyrazole, NADP monosodium salt, and glucose-6-phosphate. Glucose-6-phosphate dehydrogenase was purchased from Sigma Aldrich (St. Louis, MO). Methanol and 200 proof ethanol were purchased from VWR (Radnor, PA).

2.2 Zebrafish Exposures

Laboratory-reared *Danio rerio* (Tropical 5D) were maintained in a recirculating AHAB system (Aquatic Habitats, Inc., Apopka, FL, USA) on a 14:10h light-dark cycle. Water quality was maintained at 28°C, pH 7.0–7.5, 60ppm artificial seawater (Instant Ocean, Foster & Smith, Rhinelander, WI). The fish were fed twice daily *ad libitum* with live hatched brine shrimp and a mix of Cyclop-eeze (Argent Chemical Laboratories, Inc., WA) and Zeigler's Adult Zebrafish Complete Diet (Zeigler Bros., Inc., Gardners, PA). All animal handling was approved by the Duke University Institutional Animal Care & Use Committee.

Forty healthy adult male zebrafish (>6 months in age) were selected for the following ethanol exposure. Zebrafish were exposed to tank water (control) or 0.5% ethanol (109mM) in tank water. Exposures were carried out at a density of 5 fish per 9.4 L aquarium (Pacific Coast Distributing, Inc., Phoenix, AZ, USA) with hanging filters, with 4 replicate tanks per treatment group (n=20 fish per treatment group). After 24 hours, each fish was individually anesthetized in ice water and euthanized by cervical dislocation. Heart, brain, and liver dissections were performed as previously described (Jayasundara et al., 2015). Samples were immediately weighed, pooled with their respective tissue type (n=20 specimens per pooled sample), and placed on ice in homogenization buffer until all dissections were completed.

2.3 Preparation of Tissue Homogenate and Subcellular Fractionation

Subcellular fractionation from liver, heart, and brain soft tissue was carried out as previously described for mammalian liver (Hartman et al., 2017). Briefly, tissues were homogenized in buffer containing 2.5mM MOPS, pH 7.4, 2mM EDTA, 70mM sucrose, and 220mM mannitol and pulverized using a 2mL Tenbroeck tissue grinder. Homogenate was centrifuged at 4°C, 600×g for 10 min, and the resulting supernatant was centrifuged a second time at 4°C, 9000×g for 20 min to pellet a crude mitochondrial fraction. The mitochondrial pellet was rinsed and pelleted again at 9000×g for 20 min. Supernatant (S9) was then centrifuged at 4°C, 100,000×g for 60 min to isolate the microsomal fraction containing the endoplasmic reticulum. Protein content was obtained using the BCA Assay Kit (Pierce, Thermo Fisher, Waltham, MA) and fractions stored at –80°C until biochemical analysis.

2.4 Steady-state kinetics of 4-nitrophenol oxidation

In mammals, hydroxylation of 4-nitrophenol to 4-nitrocatechol is a specific and efficient reaction carried out by microsomal and mitochondrial CYP2E1. Assays were carried out as previously described (Hartman et al., 2013a; Hartman et al., 2013b); briefly, microsomal and mitochondrial proteins at 0.5mg/mL were incubated with various concentrations of 4-nitrophenol and a NADPH regenerating system in 50mM potassium phosphate buffer, pH 7.4, at 28°C for 2 hours. The reactions were then stopped by the addition of an equal volume of 0.4N trichloroacetic acid containing 25µM 4-nitroanisole (used as an internal standard in the HPLC analysis). Proteins were then removed by centrifugation at 14,000×g for 8 min. Resulting supernatants were analyzed by HPLC to quantify the formation of 4-nitrocatechol using a standard curve. HPLC analysis was carried out on an Agilent 1100 HPLC with UV absorbance detection at 360nm. Compounds were separated with isocratic conditions of 47.5% methanol and 52.5% 0.1% acetic acid in water on a Waters Symmetry 5µm 150×4.6mm C18 column. All compounds eluted within 8 min.

3. Results and Discussion

3.1 Zebrafish hepatic microsomes and mitochondria possess an ethanol-inducible 4-nitrophenol hydroxylase

Zebrafish brain, heart, and liver microsomes and mitochondria from control and ethanol-exposed fish were first tested for 4-nitrophenol hydroxylation (lowest detectable rate under assay conditions is 1pmol/min/mg protein). Only liver showed activity, with ethanol-induced fish possessing ~1.5 to 2-fold higher activity compared to controls (Figs. 1A–B). This is a much lesser induction of CYP2E1-like activity compared to previously reported ethanol induction of Cyp2y3, and Cyp2p6 (Tsedensodnom et al., 2013). That study used a much higher concentration of ethanol (350mM); however, in our hands, 1% (218mM) ethanol was not tolerated by the fish for a 24-hour exposure (data not shown) and the experiment had to be discontinued due to significant lethality as early as 1-hour.

Notably, 4-nitrophenol hydroxylase activity in basal and ethanol-induced conditions was completely insensitive to 4-methylpyrazole inhibition at 40µM 4-methylpyrazole (Figs. 1A–B), well above the reported K_i for mammalian CYP2E1 inhibition (1–2µM) (Hartman et al., 2013b). Thus, the overlapping specificity of the 4-nitrophenol hydroxylase enzyme(s) in zebrafish is incomplete, and 4-methylpyrazole is not an appropriate inhibitor for zebrafish 4-nitrophenol hydroxylase.

3.2 Zebrafish hepatic 4-nitrophenol hydroxylase is a low-affinity enzyme

CYP2E1 is a highly conserved mammalian enzyme, with high sequence identity and very similar catalytic activity among species. For 4-nitrophenol oxidation, rabbit, rat, mouse, and human CYP2E1 activity in microsomal fractions have K_m values around 25–50µM (Collom et al., 2008; Koop, 1986), which represents relatively high affinity compared to other CYP2E1 substrates; in our study, rat liver microsomes had a K_m value of 25 µM. By contrast, zebrafish microsomes and mitochondria displayed K_m values >10 times higher than the mammalian enzyme (Fig 1C, Table 1). This suggests that the enzyme activity present in zebrafish is much lower affinity compared to mammalian CYP2E1.

Furthermore, mammalian CYP2E1 in liver microsomes displays dramatic substrate inhibition, wherein the rate of 4-nitrophenol metabolism increases at low substrate concentrations and then decreases again at higher substrate concentrations (Fig. 1E). However, zebrafish liver microsomes display simple Michaelis-Menten kinetics (Fig. 1C), indicating a difference in the mechanism of oxidation between the zebrafish hepatic 4-nitrophenol hydroxylase and mammalian CYP2E1. Finally, both rat mitochondria (Hartman et al., 2015) and zebrafish mitochondria (Fig. 1D) display Michaelis-Menten kinetics during their metabolism, indicating similar kinetic mechanisms between the mitochondrial activities. However, the affinity is much lower in the zebrafish.

3.3 Concluding remarks

Zebrafish are an attractive model for toxicology due to low cost and ease of maintenance compared to rodent models. However, one of the most significant challenges in translating animal models to humans are differences in metabolism. CYP2E1 is a critical enzyme for toxicology, and there are clearly enzymes in the zebrafish which overlap specificity with mammalian CYP2E1. However, care must be taken to characterize the metabolism of each substrate and understand how differences in enzyme specificity may impact toxicological outcomes and translation to other species. For studies of alcohol, tobacco smoke, diesel exhaust, and other exposures containing CYP2E1-activated substrates, biochemical studies are needed to determine what activities are present. In the future, it would be advantageous to create a transgenic mammalian CYP2E1 zebrafish, which may not even require knockdown of background 4-nitrophenol hydroxylase activities due to low affinity of those enzymes for CYP2E1 substrates.

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References

- Bailey J, Oliveri A, Levin ED. Zebrafish model systems for developmental neurobehavioral toxicology. *Birth Defects Res C Embryo Today*. 2013; 99:14–23. [PubMed: 23723169]
- Bailey JM, Oliveri AN, Levin ED. Pharmacological analyses of learning and memory in zebrafish (*Danio rerio*). *Pharmacol Biochem Behav*. 2015; 139(Pt B):103–111. [PubMed: 25792292]
- Cederbaum AI. Cytochrome P450 2E1-dependent oxidant stress and upregulation of anti-oxidant defense in liver cells. *J Gastroenterol Hepatol*. 2006; 21(Suppl 3):S22–25. [PubMed: 16958665]
- Collom SL, Laddusaw RM, Burch AM, Kuzmic P, Perry MD Jr, Miller GP. CYP2E1 substrate inhibition. Mechanistic interpretation through an effector site for monocyclic compounds. *The J Biol Chem*. 2008; 283:3487–3496. [PubMed: 18056994]
- Dai YJ, Jia YF, Chen N, Bian WP, Li QK, Ma YB, Chen YL, Pei DS. Zebrafish as a model system to study toxicology. *Environ Toxicol Chem*. 2014; 33:11–17. [PubMed: 24307630]
- Garcia GR, Noyes PD, Tanguay RL. Advancements in zebrafish applications for 21st century toxicology. *Pharmacol Ther*. 2016; 161:11–21. [PubMed: 27016469]
- Hartman JH, Boysen G, Miller GP. Cooperative effects for CYP2E1 differ between styrene and its metabolites. *Xenobiotica*. 2013a; 43:755–764. [PubMed: 23327532]

- Hartman JH, Bradley AM, Laddusaw RM, Perry MD Jr, Miller GP. Structure of pyrazole derivatives impact their affinity, stoichiometry, and cooperative interactions for CYP2E1 complexes. *Arch Biochem Biophys*. 2013b; 537:12–20. [PubMed: 23811196]
- Hartman JH, Martin HC, Caro AA, Pearce AR, Miller GP. Subcellular localization of rat CYP2E1 impacts metabolic efficiency toward common substrates. *Toxicology*. 2015; 338:47–58. [PubMed: 26463279]
- Hartman JH, Miller GP, Caro AA, Byrum SD, Orr LM, Mackintosh SG, Tackett AJ, MacMillan-Crow LA, Hallberg LM, Ameredes BT, Boysen G. 1,3-Butadiene-induced mitochondrial dysfunction is correlated with mitochondrial CYP2E1 activity in Collaborative Cross mice. *Toxicology*. 2017; 378:114–124. [PubMed: 28082109]
- Howarth DL, Passeri M, Sadler KC. Drinks like a fish: using zebrafish to understand alcoholic liver disease. *Alcohol Clin Exp Res*. 2011; 35:826–829. [PubMed: 21284674]
- Jayasundara N, Kozal JS, Arnold MC, Chan SS, Di Giulio RT. High-Throughput Tissue Bioenergetics Analysis Reveals Identical Metabolic Allometric Scaling for Teleost Hearts and Whole Organisms. *PLoS One*. 2015; 10:e0137710. [PubMed: 26368567]
- Koop DR. Hydroxylation of p-nitrophenol by rabbit ethanol-inducible cytochrome P-450 isozyme 3a. *Mol Pharm*. 1986; 29:399–404.
- Lassen N, Estey T, Tanguay RL, Pappa A, Reimers MJ, Vasiliou V. Molecular cloning, baculovirus expression, and tissue distribution of the zebrafish aldehyde dehydrogenase 2. *Drug Metab Dispos*. 2005; 33:649–656. [PubMed: 15703303]
- MacRae CA, Peterson RT. Zebrafish as tools for drug discovery. *Nat Rev Drug Discov*. 2015; 14:721–731. [PubMed: 26361349]
- Miller GP. Advances in the interpretation and prediction of CYP2E1 metabolism from a biochemical perspective. *Expert Opin Drug Metab Toxicol*. 2008; 4:1053–1064. [PubMed: 18680440]
- Novak RF, Woodcroft KJ. The alcohol-inducible form of cytochrome P450 (CYP 2E1): role in toxicology and regulation of expression. *Arch Pharm Res*. 2000; 23:267–282. [PubMed: 10976571]
- Ota S, Kawahara A. Zebrafish: a model vertebrate suitable for the analysis of human genetic disorders. *Congenit Anom*. 2014; 54:8–11.
- Saad M, Cavanaugh K, Verbueken E, Pype C, Casteleyn C, Van Ginneken C, Van Cruchten S. Xenobiotic metabolism in the zebrafish: a review of the spatiotemporal distribution, modulation and activity of Cytochrome P450 families 1 to 3. *J Toxicol Sci*. 2016; 41:1–11. [PubMed: 26763387]
- Tsedensodnom O, Vacaru AM, Howarth DL, Yin C, Sadler KC. Ethanol metabolism and oxidative stress are required for unfolded protein response activation and steatosis in zebrafish with alcoholic liver disease. *Dis Model Mech*. 2013; 6:1213–1226. [PubMed: 23798569]
- Zhou SF. Drugs behave as substrates, inhibitors and inducers of human cytochrome P450 3A4. *Curr Drug Metab*. 2008; 9:310–322. [PubMed: 18473749]

Highlights

- Zebrafish represent an increasingly popular model organism for toxicology and pharmacology, including studies that involve CYP2E1 as a critical enzyme in the mechanism of clearance or bioactivation.
- CYP2E1 is a mammalian cytochrome P450 and is not known to be expressed in fish; however, some studies report that CYP2E1 activity exists in zebrafish.
- Herein, we show that there is a 4-nitrophenol hydroxylase in zebrafish liver that is inducible 1.5- to 2-fold by ethanol; however, this activity is not CYP2E1-like based on substrate affinity or sensitivity to the CYP2E1 inhibitor 4-methylpyrazole.

Figure 1. Zebrafish possess an ethanol-inducible, low-affinity hepatic 4-nitrophenol hydroxylase that is insensitive to 4-methylpyrazole inhibition

Ethanol induction of 4-nitrophenol hydroxylation in liver microsomes (Panel A) and mitochondria (Panel B) resulting from a 24-hour exposure to 0.5% ethanol is shown as a percentage of control (non-induced, uninhibited) rates. Red bars represent control animals, blue bars represent ethanol-treated animals. Darker shading indicates presence of 40 μ M 4-methylpyrazole, a specific CYP2E1 inhibitor. Steady-state kinetics are shown for zebrafish microsomes (Panel C) and mitochondria (Panel D) for 4-nitrophenol hydroxylation. The black line represents uninhibited reactions, while the grey line indicates reactions performed in the presence of 40 μ M 4-methylpyrazole. For comparison, rat liver microsomal 4-nitrophenol oxidation is shown with and without 4-methylpyrazole in Panel E.

Table 1

Kinetic parameters of 4-nitrophenol oxidation by rat liver microsomes and zebrafish liver microsomes and mitochondria

Enzyme Source	4MP ^a	K _m (μM)	K _i (μM) ^b	k _{cat} (nmol/min/mg protein)
Rat microsomes	–	25 (3.6 to 46)	3600 (1200 to 6000)	1.3 (1.1 to 1.5)
Rat microsomes	+	35 (16 to 53)		0.53 (0.48 to 0.58)
ZF microsomes	–	730 (650 to 800)		0.29 (0.28–0.30)
ZF microsomes	+	620 (510 to 720)		0.27 (0.25 to 0.29)
ZF mitochondria	–	770 (650 to 890)		0.60 (0.56 to 0.64)
ZF mitochondria	+	740 (680 to 810)		0.61 (0.59 to 0.63)

^a4MP indicates the presence or absence of 40μM 4-methylpyrazole, a specific inhibitor of mammalian CYP2E1 with a K_i around 1μM.

^bK_i represents inhibition equilibrium constant in substrate inhibition model, preferred mechanism for 4-nitrophenol oxidation by rat liver microsomes >99.5%, Akaike Information Criterion.