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Expression of CYP1C1 and CYP1A in *Fundulus heteroclitus* during PAH-induced carcinogenesis

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Abstract

CYP1C1 is a relatively newly identified member of the cytochrome P450 family 1 in teleost fish. However, CYP1C1's expression and physiological roles relative to the more recognized CYP1A in polycyclic aromatic hydrocarbons (PAHs) induced toxicities are unclear. *Fundulus heteroclitus* fry were exposed at 6–8 days post-hatch (dph) and again at 13–15 dph for 6 hr to dimethyl sulfoxide (DMSO) control, 5 mg/L benzo[a]pyrene (BaP), or 5 mg/L dimethylbenzanthracene (DMBA). Fry were euthanized at 0, 6, 18, 24 and 30 hr after the second exposure. In these groups, both CYP1A and CYP1C1 protein expression were induced within 6 hr after the second exposure. Immunohistochemistry (IHC) results from fry revealed strongest CYP1C1 expression in renal tubular and intestinal epithelial cells. Additional fish were examined for liver lesions eight months after initial exposure. Gross lesions were observed in 20% of the BaP and 35% of the DMBA-treated fish livers. Histopathologic findings included foci of cellular alteration and neoplasms, including hepatocellular adenoma, hepatocellular carcinoma and cholangioma. Strong CYP1A immunostaining was detected diffusely in altered cell foci and on the invading margin of hepatocellular carcinomas. Lower CYP1A expression was seen in central regions of the neoplasms. In contrast, CYP1C1 was only detectable and highly expressed in proliferated bile duct epithelial cells. Our CYP1C1 results suggest the potential for tissue specific CYP1C1-mediated PAH metabolism but not a more chronic role in progression to liver hepatocellular carcinoma.

Keywords

PAHs; CYP1C1; CYP1A; liver lesions; *Fundulus heteroclitus*

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed during the incomplete burning of coal, gas, wood, or other organic substances and generally occur as complex mixtures with a ubiquitous distribution in the air, water and soil or sediment (Collier et al. 1993; Gustafson & Dickhut 1997; Liebens et al. 2007; van Metre et al. 2000). Benzo[a]pyrene (BaP) is one of the most studied environmentally relevant carcinogenic PAHs. 7,12-Dimethyl

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benzanthracene (DMBA) is an extremely toxic synthetic PAH, and it remains a valuable tool in studying chemically induced carcinogenic effects.

CYP1 isozymes represent the main cytochrome P450 family involved in the metabolism of environmental carcinogens, including PAHs, and are under the transcriptional regulation of the aryl hydrocarbon receptor (AhR) (Lin et al. 2003; Murray et al. 2001; Santostefano et al. 1993; Shehin et al. 2000). CYP1A1 was initially thought to be the only CYP1 enzyme responsible for activation of most PAHs in both experimental animal models and humans (Conney 1982; Heilmann et al. 1988; Shimada & Fujii-Kuriyama 2004) until the early 1990s when CYP1B1 was first identified in rat gonad (Otto et al. 1992) and mouse embryonic fibroblast cells (Savas et al. 1994). Compared with CYP1A, recombinant CYP1B1 showed similar catalytic specificities toward procarcinogens, including activation of BaP and DMBA to their respective proximate toxicants (Shimada et al. 2001; Shimada & Fujii-Kuriyama 2004). The new vertebrate CYP1 subfamily was first described when CYP1C1 and CYP1C2 expression were detected in scup (*Stenotomus chrysops*) liver and head kidney (Godard et al. 2005) and CYP1C1 gill expression was identified in carp (*Cyprinus carpio*) (Itakura et al. 2005). More recently, a full-length CYP1C1 was cloned and mRNA tissue expression quantitated by PCR in *Fundulus heteroclitus* (Wang et al. 2006). To date, a CYP1C subfamily has not been identified in mammals. Sequence analysis of *Fundulus* CYP1C1 suggested that fish CYP1C shared higher identity (46–47%) with mammalian/fish CYP1B compared to CYP1A (34–36%). Differences in enzyme substrate regions have been suggested to be involved in differentiating substrate specificities between the CYP1Cs and the CYP1Bs (Godard et al. 2005; Wang et al. 2006). Last year, *Fundulus* CYP1B and CYP1D1 were also sequenced (Zanette et al. 2009) and the AhR-dependence of all fish CYP1s, except CYP1D1, was established (Goldstone et al. 2009; Jonsson et al. 2007b; Jonsson et al. 2007a). While complete understanding of the teleost CYP1-isozyme specific metabolic capacity, physiological functions, and role(s) in tumor development are unclear, these early studies suggest CYP1C1 is a relevant target because it is AhR-mediated, PAH-inducible, and similar to mammalian CYP1B1. Importantly, understanding CYP1s in non-mammalian models could help to reveal subfunctionalization of mammalian orthologous genes. Our study establishes a model for investigating the role of various CYP1s in PAH-induced carcinogenicity.

Fish serve as a valuable non-mammalian *in vivo* model system for determining environmental contaminant-related carcinogenic effects in both field and laboratory studies. In previously established laboratory studies, BaP caused liver tumors in Japanese medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*) (Hawkins et al. 1988; Hawkins et al. 1990), and DMBA-induced liver tumors in guppy (*Poeciliopsis lucida* and *P. monacha*) following waterborne exposure. Although a number of studies have observed altered expression of CYP1A in liver lesions from both field studies and experimental teleost models, the relationship between carcinogen-induced hepatotoxicity and the role(s) of the newly cloned CYP1 subfamily (CYP1B/CYP1C) during tumorigenesis has not been addressed.

In our study, we hypothesized that the new CYP1C1 in fish would be induced following initial exposure and in PAH-induced liver lesions. While expression of both CYP1s were significantly induced immediately following exposure, only CYP1A protein expression was associated with classic pre- and neoplastic hepatocellular lesions. In contrast, CYP1C1 expression was more isolated and associated with proliferated bile duct epithelial cells.

2. Materials and methods

2.1 Fish care

A parental population of *F. heteroclitus* collected from an uncontaminated site at the Newport River near Beaufort Inlet, NC was raised under the University Institutional Animal Care and Use Committee approved conditions as previously described (Wang et al. 2006). First generation offspring, from wild parents, were used for the studies described here. Fish were fed with tropical flake fish food (Tetramin, Tetra Werke, Germany) and supplemented with live brine shrimp twice daily.

2.2 Chemical exposure

Stock solutions of BaP and DMBA (Sigma, St. Louis, MO) were 10 mg/mL in DMSO. *Fundulus* fry at 6–8 days post hatch (dph) were divided into 3 treatment groups in beakers containing 1 L water (salinity ~22 ppt) and exposed to: Control (500 μ L/L DMSO), 5 mg/L BaP (500 μ L BaP stock/L), and 5 mg/L DMBA (500 μ L DMBA stock/L). Exposures were carried out in the dark for 6 hr after which fry were removed to 1 L non-contaminated water (salinity ~22 ppt). Identical exposures were repeated after 7 days. Water samples (10 mL) were collected 20 min after dosing to confirm concentrations of BaP and DMBA using gas chromatography/mass spectrometry in selected ion monitoring mode (Patel et al. 2006; Wang et al. 2006).

2.3 Quantitative real-time PCR and western blotting and measurement

Fundulus fry were anaesthetized with MS-222 at 0 (right after exposure), 6, 18, and 24 hr following the second exposure for protein and RNA extraction (5 to 10 fry per pool per treatment, n=3 to 5 replicates) or moved to 5 L tanks (n= ~56 per treatment) and maintained at a light/dark cycle of 14/10 hr, ~20–25 °C and grown up to eight months for histological examination. The detailed RNA extraction method and real time PCR procedure has been described in our previous work (Wang et al. 2006). For protein analysis, fry were homogenized in 200 μ L phosphate buffer (10 mM potassium phosphate, 100 mM potassium chloride, 1 mM EDTA, pH=7.47) with proteinase inhibitor 0.25 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) and 0.1 mM dithiothreitol (DTT, Fisher Scientific, Pittsburgh, PA). The protein of a 10,000 $\times$ g supernatant was quantitated by the Bradford assay, and used for western blot analysis. Briefly, 200 μ g of total protein were separated on SDS-PAGE gel using 18 well 10% Tris-HCl Criterion precast gel (Bio-RAD, Hercules, CA). After transferring to PVDF membrane (0.45 μ m, VWR, West Chester, PA), a blotting step was performed by the WesternBreeze Chemiluminescent Immunodetection Kit (Invitrogen) with either rabbit anti-fish CYP1A antibody (BioSense, Bergen, NO distributed by Cayman Chemical, Ann Arbor, MI) (1:120) or our anti-*Fundulus* CYP1C1 antibody (Spring Valley Laboratories, Inc. Woodbine, MD) (1:5000) with alkaline phosphatase(AP)-linked goat-anti rabbit IgG antiserum as the secondary antibody (1:2000, Bio-RAD). The polyclonal anti-*Fundulus* CYP1C1 antibody epitope sequence was selected according to immune reactivity and highest similarity with mammalian CYP1B1, but not *Fundulus* CYP1A (Sequence: N terminal-GRSLTFTNYSKQWKAH- C terminal). Recombinantly expressed *Fundulus* protein for both CYP1C1 and CYP1A (Zhu, 2007) were used as positive and negative controls, respectively. The antibody peptide sequence showed low percent identity with *Fundulus* CYP1A (25%), CYP1B1 (43%) and higher identity with CYP1C2 (71%) AAs. β -actin blotting (1:1000, Abcam, MA) was used as loading control to validate the transfer of proteins. CDP-Star Chemiluminescence Reagent (PerkinElmer Life and Analytical Sciences, Boston, MA) was used for detection of AP. Imaging analysis was accomplished by the VersaDoc 3000 Imaging System and Quantity One analysis software (Bio-RAD). Optical density was given as a Trace Quantity with the unit of intensity $\times$ area. Relative band intensities were quantified by dividing CYP1 and β -actin band intensities. All data was

analyzed by GraphPad Prism 4.0 and one-way ANOVA followed by Student-Newman-Keuls post hoc test ($p < 0.05$) was used for all tests.

2.4 Histological procedures

For histopathological examination, 8 month old fish were euthanized with MS-222, and their weights and lengths recorded. Livers were examined grossly, fixed into 10 mL of 4% (w/v) paraformaldehyde for 24 hr, and then processed routinely by dehydration in a graded series of ethanol of increasing strength. After clearing in Clearify™ (American Master Tech Scientific, Lodi, CA), tissues were embedded in molten paraffin (Paraplast embedding media X-tra, Sigma). Livers were sectioned longitudinally through the dorso-ventral plane using an Olympus Cut 4055 microtome (Olympus American Inc., San Jose, CA) at 5 μ m. A total of 112 fish livers ($n=20$ to 47 per treatment group) were sectioned, and stained with hematoxylin and eosin (HE) for treatment blind microscopic evaluation.

2.5 Immunohistochemistry

All immunohistochemistry procedures were performed on paraffin sections mounted to glass histological slides. Tissue sections were deparaffinized in Clearify™, hydrated in a gradient ethanol series, and washed in phosphate-buffered saline (PBS). Endogenous peroxidase and biotin activity were quenched by 0.3% H_2O_2 in methanol for 20 min at room temperature and followed by washing in PBST (PBS + 0.1% Triton X-100). Antigen retrieval was carried out by 10 μ g/mL proteinase K in PBS for 15 min at room temperature. Nonspecific binding was blocked with 3% bovine serum albumin (BSA, Sigma) in PBS for 1 hr. Primary anti-CYP1A Mab 1-12-3 (kindly donated by Dr. John Stegeman) (1:2000), anti-*Fundulus* CYP1C1 polyclonal antibody (1:100) or monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (clone PC10, Sigma, 1:1000) in 3% BSA, 400 μ L per slide, was applied directly and incubated at 4 °C overnight. The next day after washing in PBS and PBST, sections were blocked in 3% BSA for 1 hr at room temperature. Sections were then incubated with biotin labeled horseradish peroxidase (HRP)-linked goat anti-rabbit secondary antibody for CYP1C1 (1:1000, Sigma) or biotin labeled HRP-linked horse anti-mouse IgG for CYP1A and/or PCNA antibody (1:1000, Thermo Fisher Scientific, Waltham, MA) for 90 min. Following incubation with avidin-biotin complex (Vector ABC kit, Vector Laboratories, Burlingame, CA) for 40 min at room temperature, staining was developed with 3, 3'-diaminobenzidine (DAB, Sigma) as the substrate (0.5 μ g/ μ L DAB with 0.5% H_2O_2) and incubated for 1–2 min at room temperature. Slides were rinsed with PBS, counterstained with methyl-green for 15 min, dehydrated in 100% ethanol and cover slipped with mounting medium (Thermo Fisher Scientific). Negative controls were performed by replacing the primary antibody with 3% BSA. Slides were examined by light microscopy (BX40, Olympus) and findings recorded with a digital camera (Optronics, Goleta, CA).

3. Results

3.1 Water concentrations

Actual water concentrations in tanks were 4.1 ± 0.25 mg/L and 6.55 ± 0.52 mg/L for BaP and DMBA, respectively. Mortalities were similar between groups during the exposure phase or grow-out period. The percentages surviving eight months after initial exposure were: Control (~80%), DMBA (~89%), and BaP (~89%).

3.2 CYP1 mRNA expression in *Fundulus* fry

RNA was isolated from *Fundulus* fry at 0 (right after end of exposure), 6, 18, 24 and 30 hr following the end of the second 6 hr exposure. There was significant CYP1A induction (~43-fold in DMBA and ~46-fold in BaP groups, ANOVA $p < 0.01$) and CYP1C1 induction

(~23-fold in DMBA and ~31-fold in BaP groups, ANOVA $p < 0.001$) at 0 hr after the exposure relative to the DMSO control group (Figure 1a). However, expression rapidly decreased within 24 hr. CYP1A was more highly expressed compared with CYP1C1 at all time points. The highest expression of CYP1A relative to CYP1C1 was found in the BaP group at 18 hr (3.46-fold), 24 hr (3.53-fold) and 30 hr (3.14-fold) (Figure 1b).

3.3 CYP1 protein expression in *Fundulus* fry

CYP1A protein expression was elevated relative to controls between 0 to 18 hr after the second exposure and peak expression was seen at 6 hr in both DMBA and BaP groups (Figure 2). In contrast, weak CYP1C1 protein expression was detectable only at 0 hr (right after the second exposure) by western blot (Figure 2). No statistical significance was observed in CYP1A expression western blot quantification by one-way ANOVA. To determine the tissue specificity of CYP1 expression, immunohistochemistry was performed on 0 hr fry sections with both CYP1A and CYP1C1 antibodies. As expected, CYP1A induction by both PAH treatments was observed in hepatocytes, biliary epithelium, renal tubule epithelium, and intestinal epithelium, as well as vascular endothelium of the liver, gill, heart, and brain (Figure 3a). CYP1C1 was highly expressed constitutively and induced moderately in renal tubule and gut mucosa (Figure 3b).

3.4 Histopathological observations and analysis

A total of 146 exposed *Fundulus* were grown out for 8 months and necropsied blind to treatment. Gross lesions were observed in 20% (10/50) of the BaP-treated group and 35% (18/51) of the DMBA-treated group. None were found in the control (DMSO, $n = 45$) group. Lesions were pale pink and sharply demarcated from normal tissues (Figure 4a). There was no significant difference in liver versus body weight ratio between DMBA or/and BaP-treated fish relative to controls (data not shown). Twenty to 47 livers per treatment were examined for histopathological changes using criteria described by previous studies (Blazer et al. 2006; Boorman et al. 1997). Both control and treatment livers exhibited the presence of extensive clear or ill-defined hepatocellular vacuolation consistent with the excessive storage of fat and glycogen, respectively, commonly seen in cultured fish reared on artificial diets (Blazer et al. 2006; Evenson 2006). Histopathologic findings are summarized in Table 1. Most hepatic lesions were classified as pre-neoplastic (eosinophilic or basophilic altered cell foci) and occurred in 83% of DMBA-treated and 62% of BaP-treated fish livers. One hepatocellular adenoma and three hepatocellular carcinomas were found in the BaP and DMBA treatments, respectively (Figure 4b). Bile duct hyperplasia was observed in fish from both PAH treatment groups and one cholangioma was found in a DMBA-treated fish.

3.5 Immunohistochemistry in 8 month liver sections

Strong CYP1A, but not CYP1C1, expression was detected in altered cell foci. In these lesions, hepatocytes and vascular endothelia were most immunoreactive to the CYP1A MAb 1-12-3. PCNA signals were increased in altered cell foci as well. In hepatocellular carcinomas, CYP1A staining was more frequently in association with cells localized at the invasive border of the tumors (Figure 5a). In contrast, CYP1C1 expression was not observed in hepatocellular carcinomas but was highly expressed in neoplastic biliary epithelial cells as well as granulomas. Interestingly, CYP1C1 expression was only concentrated at the apical cytoplasm of these ductal epithelial cells (Figure 5b).

4. Discussion

In this study, we used a standard protocol (Hawkins et al. 1988; Hawkins et al. 1990) for generating tumors in laboratory fish using a short but higher PAH dosage than occurs in the environmentally relevant exposures. Histopathologic observation suggests a positive

relationship between both BaP and DMBA exposure and the formation of hepatic lesions. The total incidence of pre-neoplastic and neoplastic lesions in our study (91% of DMBA and 64% of BaP-treated fish) is similar to a field study from a creosote-contaminated (85% mixed-PAHs) site in the Elizabeth River, VA where 93% of *Fundulus* had hepatic lesions (Stine et al. 2004; Vogelbein et al. 1990). In a recent lab study, hepatocellular lesion incidence was investigated in *Fundulus* fry from Elizabeth River parents compared to fry from Kings Creek parents, a reference site, following two 200 or 400 µg/L 24 hr BaP exposures. Hepatic lesion incidence included cellular foci and hepatocellular adenomas and carcinomas and was 30 and 6% for Kings Creek and Elizabeth River fry, respectively when assessed 9 months after exposure (Wills et al. 2010a). The higher incidence of lesions reported in our study compared to the Kings Creek fry is likely related to the higher dose of BaP (5 mg/L), albeit shorter exposure time, used to initiate lesions. Higher lesion incidences in both guppy (Hawkins et al. 1988) and *Fundulus* after DMBA exposure confirmed DMBA to be a more potent carcinogen than BaP and justified its use as a positive control in our study. Interestingly, in BaP-exposed Japanese medaka, 27% and 8% of fish had adenomas and carcinomas, respectively, but no altered cell foci were reported, suggesting increased progression from pre-neoplastic foci in medaka (Hawkins et al. 1988). However, the criteria for diagnosis could vary among individual pathologists and is sometimes difficult to compare between species. For example, in the medaka and guppy studies, it was suggested that the difference between a basophilic focus and adenoma was a matter of lesion size. In other words, what was reported to be an adenoma was equivalent to what an earlier rainbow trout study reported to be a basophilic focus (Hendricks et al. 1984). Subjective differences in interpretation could therefore account for the higher incidence of adenomas observed in the medaka and guppy study versus the altered cell foci reported here.

A number of studies have correlated CYP1A induction with PAH metabolites, DNA adducts, immune suppression, and tumor formation in wild fish and following laboratory exposures (Carlson et al. 2004; Collier et al. 1992; Willett et al. 1995; Wirgin & Waldman 1998). Based on wild *Fundulus* field studies (Van Veld et al. 1992; Vogelbein et al. 1990), it was hypothesized that a decreased amount of CYP1A provided an advantage for reducing PAH toxic metabolite generation. However, the roles of other CYPs in PAH-mediated fish toxicity have only recently begun to be explored. Considering the low-cost, shorter life cycle, high sensitivity and incidence of tumorigenesis (reviewed in (Bailey et al. 1996; Bunton 1996)), there is increased value in using fish as models in chemical carcinogenesis studies. However, there is a void in the study of other CYP1 genes in this non-mammalian model.

We used quantitative PCR and an anti-*Fundulus* CYP1C1 antibody to detect mRNA and protein expression after tumor initiation exposure. In our previous studies, CYP1C1 protein expression was detected only in livers following a 15 day waterborne exposure in adult fish (3.7-fold in 10 µg/L BaP and 3.4-fold in 100 µg/L BaP treated groups) (Wang, 2009) and CYP1C1 mRNA was similarly induced ~5-fold by 10 µg/L BaP (Wang et al. 2006). In larvae following BaP or DMBA exposures, both CYP1A and CYP1C1 mRNA were induced immediately after the second exposure (0 hr), but peak expression rapidly decreased and remained level between 6 and 30 hr after exposure. Compared to mRNA, CYP1 protein induction was maximal at 6 hr after the exposure, but by 24 hr protein expression had also decreased. The delay in maximal protein expression relative to message expression may reflect the time necessary for protein translation after mRNA induction. Detection of CYP1C1 protein expression was very low or undetectable by western blot. The low expression may be because the proteins were not concentrated into the microsomal fraction. The 10,000 × g supernatants were used instead of microsomal fractions because the latter would have necessitated exposing significantly more fish to isolate enough microsomal protein.

To more directly measure tissue and cellular expression of CYP1s, IHC was used. The CYP1A expression and induction pattern was consistent with earlier reports (Van Veld et al. 1997). Strong CYP1C1 protein expression was detected in *Fundulus* renal tubular and intestinal epithelium at constitutive levels and was moderately induced after acute BaP exposure. It should be noted that the polyclonal anti-*Fundulus* CYP1C1 antibody epitope showed 71% identity with *Fundulus* CYP1C2, so the antibody could potentially recognize both CYP1Cs. However, the IHC results of CYP1C1 expression were consistent with our previous report that CYP1C1 mRNA is constitutively expressed at high levels in adult *Fundulus* kidney. Both the kidney and intestine are involved in xenobiotic metabolism and are considered target organs of chemical-induced carcinogenesis.

Since the new teleost members of the CYP1 family were identified, it has been hypothesized that they could have a role in PAH metabolism and contribute to either toxication or detoxication pathways. BaP genotoxicity requires microsomal cytochrome P450 to convert it into a variety of highly electrophilic metabolites such as BaP-diol epoxides, BaP-quinones or BaP radicals that either bind covalently with DNA or oxidatively damage DNA bases. Among the various metabolites, the ($\pm$)-anti-7 β ,8 α -dihydroxy-9 α ,10 α -epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (anti-BPDE) which is derived from BaP-7,8-dihydrodiol, is considered as the principle toxic intermediate that forms DNA adducts and can lead to tumor initiation (Venkatachalam et al. 1995; Venkatachalam & Wani 1994; Wani et al. 2000). In order to determine the role of the individual CYP1s in the activation of PAHs, metabolism studies with recombinantly expressed protein in isolation are necessary. While not *Fundulus*, recombinantly expressed zebrafish CYP1A and CYP1C1 predominantly produced BaP-7,8-dihydrodiol and 3-hydroxyBaP, each accounting for about 25% of the total BaP metabolism. Zebrafish CYP1B1 and CYP1C2 also showed high capacity to metabolize BaP *in vitro*, but both enzymes produced 3-hydroxy BaP as the prominent metabolites (Scorniaenchi et al. submitted). While there is precedent for *Fundulus* adults following BaP exposure to generate and excrete the BaP-7,8-dihydrodiol in the bile (Zhu et al. 2008), we have not been able to detect the metabolite in BaP exposed embryos (Wills et al. 2009). When comparing embryos from the Kings Creek reference site and the creosote contaminated Elizabeth River, Kings Creek embryos exposed to BaP have highly inducible CYP1A, CYP1B1 and CYP1C1 (Wills et al. 2010b). Exposed Kings Creek larvae have more DNA damage, more BaP excretion, and, as described previously, higher liver lesion incidence compared to larvae with Elizabeth River parents (Wills et al. 2010a). Together these studies suggest that CYP1s are having a role in tumor initiation and toxic metabolite formation but additional *in vivo* studies and isoform specific CYP1 inhibitors are needed.

The structural anatomy and essential functions of the fish liver associated with chemically induced toxicity, as well as the comparisons with mammalian liver physiology, have been well described (Hampton et al. 1985; Hampton et al. 1988; Hinton & Couch 1998). These fundamental studies have provided a strong background for investigating the role of CYP1C1 versus CYP1A in PAH-induced liver toxicity. CYP1A altered expression in pre-neoplastic and neoplastic lesions has been reported in various fish species, including *Fundulus*. Previously, decreased CYP1A expression and EROD activity was found in neoplasms and/or cellular alterations in *Fundulus* (Van Veld et al. 1992). However, in altered cell foci, we observed a strong increase in CYP1A immunostaining relative to normal areas. The difference in CYP1 expression may be related to the continuous and PAH mixture exposure in the wild fish compared to our 2-hit larval dosing regimen. Furthermore, a more uniform CYP1A signal was detected in invading cancer cells which were localized at the border of the tumor (but not the edge of a section) relative to the rest of the neoplasm. Cell proliferation was confirmed by PCNA immunostaining in those lesion areas. The significance of altered CYP1A expression is not clear and it may be due to gene regulatory changes in proliferating cells.

In contrast to CYP1A, CYP1C1 was only detected in hyperplastic and neoplastic bile duct epithelial cells (cholangiocytes). Interestingly, CYP1C1 expression was observed only at the apical membrane instead of across the entire cytoplasm. Cholangiocytes directly interact with bile and have a function in maintaining bile flow and pH (Fitz 2002). In order to support their function, cholangiocytes appear to have polarity, the basal surface connects with the basement membrane and the apical surface forms the luminal space providing a pathway for bile and lipid. Although numerous genes including protein kinase C, F-actin and apical sodium bile acid co-transporter have been detected at the bile duct apical membrane, no cytochrome P450 expression has ever been reported at this location (Tanimizu et al. 2007; Xia et al. 2006). Eight months after PAH exposure, induced CYP1 expression should not be due to the presence of parental compounds or their metabolites. However, after acute exposure, toxic intermediates may have altered cholangiocyte apoptosis and differentiation and, in turn, lead to proliferation and chronic inflammatory conditions known to alter gene regulatory pathways. The biological significance of CYP1C1 expression in cholangiocytes, but not hepatocytes is unknown, although it has been proposed that a stem cell in the liver could be activated following hepatocarcinogen exposure and liver injury (Sell 1990). In fish, biliary preductular epithelial cells could be the stem cell counterparts to rodent oval cells (Hinton & Couch 1998). This hypothesis could explain why CYP1C1 was only detected in proliferated biliary epithelial cells and not the hepatocyte-derived neoplasms.

5. Conclusions

In conclusion, we have established a model system wherein acute water-borne PAH exposure results in diverse hepatic lesions after only eight months. The protein expression of a new CYP1 member, CYP1C1, has been addressed in early and late tumor development. CYP1C1 was induced after PAH exposure in fish fry and high expression was detected in immune-related organs. Contrary to our hypothesis, CYP1C1 upregulation was detected in hyperplastic biliary tissue, but not any pre-neoplastic foci or hepatocellular carcinomas. In addition to the traditional CYP1A biomarker, our study suggests CYP1C1 should be further investigated for its relative role in generating genotoxic BaP metabolites *in vivo*.

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Figure 1.

RT-PCR results for CYP1 expression in the tumor initiation study with *Fundulus* fry. Fry 6–8 days post hatch were exposed for 6 hr and after 7 days were exposed for a second 6 hr. Three to 5 pools of larvae per treatment were collected after second exposure (5 to 10 larvae per pool). **a)** CYP1A and CYP1C1 mRNA expression in both DMBA and BaP treatments relative to control (DMSO) at 0, 6, 18, 24 and 30 hr after the second exposure. Statistically significant inductions appear at 0 hr (right after second exposure) for both CYP1s (ANOVA done on $2^{-\Delta C_T}$ value, $p < 0.001$ for both CYP1A and CYP1C1 in both PAH groups relative to control group). **b)** CYP1A was more highly expressed compared with CYP1C1 at all time points. The highest expression of CYP1A relative to CYP1C1 was found in the BaP group at 18, 24 and 30 hr, but no statistical significance was detected.

Figure 2.

Western blot results in *Fundulus* fry (5 to 10 per pool, n=3 to 5). Both CYP1A (left) and CYP1C1 (right) protein were induced within 6 hr following the second exposure. CYP1A induction decreased after 18 hr. Weak CYP1C1 expression was only seen at 0 hr after the exposure (r= recombinant expressed protein; M= marker).

Figure 3.

IHC of CYP1 immunostaining on fry sections at 0 hr after exposure. **a)** BaP-induced CYP1A expression in liver hepatocytes, biliary epithelium and endothelial cells of arterioles and veins, gill pillar cells and epithelium, renal tubules epithelium, heart endothelium, brain vascular endothelium and gut mucosal epithelium. **b)** Strong CYP1C1 basal expression and moderate induction was found in renal tubules and gut mucosal epithelium. Brown color indicates positive signal; blue color is the counterstaining with methyl green. (–) Controls were done by the same methods but without primary antibody incubation. All pictures at same magnification (bar= 100 μ m).

Figure. 4a

Figure. 4b

Figure 4.

Fundulus livers 8 months after PAH exposure. **a)** Gross lesions were observed in 35% of DMBA-treated and 20% of BaP-treated groups. **b)** Histopathological findings in liver sections. Altered cell foci were characterized by variable tinctorial properties, discrete borders that merged inconspicuously with adjacent parenchyma, preservation of normal tissue architecture, and lack of cellular atypia. Eosinophilic foci (A) had more regular borders and were composed of slightly larger, more heavily vacuolated cells compared to basophilic foci (B). Adenomas (C) were expansive and more densely cellular but lacked atypia and did not compress adjacent tissue. Hepatocellular carcinomas (D) exhibited invasion of adjacent tissue, with mild cellular pleomorphism and nuclear atypia (bar=100 μ m. Harris' hematoxylin and eosin staining).

Figure 5.

Immunohistochemistry results for 8 month *Fundulus* liver sections. Brown color indicates positive signal; blue color is the counterstaining with methyl green. **a)** Red squares in the foreground indicate the region of higher magnification compared to the background (bar=100 μ m). CYP1A and PCNA expression were detected in both cellular foci and HCC. Letters in HCC stand for different locations, A= tumor border; B= inner parenchyma. **b)** CYP1C1 was highly expressed in cholangiolar or ductular epithelial cells in the bile duct hyperplasia as well as granulomas with fibrosis tissue. CYP1C1 expression was only concentrated at the apical cytoplasm of these ductal epithelial cells (bar=100 μ m).

A total of 146 exposed *Fundulus* were grown out for 8 months and 20 to 47 per treatment liver were sectioned and stained with hematoxylin and eosin for microscopic evaluation. Both necropsy and pathologic characterization were done blind to treatment.

Table 1

	Altered Cell Foci	Hepatocellular Adenoma	Hepatocellular Carcinoma	Cholangioma	Granuloma
Control	5% (1/20)	0 (0/20)	0 (0/20)	0 (0/20)	5% (1/20)
DMBA	83% (39/47) ^a	0 (0/47)	6% (3/47)	2% (1/47)	9% (4/47)
BaP	62% (28/45) ^b	2% (1/45)	0 (0/45)	0 (0/45)	9% (4/45)

^a 14 had eosinophilic foci, 10 had basophilic foci, 15 had both types.

^b 18 had eosinophilic foci, 7 had basophilic foci, 3 had both types.