Published in final edited form as:

Comp Biochem Physiol C Toxicol Pharmacol. 2010 January ; 151(1): 107–112. doi:10.1016/j.cbpc.2009.09.003.

Identification of mitochondrial cytochrome P450 induced in response to polycyclic aromatic hydrocarbons in the mummichog (*Fundulus heteroclitus*)

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Abstract

Increasing evidence suggests that polycyclic aromatic hydrocarbons (PAHs) such as benzo[a]pyrene (BaP) are localized to the mitochondria. Because the toxic effects of many PAHs are the result of metabolism by cytochrome P4501A (CYP1A), it is important to investigate whether active forms of these enzymes can be identified in the mitochondria. In this study, we identified mitochondrial P450s with a monoclonal antibody against scup (*Stenotomus chrysops*) CYP1A in the isolated mitochondrial fraction of liver from adult male mummichog (*Fundulus heteroclitus*) livers. The size of the protein in the mitochondria was the similar to that of microsomal CYP1A. Fish dosed with 10 mg/kg BaP had increased EROD activity in the mitochondrial fraction compared to controls. In mummichog larvae dosed with 100 µg/L BaP and 100 µg/L benzo[k]fluoranthene, CYP1A protein levels as well as enzyme activity were elevated. However, fish from a PAH-polluted Superfund site (Elizabeth River, Portsmouth VA) showed recalcitrant mitochondrial CYP1A protein levels and enzyme activity in a similar manner to microsomal CYP1A.

Keywords

Fundulus heteroclitus; cytochrome P450 1A; mitochondria; polycyclic aromatic hydrocarbon; Elizabeth River Superfund site

Introduction

Cytochrome P450 proteins (CYPs) are hemoproteins that exist in most organisms from bacteria to humans. There are 57 known families of cytochrome P450 proteins (CYPs) in humans (Furge et al. 2006; Myasoedova 2008). These proteins are involved in a variety of cellular functions such as drug metabolism, fatty acid metabolism, and bile acid biosynthesis. Within these protein families are the CYP1 family of proteins regulated by the aryl hydrocarbon receptor (AhR) pathway. These proteins are involved in the metabolism of planar organic carbons such as polycyclic aromatic hydrocarbons (PAHs) (Hahn 1998; Denison et al. 2003; Nebert et al. 2004).

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While most CYPs are located and function in the endoplasmic reticulum (microsomes), some CYP proteins are localized to the mitochondria. The existence of mitochondrial CYPs in vertebrates has been known for over forty years (Harding et al. 1964; Omura 2006). These proteins are involved in the synthesis of steroid hormone and bile acid, and in the conversion of Vitamin D to its active form (Omura 2006). Nevertheless, less is known regarding the presence and function of mitochondrial CYPs that in microsomes are involved in the metabolism of xenobiotic chemicals. Since the 1980s, several studies in mammalian systems have shown that several CYP proteins with 'microsomal characters' (i.e. CYPs involved in the metabolism of xenobiotic chemicals) are present in the mitochondria (Niranjan et al. 1984; Niranjan et al. 1985; Anandatheerthavarada et al. 1997).

Mitochondrial CYPs that can be detected with antibodies against microsomal CYP1, CYP2, and CYP3 families have been identified in various tissues in mammals (Niranjan et al. 1985; Bhagwat et al. 1995; Anandatheerthavarada et al. 1997; Genter et al. 2006). Further analyses have shown that these proteins are induced by the same xenobiotic compounds that induce CYPs in the microsome (Niranjan et al. 1985; Niranjan et al. 1988; Boopathi et al. 2000). It is now accepted that these proteins are transcribed by the same genes that code for the microsomal CYP proteins, but are post-translationally modified in the cytosol, either by phosphorylation or truncation of the N-terminus, and targeted to the mitochondria (Addya et al. 1997; Genter et al. 2006).

In several teleost fish species, microsomal CYP proteins involved in xenobiotic metabolism, especially as part of the AhR pathway, have been investigated in detail (Stegeman et al. 1991; Williams et al. 1998; Wirgin et al. 2004). At the same time, mitochondrial CYPs that are also involved in steroid hormone and bile acid synthesis have also been identified (Leusch et al. 2003; Hagen et al. 2006). However, unlike the mammalian system, the existence of mitochondrial CYPs involved in xenobiotic metabolism has not been reported.

As ectotherms, fish have to respond to changes in external temperature (Wilhelm Filho 2007; Fangue et al. 2009). In addition, frequent episodes of hypoxia can increase oxidative stress in aquatic organisms (Abele et al. 2004). Therefore, mitochondrial integrity is particularly important in fish. It is known in mammals that PAHs are localized to the mitochondria (Zhu et al. 1995), and PAHs have been shown to adversely impact mitochondrial function (Li et al. 2003; Ko et al. 2004; Xia et al. 2004; Huc et al. 2006). Hence, identification of functionally active CYP1A proteins that have the potential to metabolize PAHs and affect mitochondrial function in fish may be important in understanding the effect of such xenobiotics in aquatic organisms. This would further enhance our understanding of how pollutants that are metabolized by the AhR pathway may affect the organism's energy metabolism, thermoconformation, and response to hypoxia.

The mummichog (*Fundulus heteroclitus*) is a well-established environmental model in the field of aquatic toxicology (Burnett et al. 2007). The AhR pathway in response to contaminants in the environment have been examined in detail in this species (Binder et al. 1985; Prince et al. 1995a; Prince et al. 1995b; Hahn 1998; Nacci et al. 1999; Bello et al. 2001; Meyer et al. 2002; Nacci et al. 2002; Powell et al. 2004; Wassenberg et al. 2004; Matson et al. 2008). Several populations of mummichog inhabiting areas with high levels of PAHs and dioxin-like compounds show refractory CYP1A induction in responses to these chemicals (Prince et al. 1995a; Elskus et al. 1999; Nacci et al. 1999). Previous studies from one such population with this refractory CYP1A expression, the mummichog inhabiting the Atlantic Wood Superfund site at the Elizabeth River in Portsmouth, Virginia (U.S.A), indicate that these fish may have impaired mitochondria compared to fish from uncontaminated sites. These fish have higher tolerance to oxidative stress and upregulated antioxidant defense, such as manganese superoxide dismutase, (Meyer et al. 2003b; Bacanskas et al. 2004), greater sensitivity to

hypoxia (Meyer et al. 2003a), indication of more reliance on anaerobic metabolism (Meyer et al. 2005), and higher levels of mitochondrial DNA damage (Jung et al. 2009a).

In this study, we confirmed the presence of mitochondrial CYP1A proteins in the mummichog and examined the induction of mitochondrial CYP1A proteins in adult and larval mummichog. In addition, we compared the protein level and enzyme activity of mummichog from the Elizabeth River Superfund site to fish from a reference site to test whether the Elizabeth River fish exhibit refractory mitochondrial CYP1A protein level and activity, as is the case for microsomal CYP1A in this population.

Materials and Methods

1. Fish care

Adult mummichog were captured from King's Creek (KC), a tributary of the Severn River in Gloucester County, VA, USA (37°17'52.4"N, 76°25' 31.4"W), and from the Atlantic Wood Superfund Site at the Elizabeth River (ER) in Portsmouth, VA, USA (36°48'27.4" N, 76°17' 36.1" W) using baited minnow traps. The fish were moved to Duke University Ecotoxicology Laboratory and reared in lab as described previously (Wills et al. 2009). The fish were kept in controlled conditions for at least four weeks before experiments were conducted.

Fish eggs were collected from egg boxes stationed in each tank. Eggs were plated out and incubated at 27 °C for 14 days. Fish were manually hatched by adding 20 ppt artificial sea water (ASW, Instant Ocean®, Aquarium Systems, Rhinelander, WI, USA) to the plates and shaking for 30 min. Hatched larvae were kept in 2-L beakers until initiation of experiments. Larvae were fed brine shrimp (Brine Shrimp Direct, Ogden, UT, USA) daily, and water was changed every other day during maintenance.

2. Chemical treatment

Adult male fish were moved to 3-L tanks 24 h prior to dosing. In the treatment group, fish were dosed with 10 mg/kg benzo[a]pyrene (BaP, Sigma-Aldrich, St. Louis, MO, USA) dissolved in corn oil via intraperitoneal injection. An equal volume per mass of corn oil (5 ml/kg) was injected in the control group. The water in each tank was statically renewed every other day and the fish were fed Tetramin® Tropical Fish Food (Tetra Systems, Blacksburg, VA, USA) everyday. At the end of 3d, fish were sacrificed via cervical dislocation. Liver was harvested from each individual, flash frozen and stored at -80 °C until further analysis.

Larvae were dosed 10 d after hatching with either 100 µg/L BaP (Absolute Standards, Inc., Hamden, CT, USA) or 100 µg/L benzo[k]fluoranthene (BkF, Absolute Standards, Inc.), a potent CYP1A inducer, via water-borne exposure. Fish were dosed in groups of ten in 100 mL of 20 ppt ASW. Larvae were fed brine shrimp daily during exposure. The dosing solution was statically renewed every other day throughout the exposure period. At the end of 5 d, fish from each group was flash frozen together, and kept at -80 °C until further analysis.

3. Mitochondria and microsome isolation

Mitochondrial and cytosolic fractions were isolated according to a protocol modified from Harada and Omura (1980), Ivanina and Sokolova (2008), and Dong et al. (2009). In short, isolated livers or whole embryos were blotted and washed with homogenization buffer (300 mM sucrose, 50 mM KCl, 50 mM NaCl, 8 mM EGTA, 30 mM HEPES, pH 7.5). Tissues were then homogenized in homogenization buffer with 1 mM PMSF (phenylmethanesulfonyl fluoride, Sigma-Aldrich), 1 µg/mL leupeptin (Sigma-Aldrich), and 1 µg/mL aprotonin (Sigma-Aldrich) with a hand-held tissue homogenizer. The homogenate was centrifuged at 500 g for 15 min at 4°C. Supernatant was moved to a new tube, and the pellet was homogenized again

and centrifuged at 500 g for 15 min at 4 °C. Supernatant from the second run was combined with the supernatant from the first run and spun at 1,000 g for 15 min at 4 °C. The supernatant was moved to a new tube and centrifuged at 10,000 g for 15 min at 4 °C. The pellet was resuspended with wash buffer (30 mM HEPES, 500 mM sucrose, pH 7.5) and centrifuged again at 10,000 g for 15 min at 4 °C. This procedure was repeated three more times. After the fifth run, the pellet (mitochondria) was resuspended with suspension buffer (0.25 M sucrose, 1 mM EDTA, 0.1 M Tris-HCl in 20 % glycerol, pH 7.4), aliquoted, and flash frozen in liquid nitrogen, and stored at –80 °C.

For adult samples, the supernatant after the first 10,000 g centrifugation was moved to a new tube and centrifuged at 18,000 g for 30 min at 4 °C, and the pellet was discarded. The supernatant was spun at 105,000 g for 60 min at 4 °C, and the pellet (microsomes) was washed with wash buffer, resuspended in suspension buffer, aliquoted, and stored as mentioned above.

4. Western blot

Proteins were isolated by SDS-PAGE loaded with equal volumes of protein (10–30 µg) and transferred to polyvinylidene fluoride (PVDF) membranes. Blots were first probed with monoclonal antibody 1-12-3 (3 µg/mL) against scup (*Stenotomus chrysops*) CYP1A (Park et al. 1986; Kloepper-Sams et al. 1987), then probed with goat anti-mouse IgG horseradish peroxidase secondary antibody (1:10,000 dilution, Jackson laboratory, Bar Harbor, ME, USA). Additional blots were also probed for monoclonal antibody against the endoplasmic reticulum marker protein, bovine liver protein disulfide isomerase (anti-PDI) (1:1000 dilution, Stressgen, Ann Arbor, MI, USA), and monoclonal antibodies against two mitochondrial marker proteins, cytochrome c oxidase subunit I (anti-COX I, 1: 1000 dilution, MitoScience, Eugene, OR, USA) and cytochrome c oxidase subunit IV (anti-COX IV, 1: 1000 dilution, MitoScience) and with secondary antibody as described above. The blots were visualized on x-ray film by enhanced chemiluminescence (SuperSignal® West Pico Chemiluminescence Substrate, Thermo Scientific, Rockford, IL, USA) following the manufacturer's protocol. Band intensity was measured using Image J (Abramoff et al. 2004).

5. Enzyme activity assays

The glucose 6-phosphatase assay was conducted using the protocol modified from Greenawalt (1974). Briefly, triplicates of 20 – 30 µg of protein from samples were incubated with assay buffer (200 mM imidazole-HCl, 500 mM glucose-6-phosphate, 50 mM NAD⁺, 100 mM EDTA, 0.1 IU mutarotase, 1 IU glucose dehydrogenase) in a 96-well microplate and the increase in NADH was measured at 340 nm. Absorbance was converted to activity using 6.3 mM⁻¹·cm⁻¹ as the molar extinction coefficient for NADH.

The *in vitro* ethoxyresorufin-*O*-deethylase (EROD) assay was conducted on isolated mitochondrial and microsomal fractions according to the protocols of Meyer et al. (2002) and Kennedy et al. (1993). Three replicates of 20 to 50 µg of protein from each sample were incubated with 2.5 µM 7-ethoxyresorufin in cofactor buffer (100 mM HEPES, NADPH (102 µM NADPH, 120 µM NADH, and 5 mM MgSO₄) in a 96-well microplate. The fluorescence of resorufin (reaction product) was measured at 530/590 nm and molar specific activity (pmols resorufin per mg protein per minute) was calculated.

6. Phosphorylation site prediction

Potential phosphorylation site in the N-terminal region of the mummichog CYP1A protein was predicted using the NetPhosK 1.0 server, <http://www.cbs.dtu.dk/services/NetPhosK/> (Blom et al. 2004), with the *Fundulus heteroclitus* CYP1A protein sequence (GenBank accession no. AAD01809)

7. Statistics

Statistical analyses were performed using SPSS, version 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Student's *t* test or analysis of variance (ANOVA) with Fisher's Protected Least-Significant Differences (LSD) were used where appropriate ($\alpha = 0.05$).

Results

1. Verification of isolation method

To confirm that the mitochondrial fractions isolated were not contaminated by microsomes, we performed Western blots with an antibody against protein disulfide isomerase (PDI), commonly used as a microsomal biomarker, and an antibody against cytochrome c oxidase subunit I (COXI), a part of the mitochondrial respiratory chain proteins. We could detect the COXI polypeptide in the mitochondrial fraction, but not in the microsomal fraction. Similarly, PDI was detected in the microsomal fraction only (Figure 1). In addition, we tested the activity of glucose 6-phosphatase, another common method utilized to identify the presence of microsomes. The enzyme activity in the mitochondrial fraction was about 8% of the enzyme activity in the microsomal fraction. These results confirmed that there was minimal cross contamination between mitochondria and microsomes.

2. Adult liver CYP1A expression and in vitro EROD activity

In adult male fish from our reference site, CYP1A protein was detected in the mitochondria (Figure 2). CYP1A levels were increased in fish treated with 10 mg/kg BaP by roughly 2.11 fold. In contrast, another nuclear-transcribed mitochondrial protein, COX IV, was not different between treatment groups, which demonstrates that the increase in CYP1A protein was specifically due to BaP treatment. BaP-treated fish liver mitochondria showed significantly higher EROD activity level compared to the control group (Figure 3, $p = 0.001$). The level of increase was similar to that of microsomal CYP1A activity.

3. Comparison of CYP1A protein expression and activity in adapted and reference populations

Compared to larval mummichog from the reference site (KC), larval Elizabeth River mummichog (ER) showed refractory mitochondrial EROD activity as well as refractory protein induction when dosed with 100 $\mu\text{g/L}$ BaP (Figure 4A). Only KC fish treated with BaP showed increased protein level and activity ($p < 0.001$). Similar results were seen in fish treated with 100 $\mu\text{g/L}$ BkF, a more potent CYP1A inducer (Figure 4B). Only the BkF-treated KC larvae showed increased protein levels and activity ($p < 0.001$).

4. Prediction of potential phosphorylation site

Analysis of the mummichog CYP1A protein sequence for potential PKC-mediated phosphorylation site using NetPhosK 1.0 identified Thr-31 to be a probable PKC phosphorylation site (score = 0.72).

Discussion

Previous studies have shown that BaP can be metabolized in the mitochondria *in vitro* and subsequently induce mitochondrial DNA damage (Niranjan et al. 1984; Niranjan et al. 1985), indicating that mitochondrial CYPs can be involved in the activation of BaP in the mitochondria. However, Raza and Avadhani (1988) showed that BaP metabolism by mitochondrial CYP1A1 *in vitro* was only about 10% of microsomal CYP1A1 by measuring the metabolite concentration of [^3H] BaP incubated with subcellular fractions. Similarly, a recent study by Dong et al (2009) using knock-in mice showed that mice lacking signals for

endoplasmic reticulum-targeting in the *Cyp1a1* gene showed similar BaP-induced toxicity symptoms as *Cyp1a1*($-/-$) knockout mice, whereas mice lacking the mitochondria-targeting signal were not much different from wild type mice. Therefore, the authors concluded that most of the detoxification of orally administrated BaP was achieved by microsomal CYP1A1, and that mitochondrial CYP1A1 had only minor role in the detoxification process. Although it seems that mitochondrial CYP1A may not play a great role in the acute toxicity of BaP in the organismal level, the role these proteins play in terms of affecting mitochondrial function remains unclear. This is especially true in light of our results. *In vitro* EROD activity in the mitochondria of BaP dosed fish was not significantly lower than that of the microsome, suggesting that the mitochondrial CYP1A protein is as active as the microsomal protein. Further studies on actual metabolism of BaP in the two cellular components of the mummichog will be necessary to understand the role of mitochondrial CYP1As in the mummichog model.

The Elizabeth River Superfund site mummichog population seems to have lowered energy metabolism level as well as increased antioxidant defense mechanism (Meyer et al. 2003b; Bacanskas et al. 2004; Meyer et al. 2005). At the same time, this fish population is more vulnerable to low oxygen levels compared to reference site mummichog (Meyer et al. 2003a). In addition, previous studies in our laboratory show that the basal mitochondrial DNA (mtDNA) damage level is higher in both wild-caught fish and larval F1 fish that were fertilized in the laboratory (Jung et al. 2009a, Jung et al. 2009b). Interestingly, although we saw that BaP treatment to KC fish results in increased level of mitochondrial DNA damage, the same treatment did not induce higher level of mitochondrial DNA damage compared to the control group in the ER population. These studies suggest that adaptation of the ER mummichog to the PAH-contaminated habitat may have affected the efficiency of mitochondrial function.

The ER mummichog show refractive microsomal CYP1A induction in response to PAH treatment in both the adult fish and the F1 generation fertilized in laboratory (Elskus et al. 1999; Nacci et al. 1999; Meyer et al. 2002; Meyer et al. 2003c). In the present study, we verified that these fish also have refractive CYP1A protein levels and activity in the mitochondria. The recalcitrant CYP1A proteins in the ER mummichog occur at the transcriptional level (Meyer et al. 2003c). Since it is accepted that CYP1A translocation into the mitochondria occurs after post-translational modification, it is not surprising to see this refractory characteristic in the mitochondria as well. It is quite likely that the quantity of CYP proteins targeted to the mitochondria would also be reduced in this population due to the lower total amount of the gene product. Since genes in the AhR pathway are not induced by PAHs in these fish, the proportion that are translocated into the mitochondria are also not increased compared to the KC fish. Therefore, this recalcitrant phenotype is probably not a protective mechanism directly related to the mitochondria, but is another result of the repression of the AhR pathway as a whole. However, how this refractive character affects BaP-mediated toxicity in the mitochondria is an intriguing question that has yet to be explored. If PAHs, such as BaP, are metabolized and activated in the mitochondria by mitochondrial CYP1As (Niranjan et al. 1984; Niranjan et al. 1985), the smaller amount of these proteins in the ER mummichog could protect from activation of BaP in the mitochondria. This hypothesis is somewhat supported by our previous study that examined mtDNA damage (Jung et al. 2009b). We saw that BaP treatment resulted in significant increase in the level of mtDNA damage in the KC mummichog. However, no difference was seen between the control and the BaP-treated group in the ER mummichog.

There is evidence that translocation of CYP1A to mitochondria can be signaling-mediated through endoprotease activity or protein kinase C (PKC) activity (Dasari et al. 2006). More specifically, the N-terminal of the CYP1A protein consists of endoplasmic reticulum-targeting sequences. When this sequence is cleaved by endoprotease, a cryptic mitochondria-targeting sequence is revealed. Alternatively, phosphorylation of Thr-35 by PKC in mammals diminishes

the protein's affinity for the endoplasmic reticulum. Genter et al. (2006) speculated that events such as reactive oxygen species (ROS) production may trigger this signaling event. In populations of fish adapted to sites such as the Elizabeth River where fish are continuously exposed to a complex mixture of chemicals (Hartwell et al. 2007; Vogelbein et al. 2008), alterations in cellular pathways in addition to the AhR pathway are not entirely unexpected. Our results suggest that there is a potential PKC-mediated phosphorylation site at Thr-31 in the mummichog CYP1A protein. Nevertheless, alterations in the PKC signaling pathway in the ER mummichog have not been examined. More in-depth investigation into the mechanism behind translocation of mitochondrial CYP1As in this mummichog population may be worthwhile.

In conclusion, we have identified for the first time in teleost fish, mitochondrial CYP proteins that are induced by AhR agonists and involved in xenobiotics metabolism. Our finding implies that PAH metabolism and its consequences should also be examined in the mitochondria, when considering the aquatic ecosystem. In addition, we have confirmed that the ER mummichog show recalcitrant mitochondrial CYP1A protein level and activity in response to BaP treatment. Further investigation into the implications this has for the function of mummichog mitochondria and how this affects the survival of the animals in the wild is warranted.

Acknowledgments

We thank Dr. Inna Sokolova and Autumn Bernal for their valuable input. We also thank Drs. John Stegeman and Mark Hahn for providing the MAb 1-12-3 antibody. This research was supported by the Superfund Basic Science Research Center (P42 ES10356) and NIEHS Integrated Toxicology and Environmental Health Program (T32ES07031).

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Figure 1. Expression of mitochondrial and microsomal marker proteins

Equal amounts of proteins loaded for SDS-PAGE followed by probing for mitochondrial and microsomal fractions with antibodies specific to mitochondria (COX I) and endoplasmic reticulum (PDI). There is minimal cross contamination between mitochondria and microsome. mt: mitochondria, ms: microsomes, cytochrome oxidase subunit I: COX I, protein disulfide isomerase: PDI.

Figure 2. CYP1A and COX IV protein expression in mitochondria of adult killifish

Adult male killifish were dosed with corn oil or BaP (10 mg/kg) and mitochondria from the liver of each individual was isolated 72 hrs after treatment. There is increased CYP1A expression in response to BaP injection. However, COX IV protein, a nuclear-encoded mitochondrial protein does not show difference between treatment groups. ctl: control, BaP: BaP treated, COX IV: cytochrome oxidase subunit IV.

Figure 3. *In vitro* EROD activity level in mitochondrial and microsomal fractions of adult killifish. Proteins were isolated from livers of adult male killifish dosed with corn oil (control) or BaP (10 mg/kg). *In vitro* EROD activity was increased in the BaP-treated fish. Mitochondrial EROD activity of control fish was set as reference. $n = 5$ per treatment. Error bars represent standard error of means.

Figure 4. *In vitro* EROD activity level and CYP1A protein expression in larval killifish mitochondria

Protein isolated from the mitochondria of pooled individuals of 10 day-old larvae dosed with DMSO or 100 μ g/L BaP (A) and DMSO or 100 μ g/L BkF (B) were examined for EROD activity and CYP1A protein expression. *In vitro* EROD activity and CYP1A protein level were increased only in the BaP-treated King's Creek fish. KC: King's Creek fish, ER: Elizabeth River fish, ctl: control, BaP: BaP treated, mtCYP1A: mitochondrial CYP1A. n = 3 to 4 of pooled samples of 10. Error bars represent standard error of means. * indicates $p < 0.001$.