Interactive effects of hypoxia and PCB co-exposure on expression of CYP1A and its potential regulators in Atlantic croaker liver

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Short title: Effects of hypoxia and PCB on hepatic CYP1A regulation in fish

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Abstract

Although marine and coastal environments which are contaminated with xenobiotic organic compounds often become hypoxic during the summer, the interactive effects of hypoxia and xenobiotic exposure on marine species such as teleost fishes remain poorly understood. The expression and activity of monooxygenase enzyme cytochrome P450-1A (CYP1A) in fishes are upregulated by exposure to polychlorinated biphenyls (PCBs), whereas they are down-regulated during hypoxia exposure. We investigated the interactive effects of hypoxia and PCB co-exposure on hepatic CYP1A expression in Atlantic croaker and on potential regulators of CYP1A. Croaker were exposed to hypoxia (1.7 mg/L dissolved oxygen), 3,3',4,4'-tetrachlorobiphenyl (PCB 77, dose: 2 μg/g and 8 μg/g body weight), and Aroclor 1254 (a common PCB mixture, dose: 0.5 μg/g and 1 μg/g body weight), alone and in combination for 4 weeks. PCB 77 exposure markedly increased hepatic CYP1A mRNA and protein expression, and ethoxyresorufin-*O*-deethylase (EROD, an indicator of CYP1A enzyme) activity and increased endothelial nitric oxide synthase (eNOS) protein expression. PCB 77 treatment also increased interleukin-1β (IL-1β, a cytokine) mRNA levels and protein carbonyl (PC, an indicator of reactive oxygen species, ROS) contents. These marked PCB 77- and Aroclor 1254-induced increases in CYP1A mRNA levels and EROD activity were significantly attenuated by co-exposure to hypoxia, whereas the increases in hepatic eNOS protein and IL-1β mRNA expression, and PC contents were augmented by hypoxia co-exposure. The results suggest that biotransformation of organic xenobiotics by CYP1A is reduced in fish during co-exposure to hypoxia and is accompanied by alterations in eNOS, ROS and IL-1β levels.

1. INTRODUCTION

Marine organisms are frequently exposed to hypoxia due to natural seasonal and/or diurnal fluctuations in dissolved oxygen (DO) levels.[1-3] Hypoxia in coastal regions is frequently exacerbated as a result of eutrophication caused by anthropogenic inputs of nutrients in runoff from agricultural land treated with fertilizers, feed lots and landfill leachates.[3] In addition to hypoxia exposure, marine organisms in coastal environments are often also exposed to a variety of anthropogenic pollutants including polycyclic aromatic hydrocarbons (PAH), and planar halogenated aromatic hydrocarbons (PHAH), such as polychlorinated biphenyls (PCBs).[4-7] Thus, organisms in coastal regions are frequently exposed to hypoxia and anthropogenic pollutants simultaneously.[2,8]

PCBs were produced in many countries since the 1930s for various commercial applications such as dielectric and coolant fluids in transformers and in other electrical and hydraulic equipment.[9,10] Due to their high bioaccumulation in biota and toxicity, PCB production and use were banned in the United States in the late 1970s, and in most of Europe, and several countries in Asia in the 1980s.[9,11] However, PCBs are resistant to degradation, so they are persistent and ubiquitous contaminants that remain a major environmental concern.[9,10] PCBs display a broad spectrum of pathophysiological effects in fish, often causing declines of growth, neuroendocrine disruption and reproductive impairment, disruption of spawning rhythms and larval recruitment, and in extreme cases, mortality.[4,12,13] Many adverse effects of coplanar PCBs in fish have been shown to be mediated through the aryl hydrocarbon receptor (AhR).[14] However, some of the toxic effects of coplanar PCBs such as apoptosis, immune suppression and reactive oxygen species (ROS) generation have also been reported in mammals and fish exposed to

nonplanar PCBs, indicating the importance of nonAhR-mediated mechanisms in PCB toxicity.[15-17]

Monooxygenase enzymes such as cytochrome P450-1A (CYP1A) catalyze the biotransformation of various organic compounds including PCBs.[4,18-20] CYP1A is one of the earliest and most commonly used toxicological indicators of exposure to PAHs and PHAHs in tetrapods and teleost fishes, and is upregulated by a wide range of these pollutants.[19,20] *In vivo* treatments with 3,3',4,4'-tetrachlorobiphenyl (PCB 77, a co-planar PCBs) significantly increased CYP1A mRNA and protein levels, and activity of ethoxyresorufin-*O*-deethylase (EROD, a measure of CYP1A enzyme activity) in hepatic tissues of marine teleost fishes such as scup, rainbow trout, and yellow bullhead.[21-23] Similarly, a PCB mixture such as Aroclor 1254 treatment drastically increased CYP1A activity in hepatic tissues of freshwater fish such as common carp.[22] Moreover, *in vitro* studies on rainbow trout hepatocytes and on scup liver microsomes have shown that PCB 77 treatment increases CYP1A mRNA levels and EROD activity.[24,25] These results suggest that the mRNA levels and enzymatic activity of CYP1A are highly susceptible to induction by exposure to PCBs in teleost fishes.

Hypoxia exposure also influences CYP1A activity in teleost fishes. Hypoxia exposure significantly decreased CYP1A mRNA levels in hepatic tissues of Atlantic cod, and EROD activity in sheepshead minnow and zebrafish.[26-28] Recently, we have shown that hypoxia exposure markedly decreases CYP1A mRNA and protein levels in hepatic tissues of Atlantic croaker (croaker, a marine fish).[29] In addition, our *in vivo* studies have shown that the NO-donor, *S*-nitroso-N-acetyl-DL-penicillamine, significantly decreases CYP1A mRNA and protein levels in croaker liver, whereas the competitive inhibitor of NOS

enzyme, N_{ω}-nitro-L-arginine methyl ester, restores CYP1A activity and expression in hypoxia-exposed fish. Moreover, exposure to hypoxia drastically increases interleukin-1β (IL-1β, a cytokine, CTK and a key mediator of inflammation) mRNA levels, endothelial NOS (eNOS) protein expression, and superoxide radical ($O^{2\bullet-}$, a ROS) production in croaker livers.[29,30] We have also shown that the increase in eNOS, IL-1β and ROS levels and decrease in CYP1A activity in croaker livers during hypoxia exposure are reversed by antioxidant treatment.[29] Collectively, these results suggest that hypoxia-induced down-regulation of hepatic CYP1A activity in croaker is influenced by alterations of hepatic eNOS, IL-1β and oxidant status.

Currently, there is a lack of information on interactive effects of hypoxia and PCBs on hepatic CYP1A activity and regulation of NOS, ROS and CTK in teleost fishes. Therefore, the present study was designed to assess the interactive effects of hypoxia and PCB exposure on CYP1A expression and NOS, ROS, and CTK regulation in croaker hepatic tissues. Croaker is an appropriate teleost model for such studies because the effects of hypoxia exposure on NOS, ROS and IL-1β levels and the role of NOS in regulation of CYP1A expression and activity have already been characterized in this species.[29] Juvenile croaker were exposed to two sublethal doses of a PCB mixture, Aroclor 1254, and a co-planar PCB (PCB 77) in their diet for a month, alone, and in combination with co-exposure to hypoxia (1.7 mg/L dissolved oxygen). Other croaker were exposed to hypoxia alone. Hepatic CYP1A mRNA and protein expression, EROD activity, and eNOS protein levels were measured in all treatment groups, and IL-1β mRNA levels and protein carbonyl (PC, an index of ROS) content were also measured after exposure to PCB 77 and hypoxia. The results show that changes in CYP1A

expression and activity after *in vivo* exposure to both the PCB and hypoxia stressors, alone and in combination, are associated with elevations of eNOS, IL-1β and PC contents in croaker livers.

2. MATERIALS AND METHODS

2.1. Chemicals

3,3′,4,4′-tetrachlorobiphenyl (PCB 77) and Aroclor 1254 were obtained from Chem Service (West Chester, PA). Molecular and analytical grade reagents were purchased from Agilent Technologies (La Jolla, CA), Pierce (Rockford, IL), Bio-Rad (Hercules, CA) and Sigma-Aldrich (St. Louis, MO).

2.2. Experimental fish

Young-of-the-year juvenile Atlantic croaker (10-11 cm total length, 12-18 g body weight, BW), collected in Texas bays, were purchased from local bait stands and transported to fish holding facilities at the University of Texas Marine Science Institute. Fish were stocked in large indoor tanks (capacity: 4,727 L) with recirculating sea water (salinity 30-32 ppt) system under ambient temperature (22±1^{o}C) and photoperiod (13D:11L) conditions. Fish were fed frozen chopped shrimp once a day (3% BW/day) and acclimated to controlled laboratory conditions for 1 month prior to hypoxia and PCB 77 or Aroclor 1254 co-exposure experiments.

2.3. Experiment: Interactive effects of hypoxia with PCB 77 or Aroclor 1254 exposure

Details of the hypoxia experimental set-up were described previously by Rahman and Thomas (2012).[28] Briefly, thirty mixed-sex fish were stocked into each of twelve tanks (capacity: 2,025 L seawater) with a recirculating system. Six tanks were assigned to normoxic conditions (6.5 mg DO/L) and the other six tanks were maintained under hypoxic conditions (1.7 mg DO/L) in each treatment group. For the combined hypoxia with PCB 77 exposure experiment, two tanks of fish at each DO level (two tanks for normoxia and two tanks for hypoxia exposure) were fed frozen chopped shrimp mixed with low dose of PCB 77 (2 μg/g BW), another four tanks (two for normoxia and two for hypoxia) were fed a high dose of PCB 77 (8 μg/g BW), and the remaining four tanks (two for normoxia and two for hypoxia) of fish were given control food (no PCB77 added to food). Similar to the PCB 77 exposure experiment, four tanks of fish (two tanks for normoxia and two tanks for hypoxia exposure) were fed chopped shrimp with a low dose of Aroclor 1254 (0.5 μg/g BW), another four tanks of fish (two for normoxia and two for hypoxia) were fed a higher sublethal dose of Aroclor 1254 (1 μg/g BW), and the third set of four tanks (two for normoxia and two for hypoxia) of fish were fed control diet (no Aroclor 1254 added to chopped shrimp). The concentrations of PCB 77 and Aroclor 1254, and experimental period in this study were based on environmental levels of PCBs and previous publications in teleost fishes including croaker.[12,13,31-34] Fish in each tank were fed the same amount of food (3% BW/day) for 4 weeks. At the end of the experiments, the fish were sacrificed under deep anesthesia using quinaldine sulphate (Sigma Aldrich) in sea water. Animal care, and hypoxia and PCB 77 or Aroclor 1254 co-

exposure experiments were conducted in the following guidelines and ethical rules approved by the University of Texas at Austin Animal Care and Use Committee (IACUC protocol #08080401). Liver tissues were quickly frozen in liquid nitrogen and stored at -80^{o}C until use for RNA extraction, enzymatic activity, and protein determination. Liver tissues were also fixed in 4% paraformaldehyde (Sigma-Aldrich) overnight at 4^{o}C for immunohistochemical detection of CYP1A protein expression.

2.4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR analyses were performed on total RNA using a one-step qRT-PCR method as described previously by Rahman and Thomas (2012).[28] Briefly, RNA was extracted from liver tissues using TRI reagent according to the manufacturer's protocol (Sigma-Aldrich) followed by DNase treatment (Promega, Madison, WI). The quality and quantity of total RNA were confirmed with a NanoDrop 2000C (Thermo Fisher Scientific, Waltham, MA). qRT-PCR analyses were performed using a one-step SYBR Green qRT-PCR master mix (Agilent Technologies) following the manufacturer's instructions. Gene-specific primers were designed according to the croaker CYP1A (GenBank accession number JQ622220), IL-1β (JQ622219) and 18S (AY866435) sequences using Primer3 software according to Untergasser et al. (2012).[35] The following gene-specific primers were used for CYP1A (forward: 5'-TCAACGATGGCAAGAGTCTG-3' and reverse: 5'-TACTCTGGGGTTGTGCCTTC-3', amplicon size: 121 bp), IL-1β (forward: 5'-CGTGA CCGACAGTGAGAAGA-3' and reverse: 5'-TCCCATCCTTATGGCAAGAG-3', amplicon size: 136 bp) and 18S rRNA (forward 5'-AGAAACGGCTACCACATCCA-3' and reverse 5'-TCCCGAGATCCAACTACGAG-3', amplicon size: 249 bp) mRNAs.

The qRT-PCR amplification cycles consisted of 50°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 30 s. At the end of each qRT-PCR run, a melting curve analysis was also included at 1 cycle of 95°C for 1 min, 50°C for 30 s, and 95°C for 30 s. The melting curve of each qRT-PCR cycle was analyzed to assess the specificity of amplification. The relative mRNA levels of CYP1A and IL-1β among control and treatment groups were calculated using $2^{-\Delta\Delta Ct}$ equation according to Livak and Schmittgen (2001).[36]

2.5. Preparation of microsomes and ethoxyresorufin-O-deethylase (EROD) activity assay

Microsomes were separated from liver tissue according to Burke and Mayer (1974).[37] Briefly, liver tissue (~1 g) was homogenized in stabilization buffer (100 mM potassium phosphate containing 20% glycerol, 1 mM dithiothreitol, 1 mM EDTA, pH 7.4; containing 10 µl of halt protease inhibitor cocktail), centrifuged at 12,000 x *g* for 11 min and collected supernatants. The resulting supernatants were further centrifuged using an ultracentrifuge at 100,000 x *g* for 60 min to yield microsomal pellets. Microsomal pellets were dissolved in 500 µl stabilization buffer, vortexed, and stored at -80°C until used for EROD activity assay and Western blot analysis. EROD activity was assayed using a fluorescence method.[37]

2.6. Western blot analysis

CYP1A and eNOS protein expression and levels were determined from hepatic microsomal and cytosolic fractions, respectively, by Western blot analysis as described

previously by Rahman and Thomas (2012).[29] Briefly, protein samples were solubilized in loading buffer by boiling, resolved on SDS-PAGE gels and transferred onto immuno-blot PVDF membranes (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in TBS-T buffer for 1 h at room temperature and probed with CYP1A and eNOS (Santa Cruz Biotechnology, Paso Robles, CA) primary antibodies (dilution: 1:1,000) overnight at 4°C. The mouse CYP1A monoclonal antibody was generated against a highly conserved amino acid sequence in rainbow trout CYP1A gene,[38] which is 89% identical to the corresponding region of amino acid sequence in croaker CYP1A gene.[29] The rabbit eNOS antibody was validated previously in croaker liver and stinging catfish ovary.[29,39] Membranes were then incubated with secondary anti-mouse antibody (1:5,000; Cell Signaling, Danvers, MA) for CYP1A and goat anti-rabbit antibody (1: 4,000; Southern Biotech, Birmingham, AL) for eNOS proteins. Actin protein was used as an internal control to normalize sample loading on the gels. The immunoreactive (IR) signals of CYP1A, eNOS and actin protein bands were visualized by the addition of chemiluminescent substrate (Pierce), photographed on Hyperfilm (GE Healthcare, Buckinghamshire, UK) and calculated by ImageJ software (National Institutes of Health, Bethesda, MD).

2.7. Immunohistochemistry

The immunofluorescent signals of CYP1A and eNOS proteins in croaker liver tissues were amplified by diaminobenzidine (DAB) peroxidase (Vector Laboratories, Burlingame, CA) and tyramide signal amplification solutions (Molecular Probes, Eugene, OR), respectively. Liver tissues were embedded in paraffin, sectioned into 7 μm section

on a microtome, deparaffinized with xylene and rehydrated with a series of ethanol. To expose the antigen, sections were rinsed in PBS and treated in a retrieval solution (10 mM citric acid, pH 6.0) for 10 min, rinsed in PBS and incubated for 1 h in blocking solution (1% BSA containing PBS). After blocking, sections were rinsed in PBS and incubated with mouse anti-CYP1A and rabbit eNOS primary antibodies (dilution: 1:100) overnight at 4°C. After incubation, sections were rinsed in PBS and incubated for 2 h with HRP-linked anti-mouse and Alexa Fluor 594 goat anti-rabbit secondary antibodies for detection of CYP1A and eNOS proteins, respectively. The IR signal of CYP1A protein was detected using DAB substrate according to the manufacturer's instructions (Vector Laboratories). The IR signals of CYP1A and eNOS proteins were captured by Cool-SNAP camera (Photometrics, Tucson, AZ) and calculated by ImageJ software.

2.7. Protein carbonyl (PC) assay

PC contents were measured in liver tissues according to the method of Levine et al. (1994).[40] Briefly, tissue samples (0.2-0.3 g) were homogenized in a glass homogenizer in ice-cold PBS buffer and centrifuged at 10,000 x *g* for 15 min at 4°C. Two hundred µl of supernatant were added in 800 µl of DNPH to the sample and 800 µl of 2.5 *N* HCl to the control. Both samples were incubated in the dark at room temperature for 1 h. The proteins were then precipitated by adding 1 ml of 20% TCA and centrifuged at 10,000 x g for 10 min at 4°C. The resulting pellet was washed three times with 1 ml of ethanol:ethyl acetate mixture (1:1). After the final wash, the protein pellet was dissolved in 500 µl of 6 M guanidine hydrochloride. PC contents were determined by absorbance at

a wavelength between 360-385 nm using a Fluoro Star spectrophotometer (BMG Lab Tech, Cary, NC) and expressed as nmol/mg of protein.

2.8. Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM). All of the experimental data were analyzed by one-way ANOVA followed by Fisher's PLSD test for multiple comparisons. Unpaired means were compared with Student's *t*-test. A P value <0.05 was considered statistically significant for all the tests.

3. RESULTS

3.1. Interactive effects of hypoxia and PCB 77 on CYP1A mRNA levels

Exposure to hypoxia (1.7 mg DO/L for 4 weeks) caused significant decreases in CYP1A mRNA levels, whereas PCB 77 treatments (low dose: 2 µg/g body weight, BW; high dose: 8 µg/g BW) for 4 weeks caused a marked elevation (~75- and ~115-fold for low- and high-dose, respectively) of CYP1A mRNA levels compared to normoxic controls (Fig. 1A). Combined exposure to hypoxia and the high dose of PCB 77 (8 µg/g BW for 4 weeks) significantly reduced CYP1A mRNA levels around 50% compared to PCB-treatment alone (Fig. 1A).

3.2. Interactive effects of hypoxia and PCB 77 on EROD activity and CYP1A protein expression

Exposure to hypoxia significantly reduced EROD activity compared to normoxic controls (Fig. 2A). PCB 77 treatments markedly elevated EROD activity ~10- to 12-fold

compared to normoxic controls, whereas the marked elevation of EROD activity was attenuated when fish were simultaneously exposed to hypoxia and the high dose of PCB 77 (Fig. 2A). Western blot analysis showed the presence of a predicted-size band of IR CYP1A protein of around 59 kDa (Fig. 2B). Immunoblot results showed that the expression of the CYP1A IR signal was reduced to around 27% of controls in hypoxia-exposed fish (1.7 mg DO/L for 4 weeks), whereas treatments with PCB 77 (low dose: 2 μg/g body weight, BW; high dose: 8 μg/g BW for 4 weeks) increased CYP1A protein levels ~3- to 4-fold compared with normoxic controls following normalization to actin protein (Fig. 2C, D). Combined exposure to hypoxia and the high dose of PCB 77 (8 μg/g BW) significantly decreased CYP1A protein levels around 14% ($P<0.05$, Student's *t*-test) compared to PCB treatments under normoxic conditions (Fig. 2C, D).

3.3. Interactive effects of hypoxia and Aroclor 1254 on CYP1A mRNA levels and protein expression

Hypoxia exposure (1.7 mg DO/L for 4 weeks) caused a decrease in CYP1A mRNA levels, whereas the Aroclor 1254 treatments (low dose: 0.5 μg/g BW, high dose: 1 μg/g BW for 4 weeks) caused marked increases in CYP1A gene expression. The combined treatments with hypoxia attenuated the CYP1A responses to both the high and low doses of Aroclor 1254 by approximately 50% (Fig. 3A).

The interactions between hypoxia and Aroclor 1254 on CYP1A protein expression in croaker livers were also examined by immunohistochemistry (IHC). The expression and intensity of IR staining of CYP1A protein decreased around 11% in hypoxia-exposed (1.7 mg DO/L for 4 weeks) fish compared to normoxic controls (Fig.

3C,D). On the other hand, treatments with Aroclor 1254 (low dose: 0.5 μg/g BW, high dose: 1 μg/g BW) for 4 weeks caused marked increases (~2.5- to 3.5-fold) in CYP1A protein expression and intensity compared to normoxic control fish. However, the increases in CYP1A IR expression and intensity were attenuated ~60- to 88% when fish were co-exposed to hypoxia and Aroclor 1254 (Fig. 3C,D).

3.4. Interactive effects of hypoxia and PCB 77/Aroclor 1254 on eNOS protein expression

In order to investigate the potential mechanisms of CYP1A regulation by hypoxia and PCB 77, we measured eNOS protein expression, PC contents and IL-1β mRNA levels in croaker livers. Western blot result showed a predicted-size band of IR eNOS protein of ~140 kDa (Fig. 4A). The expression of eNOS IR signal was increased ~2.5-fold in hypoxia-exposed fish compared with normoxic fish following normalization to actin protein (Fig. 4B, C). Treatments with PCB 77 increased eNOS protein levels ~5-fold compared to those in normoxic fish. No significant difference was observed in eNOS protein levels between the control-low dose of PCB 77 and the combined hypoxia-low dose of PCB 77 treatment (2 μg/g) groups. The combined treatment of hypoxia and high dose of PCB 77 (8 μg/g BW for 4 weeks) significantly increased eNOS protein levels compared to those in fish exposed to PCB 77 alone (Fig. 4B, C). We also analyzed the expression pattern of eNOS protein in croaker livers by IHC. The IR staining intensity of eNOS protein increased ~3-fold in liver tissues of hypoxia-exposed fish compared to normoxic controls (Fig. 4D,E). Treatments with Aroclor 1254 (low dose: 0.5 μg/g BW, high dose: 1 μg/g BW) for 4 weeks caused marked increases (~7- to 12-fold) in eNOS

protein expression and intensity compared to normoxic control fish. The increase in eNOS expression and intensity was further elevated when fish were co-exposed to hypoxia and Aroclor 1254 (Fig. 4D,E).

3.5. Interactive effects of hypoxia and PCB 77 on PC contents and IL-1β mRNA levels

Protein oxidation results showed PC contents in croaker livers were increased around 3-fold in hypoxia-exposed fish and around 5-fold in the PCB 77-exposed fish compared to normoxic controls (Fig. 5A). PC contents in the combined hypoxia and PCB 77 treated fish were further significantly increased over those exposed to these treatments alone and were increased ~7.7- to ~9.9-fold over those in the normoxic controls (Fig. 5A).

qRT-PCR results showed exposure to hypoxia caused a significant ~3-fold increase in IL-1β mRNA levels in croaker livers (Fig. 5B). PCB 77 treatments, both low- and high-dose, resulted in marked increases in IL-1β mRNA levels around 7.5- to 9.5-fold compared to those in normoxic fish, and IL-1β mRNA levels were further elevated to around 13- to 16.5-fold those in controls when fish were simultaneously exposed to hypoxia and PCB 77 (Fig. 5B).

4. DISCUSSION

The results of this study clearly show that co-exposure of croaker to hypoxia blunts the increases in hepatic CYP1A mRNA and protein expression as well as EROD activity induced by treatment with PCB 77 and Aroclor 1254. Consistent with our previous findings, chronic exposure to hypoxia alone caused significant decreases in hepatic

CYP1A activity, mRNA and protein expressions compared to fish held in normoxic conditions that were accompanied by increases in hepatic eNOS protein, IL-1β mRNA, and PC levels.[29] As predicted, treatment with the PCBs had an opposite effect on CYP1A expression and activity, dramatically increasing mRNA and protein expression and EROD activity. Interestingly though, chronic treatment with PCB exposure also elevated hepatic eNOS protein expression, PC contents and IL-1β mRNA levels, with increases significantly greater than those in the livers of fish held in hypoxic conditions. Moreover, the attenuation of the CYP1A response to PCB treatment by co-exposure to hypoxia was accompanied by further significant increases in eNOS and IL-1β expression and PC concentrations. These results suggest that an intimate but complex relationship exists between the expression and activity of CYP1A and its potential regulators, eNOS, IL-1β, and ROS, after co-exposure to hypoxia and PCBs that warrants further investigation. To our knowledge, this is the first *in vivo* study to show the interactive effects of hypoxia with PCBs on hepatic CYP1A expression and eNOS, ROS and IL-1β regulation in a teleost species.

Induction of hepatic CYP1A expression and activity by PCB exposure is a common phenomenon in tetrapods and teleost fishes.[41,42] In the present study, chronic treatment in the food with PCB 77 (2 μg/g or 8 μg/g BW) or Aroclor 1254 (0.5 μg/g or 1 μg/g BW) for 4 weeks caused marked increases in hepatic CYP1A mRNA (~40- to 115-fold) and CYP1A protein (~3-fold) expression in croaker which were accompanied by significant increases (~10- to 12-fold) in EROD activity. Similarly, a single injection with PCB 77 (1 μg/g BW) increased hepatic EROD activity around ~10-fold 7 days later in rainbow trout and around 2.5-fold in yellow bullhead.[22,23] Similar to PCB 77, a single

injection with Aroclor 1254 (10 µg/g BW) induced hepatic microsomal EROD activity around 5-fold in rainbow trout.[22]

An important finding of this study is that chronic co-exposure to hypoxia (1.7 mg DO/L for 4 weeks) causes marked decreases in PCB-induced upregulation of hepatic CYP1A mRNA and protein levels, and EROD activity in croaker. Combined effects of hypoxia and xenobiotics on hepatic CYP1A regulation have been previously reported in other teleost fishes. EROD activity in zebrafish larvae (24-72 hours post-fertilization, hpf) exposed to PCB 126, a dioxin-like PCB, is decreased ~4 to 7-fold under hypoxic (DO: 2.7 mg/L for 72 h) conditions.[43] In addition, co-exposure of zebrafish embryos (48 hpf) to 2,3,7,8-tetrachlorodinezodioxin (TCDD, a dioxin) and moderate hypoxia (DO: 3 mg/L) for 6 hours caused significant decreases (50%) in CYP1A mRNA levels.[44] In contrast, hepatic CYP1A mRNA levels were not significantly altered in juvenile grouper after combined exposure for 4 weeks to benzo[a]pyrene, an AhR agonist, and hypoxia (DO: 1.5-2 mg/L)[45], and in adult tilapia after 5 days' exposure to these stressors.[46] Collectively, these studies suggest that the reduction in the hepatic CYP1A response to PHAH and PAH exposure when fish are co-exposed to hypoxia is a common but not ubiquitous outcome in teleost fishes.

An interesting result of the present study is the overexpression of eNOS protein in croaker liver during co-exposure to hypoxia and PCB. The production of nitric oxide (NO, a reactive nitrogen species, RNS) is catalyzed by eNOS in vertebrate liver tissues.[47] NO and eNOS are both involved in regulation of hepatic CYP1A enzyme in vertebrates.[48] For example, NO enhanced the down-regulation of enzymatic activities in hepatic CYP1A1 and CYP1A2, and suppressed their mRNA and protein expression in rat

liver.[47,48] Several mammalian *in vitro* studies have been shown that treatment with PCB increases NO content in cultured rabbit and human endothelial cells.[49,50] Interestingly, treatment with NO donors such as *S*-nitroso-N-acetyl-DL-penicillamine (SNAP), and sodium nitroprusside, suppress CYP1A1 and CYP1A2 mRNA and protein expression, and also their activities in rat hepatocytes under normoxic conditions.[51] Similarly, we have recently shown that administration of NO donor, SNAP, decreases hepatic CYP1A mRNA and protein levels, and EROD activity in hypoxic fish compared to normoxic conditions.[29] Moreover, we have demonstrated that hypoxia increases hepatic eNOS protein levels and increases plasma NO metabolites (NOx, nitrite and nitrate) concentration which subsequently decreases CYP1A activity, mRNA and protein expression in croaker liver.[29,52]

Hypoxia-induced changes in gene expression are thought to be primarily regulated by a transcription factor, hypoxia-inducible factor-1α (HIF-1α), which forms a transcriptionally active dimer with HIF-1β (ARNT) under hypoxic conditions.[53-55] Upon activation by PAHs and PHAHs, the aryl hydrocarbon receptor (AhR) is translocated to the nucleus where it forms a transcriptionally heterodimer with ARNT to regulate transcription of numerous genes, including CYP1A1.[56] *In vitro* studies with mammalian and fish cell lines have shown that there is cross-talk between the HIF-1α/ARNT and AhR/ARNT pathways during co-exposure to hypoxia and AhR agonists resulting in attenuation of AhR agonist-induced CYP1A1 expression and enzymatic activity.[27,56,57] However, an analysis of AhR-mediated signaling induced by dioxin in mammalian hepatoma cell lines during hypoxia exposure revealed a lack of competition for ARNT, even though CYP1A1 mRNA was down-regulated.[58] Moreover, enzymatic activity of

CYP1A1 was shown to be inhibited by hypoxia in HIF-1α null cells which suggests that negative regulation of AhR signaling by hypoxia is not mediated by HIF-1α and competition for ARNT.[59] Therefore, the mechanisms underlining the regulation of CYP1A during co-exposure to hypoxia and PCB in croaker, and whether they involve crosstalk between HIF-1α/ARNT and AhR/ARNT pathways, remain unclear and require additional investigation.

The present findings indicate that the potential roles of eNOS, oxidative stress, and cytokines in the hypoxia-induced attenuation of the CYP1A expression and activity responses to PCB exposure also warrant further study on molecular mechanisms of CYP1A regulation in combined exposure to hypoxia and PCBs in teleost fishes. The results of a previous study using pharmacological treatments that modulated eNOS activity clearly show that eNOS is an intermediary in hypoxia-induced down-regulation of CYPIA expression and EROD activity in croaker livers.[29] Similarly, increased eNOS activity partially mediates the decrease in CYP1A activity in rat livers observed during sepsis.[48] Increased production of reactive oxygen intermediates such as ROS in rabbits during acute inflammation causes a reduction in hepatic cytochrome P450 activity.[60] In addition, cytokines such as IL-1β have been implicated in hypoxia-induced declines in hepatic cytochrome P450 activity and expression in rabbits.[61,62] These results suggest that ROS and ILβ-1 are also likely involved in hypoxia down-regulation of CYP1A expression and activity in croaker livers. Moreover, the finding that the attenuation of PCB-induced upregulation of CYP1A expression and EROD activity by co-exposure to hypoxia is accompanied by further increases in eNOS, ROS (PC content), and IL-1β expression raises the possibility that they may also mediate CYP1A down-regulation

under co-exposure conditions. A future study to test the hypothesis that the pathways regulating eNOS, CTK and ROS in response to hypoxia and PCB co-exposure antagonize each other to regulate hepatic CYP1A could clarify the interactions between these two stressors in CYP1A regulation.

5. CONCLUSION

Our *in vivo* studies provide the first clear evidence that the induction of hepatic CYP1A activity during hypoxia and PCB co-exposure in Atlantic croaker is accompanied by alterations in hepatic eNOS, ROS and CTK. The results from this study suggest that combined exposure to hypoxia and PCB could decrease xenobiotic chemical metabolism in teleost fishes that inhabit contaminated coastal waters and estuaries where fluctuations of dissolved oxygen occur frequently. This decrease in xenobiotic metabolism could alter the toxicity of some PCBs, PAHs and other AhR ligands. For example, decreased metabolism of certain PAHs such as benzo[a]pyrene which is metabolically activated to a DNA-binding carcinogen could decrease their toxicity, whereas it could increase the accumulation and toxicity of other PAHs including naphthalene and phenanthrene which are converted to more polar metabolites that can be readily excreted.[63,64] Information on the effects of hypoxia on metabolism of AhR ligands and other xenobiotic chemicals should lead to a better understanding of their toxicity and long-term impacts on wild fish populations in hypoxic environments.

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CONFLICT OF INTEREST

There is no conflict of interest to be stated.

REFERENCES

[1] Nikinmaa M. 2002. Oxygen-dependent cellular functions-why fishes and their aquatic environment are a prime choice of study. *Comp Biochem Physiol.* 2002;133A:1-16.

[2] Wu RSS. Hypoxia: from molecular responses to ecosystem responses. *Mar Pollut Bull.* 2002;45:35-45.

[3] Diaz RJ, Rosenberg R. Spreading dead zones and consequences for marine ecosystems. *Science* 2008;321:926-929.

[4] Henry TB. Ecotoxicology of polychlorinated biphenyls in fish- a critical review. *Critical Rev. Toxicol.* 2015;45:643-661.

[5] Villeneuve JP, Carvalho FP, Fowler SW, Cattini C. Levels and trends of PCBs chlorinated pesticides and petroleum hydrocarbons in mussels from the NW Mediterranean coast: comparison of concentrations in 1973/1974 and 1988/1989. *Sci Total Environ.* 1999;237/238: 57-65.

[6] Stevens JL, Northcott GL, Stern GA, Tomy GT, Jones KC. PAHs, PCBs, PCNs, organochlorine pesticides, synthetic musks, and polychlorinated n-alkanes in U.K. sewage sludge: survey results and implications. *Environ Sci Technol.* 2003;37:462-467.

[7] Minomo K, Ohtsuka N, Hosono S, Nojiri K, Kawamura K. Seasonal change of PCDDs/PCDFs/DL-PCBs in the water of Ayase river, Japan: pollution sources and their contributions to TEQ. *Chemosphere* 2011;85:188-194

[8] Mearns AJ, Reish DJ, Oshida PS, Ginn T, Rempel-Hester MA. Effects of pollution on marine organisms. *Water Environ Res.* 2011;83:1789-1852.

[9] Breivik K, Sweetman A, Pacyna JM, Jones KC. Towards a global historical emission inventory for selected PCB congeners- A mass balance approach 1. Global production and consumption. *Sci Total Environ.* 2002;290:181-198.

[10] Clarke BO, Porter NA, Marriott PJ, Blackbeard JR. Investigating the levels and trends of organochlorine pesticides and polychlorinated biphenyl in sewage sludge. *Environ Int.* 2010;36:323-329.

[11] Vorkamp K. An overlooked environmental issue? A review of the inadvertent formation of PCB-11 and other PCB congeners and their occurrence in consumer products and in the environment. *Sci Total Environ.* 2016;541:1463-1476.

[12] Khan IA, Thomas P. Disruption of neuroendocrine control of luteinizing hormone secretion by Aroclor 1254 involves inhibition of hypothalamic tryptophan hydroxylase activity. *Biol Reprod.* 2001;64:955-964.

[13] Rahman MS, Khan IA, Thomas P. Tryptophan hydroxylase: a target for neuroendocrine disruptions. *J Toxicol Environ Health B* 2011;14,473-494.

[14] Johnsson ME, Jenny MJ, Woodin BR, Hahan ME, Stegeman JJ. Role of AHR2 in the expression of novel cytochrome P450 1 family genes, cell cycle genes, and morphological defects in developing zebrafish exposed to 3,3',4,4',5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicol Sci.* 2007;100:180-193.

[15] Santiago MF, Perez-Reyes PL, Lopez-Aparicio P, Recio MN, Perez-Albarsanz MA. Differential Effects of PCBs on the induction of apoptosis machinery and PKCα translocation in rat renal tubular cultures. *Toxicol Lett.* 2006;163:91-100.

[16] Zhang J, Zhang H, Wanmin N. Oxidative stress and apoptosis of *Carassius auratus* lymphocytes induced by nonplanar (PCB 153) and coplanar PCB 169 polychlorinated congeners in vitro. *J Environ Sci.* 2009;21:1284-1289.

[17] Santoro A, Ferrante MC, Di Guidaa F, Pirozzi C, Lama A, Simeoli R, Clausi MT, Monnolo A, Mollica MP, Raso GM, Meli R. Polychlorinated biphenyls (PCB 101, 1523, 180) impair murine macrophage responsiveness to lipopolysaccharide: involvement of NFκ-B pathway. *Toxicol Sci.* 2015;147:255-269.

[18] Nebert DW, Gonzalez FJ. P450 genes: structure, evolution, and regulation. *Ann Rev Biochem*. 1987;56:945-993.

[19] Bandiera SM. Cytochrome P450 enzymes as biomarkers of PCB exposure and modulators of toxicity. Robertson, L.W., Hansen, L.G. eds., In: PCBs: Recent Advances in Environmental Toxicology and Health Effects. The University Press of Kentucky, 2001; pp.185-192.

[20] Thomas DB, Fent K. Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. *Critical Rev Environ Sci Toxicol.* 2009;25:201-268.

[21] White RD, Shea D, Solow AR, Stegeman JJ. Induction and post-transcriptional suppression of hepatic cytochrome P450 1A1 by 3,3′,4,4′-tetrachlorobiphenyl. *Biochem Pharmacol.* 1997;53:1029-1040.

[22] Melancon MJ, Lech JJ. 1983. Dose-effect relationship for induction of hepatic monooxygenase activity in rainbow trout and carp by Aroclor 1254. *Aquat Toxicol.* 1983;4:51-61.

[23] Brammell BF, Price DJ, Birge WJ, Elskus AA. Lack of CYP1A responsiveness in species inhabiting chronically contaminated habitats: two varieties of resistance? *Comp Biochem Physiol.* 2013;157C:212-219.

[24] Mortensen AS, Tolfsen CC, Arukwe A. Gene expression patterns in estrogen (nonylphenol) and aryl hydrocarbon receptor agonists (PCB-77) interaction using rainbow trout (*Oncorhynchus mykiss*) primary hepatocyte culture. *J Toxicol Environ Health A* 2006; 69:1-19.

[25] Schlezinger JJ, Struntz WDJ, Goldstone JV, Stegeman JJ. Uncoupling of cytochrome P450 1A and stimulation of reactive oxygen species production by co-planar polychlorinated biphenyl congeners. *Aquat Toxicol.* 2006;77:422-432.

[26] Olsvik PA, Kristensen T, Waagbø R, Tollefsen KE, Rosseland BO, Toften H. Effects of hypo- and hyperoxia on transcription levels of five stress genes and the glutathione system in liver of Atlantic cod *Gadus morhua*. *J Exp Biol.* 2006;209:2893-2901.

[27] Fleming CR, Billiard SM, Di Giulio RT. Hypoxia inhibits induction of aryl hydrocarbon receptor activity in topminnow hepatocarcinoma cells is an ARNT-dependent manner. *Comp Biochem Physiol.* 2009;150C:383-389.

[28] Dasgupta S, Di Giulio RT, Drollette BD, Plata DL, Brownawell BJ, McElroy AE. Hypoxia depresses CYP1A induction and enhances DNA damage, but has minimal effects on antioxidant responses in sheepshead minnow (*Cyprinodon variegatus*) larvae exposed to dispersed crude oil. *Aquat Toxicol.* 2016;177:250-260.

[29] Rahman MS, Thomas P. Effects of hypoxia exposure on cytochrome P4501A (CYP1A) expression in Atlantic croaker: molecular mechanisms of CYP1A down-regulation. *PLoS ONE* 2012;7:e40825.

[30] Rahman MS, Thomas P. Characterization of three IGFBPs in Atlantic croaker and their regulation during hypoxic stress: potential mechanisms of their upregulation by hypoxia. *Am J Physiol Endocrinol Metabol.* 2011;301:E637-E648.

[31] Khan IA, Thomas P. PCB congener-specific disruption of reproductive neuroendocrine function in Atlantic croaker. *Mar Environ Res.* 2006;62:25-28.

[32] Liu J, Pan LQ, Zhang L, Miao JJ, Wang J. Immune responses, ROS generation and the haemocyte damage of scallop Chlamys farreri exposed to Aroclor 1254. *Fish Shellfish Immunol.* 2009;26:422-428.

[33] Deshpande AD, Dockum BW, Cleary T, Farrington C, Wieczorek D. Bioaccumulation of polychlorinated biphenyls and organochlorine pesticides in young-of-the-year bluefish (*Pomatomus saltatrix*) in the vicinity of a Superfund Site in New Bedford Harbor, Massachusetts, and in the adjacent waters. *Mar Pollut Bull.* 2013;72,146-164.

[34] Feili Wu, Yao Zheng, Jiancao Gao, Shu Chen, Zaizhao Wang. Induction of oxidative stress and the transcription of genes related to apoptosis in rare minnow (*Gobiocypris rarus*) larvae with Aroclor 1254 exposure. *Ecotoxicol Environ Safety* 2014;110:254-260

[35] Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. Primer3 - new capabilities and interfaces. *Nucleic Acids Res.* 2012;40:e115.

[36] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. *Methods* 2001;25:402-408.

[37] Burke MD, Mayer RT. Ethoxyresorufin: direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metab Dispos.* 1974;2:583-588.

[38] Rice CD, Schlenk D, Ainsworth J, Goksøyr A. Cross-reactivity of monoclonal antibodies against peptide 277-294 of rainbow trout CYP1A1 with hepatic CYP1A among fish. *Mar Environ Res.* 1998;46:87-91.

[39] Tripathi V, Krishna A. Changes in nitric oxide (NO) synthase isoforms and NO in the ovary of *Heteropneustes fossilis* (Bloch.) during the reproductive cycle. *J Endocrinol.* 2008;199:307-316.

[40] Levine RL, Williams JA, Stadtman ER, Shacter E. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* 1994;233:346-357.

[41] Lubet RA, Nims RW, Beebe LE, Fox SD, Issaq HJ, McBee K. Induction of hepatic CYP1A activity as a biomarker for environmental exposure to Aroclor 1254 in feral rodents. *Arch Environ Contam Toxicol.* 1992;22:339-344.

[42] Bonfanti P, Comelli F, Assi L, Casati L, Colciago A, Villa S, Santagostino A, Costa B, Colombo A. Responsiveness of hepatic and cerebral cytochrome P450 in rat offspring prenatally and lactationally exposed to a reconstituted PCB mixture. *Environ Toxicol.* 2014;29:856-66.

[43] Fleming CR, Di Giulio RT, The role of CYP1A inhibition in the embryotoxic interactions between hypoxia and polycyclic aromatic hydrocarbons (PAHs) and PAH mixtures in zebrafish (*Danio rerio*). *Ecotoxicology* 2011;20:1300-1314.

[44] Prasch AM, Andreasen EA, Peterson RE, Heideman W. Interactions between 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and hypoxia signaling pathways in zebrafish: hypoxia decreases responses to TCDD in zebrafish embryos. *Toxicol Sci.* 2004; 78:68-77.

[45] Yu RM, Ng PK, Tan T, Chu DL, Wu RS, Ng PK, Tan T, Chu DL, Wu RS, Kong RY, 2008. Enhancement of hypoxia-induced gene expression in fish liver by the aryl hydrocarbon receptor (AhR) ligand, benzo[a]pyrene (BaP). *Aquat Toxicol.* 2008;90:235-42.

[46] Garcia-Tavera JL, Valdes-Lozano D, Poblet-Naredo I, Aalbores-Medina A, Zapata-Perez O. Bile benzo[a]pyrene concentration and hepatic CYP1A induction in hypoxic adult tilapia (*Oreochromis niloticus*). *Chemosphere* 2013;92:16-23.

[47] Khatsenko OG, Gross SS, Rifkind AB, Vane JR. Nitric oxide is a mediator of the decrease in cytochrome P450-dependent metabolism caused by immunostimulants. *Proc Natl Acad Sci USA* 1993;90:11147-11151.

[48] Eum HA, Yeom DH, Lee SM. Role of nitric oxide in the inhibition of liver cytochrome P450 during sepsis. *Nitric Oxide* 2006;15:423-431.

[49] Omori N, Fukata H, Yamazaki K, Aida-Yasuoka K, Takigami H, Kuriyama M, Ichinose M, Mori C. Polychlorinated biphenyls alter the expression of endothelial nitric oxide synthase mRNA in human umbilical vein endothelial cells. *Hum Exp Toxicol.* 2007;26:811-816.

[50] Lee HG, Yang JH. PCB126 induces apoptosis of chondrocytes via ROS-dependent pathways. *Osteoarthr Cartilage* 2012;20:1179-1185.

[51] Stadler J, Trockfeld J, Schmalix WA, Brill T, Siewert JR, Greim H, Doehmer J. Inhibition of cytochrome P4501A by nitric oxide. *Proc Natl Acad Sci USA* 1991;91:3559-3563.

[52] Rahman MS, Thomas P. Molecular characterization and hypoxia-induced upregulation of neuronal nitric oxide synthase in Atlantic croaker: reversal by antioxidant and estrogen treatments. *Comp Biochem Physiol.* 2015;185A:91-106.

[53] Semenza GL. Hypoxia-inducible factor 1: master regulator of O_2 homeostasis. *Curr Opin Genet Dev.* 1998;8:588-594.

[54] Nikinmaa M, Ress BB. Oxygen-dependent gene expression in fishes. *Am J Physiol Regul Integr Comp Physiol.* 2005;288:R1079-R1090.

[55] Thomas P, Rahman MS. Biomarkers of hypoxia exposure and reproductive function in Atlantic croaker: a review with some preliminary findings from the northern Gulf of Mexico hypoxic zone. *J Exp Mar Biol Ecol* 2009;381:S38-S50.

[56] Chan WK, Yao G, Gu YZ, Bradfield CA. Cross-talk between the aryl hydrocarbon receptor and hypoxia inducible factor signaling pathways. *J Biol Chem.* 1999;274:12115-12123.

[57] Vorrink SU, Sarsour EH, Olivier AK, Robertson LW, Goswami PC, Domann FE. PCB 126 perturbs hypoxia-induced HIF-1α activity and glucose consumption in human HepG2 cells. *Exp Toxicol Pathol.* 2014;66:377-382.

[58] Pollenz RS, Davarinos NA, Shearer TP. Analysis of aryl hydrocarbon receptor-mediated signaling during physiological hypoxia reveals lack of competition for the aryl hydrocarbon nuclear translocator transcription factor. *Mol Pharmacol.* 1999;56:1127-1137.

[59] Allen JW, Johnson RS, Bhatia SN. Hypoxic inhibition of 3-methylcholanthre-induced CYP1A1 is independent of HIF-1alpha. *Toxicol Lett.* 2005;155 :151-159.

[60] El-Kadi AOS, Bleau AM, Dumont I, Maurice H, du Souich P. Role of reactive oxygen intermediates in the decrease of hepatic cytochrome P450 activity by serum of human and rabbits with an acute inflammatory reaction. *Drug Metab Dispos.* 2000;28:1112-1120.

[61] du Souich P, Fradette C. The effect and clinical consequences of hypoxia on cytochrome P450, membrane carrier proteins activity and expression. *Expert Opin Drug Metab Toxicol.* 2011;7:1083-100.

[62] Fradette C, du Souich P. Effect of hypoxia on cytochrome P450 activity and expression. *Curr Drug Metab.* 2014;5:257-271.

[63] Stegeman JJ, Lech JJ. Cytochrome P-450 monooxygenase systems in aquatic species: carcinogen metabolism and biomarkers for carcinogen and pollutant exposure. *Environ Health Perspect.* 1991,90:101-109.

[64] Mu J, Jin F, Wang J, Wang Y, Cong Y. The effects of CYP1A inhibition on alkyl-phenanthrene metabolism and embryotoxicity in marine medaka (*Oryzias melastigma*). Environ Sci Pollut Res. 2016,23:11289-11297.

FIGURE LEGENDS:

FIGURE 1. Interactive effects of hypoxia and PCB 77 on CYP1A mRNA levels in croaker liver. Treatments with hypoxia (HYP: 1.7 mg DO/L for 4 weeks) and PCB 77 (PCB) (low dose, LD: 2 µg/g body weight, BW; high dose, HD: 8 µg/g BW) (A) on CYP1A mRNA levels, and (B) C_t values of 18S in croaker liver. Each value represents the mean±SEM (N= 7-8). Results were analyzed by ANOVA followed by Fisher's PLSD test for multiple comparisons. Different letters indicate significant differences in the Fisher's PLSD test (P<0.05) for multiple comparisons. Asterisk indicates a significant difference by Student's *t*-test (P<0.05) for unpaired means. CTL, control.

FIGURE 2. Interactive effects of hypoxia and PCB 77 on EROD activity and CYP1A protein expression in croaker liver. Effect of 4 weeks' hypoxia (HYP, 1.7 mg DO/L) and PCB 77 (PCB) (low dose, LD: 2 µg/g body weight, BW; high dose, HD: 8 µg/g BW) exposure on EROD activity (A), CYP1A protein expression from individual fish (C) and relative CYP1A protein levels (D) in croaker liver. Western blot of croaker liver microsomal fraction labeled with CYP1A and actin antibodies showing prominent band of the predicted size ~60 and ~45 kilodalton (kDa), respectively (B). Each value represents the mean±SEM (N=8-10). Results were analyzed by ANOVA followed by Fisher's PLSD test for multiple comparisons. Different letters indicate significant differences in the Fisher's PLSD test (P<0.05). Asterisks indicate a significant difference by Student's *t*-test (P<0.05) for unpaired means. CTL, control.

FIGURE 3. Interactive effects of hypoxia and Aroclor 1254 on CYP1A mRNA levels and protein expression in croaker liver. Effect of 4 weeks' hypoxia (HYP, 1.7 mg DO/L) and Aroclor 1254 (ARO) (low dose, LD: 0.5 μg/g body weight, BW; high dose, HD: 1 μg/g BW) on CYP1A mRNA (A), C_t values of 18S (B), and CYP1A protein expression (C) and immunoreactive intensity (D) in croaker liver. Representative micrographs of CYP1A protein in liver sections from fish after the various treatments (C). Scale bar= 250 μm. Each value represents the mean±SEM (N= 10-18). Results were analyzed by ANOVA followed by Fisher's PLSD test for multiple comparisons. Different letters indicate significant differences (P<0.05) in the Fisher's PLSD test. Asterisk indicates a significant difference by Student's t-test (P<0.05) for unpaired means. CTL, control.

FIGURE 4. Interactive effects of hypoxia and PCB 77 on eNOS protein expression, PC contents and IL-1β mRNA levels in croaker liver. (A) Western blot of croaker liver cytosolic fraction labeled with eNOS and actin antibodies showing prominent band of the predicted size ~140 and ~45 kilodalton (kDa), respectively. (B,C) Effect of 4 weeks' hypoxia (HYP, 1.7 mg DO/L) and PCB 77 (PCB) (low dose, LD: 2 μg/g body weight, BW; high dose, HD: 8 μg/g BW) exposure on eNOS protein expression (B) and levels (C) in croaker liver. Representative Western blot shown from individual fish. (D,E) Effect of 4 weeks' hypoxia (HYP, 1.7 mg DO/L) and Aroclor 1254 (ARO) (low dose, LD: 0.5 μg/g BW; high dose, HD: 1 μg/g BW) on eNOS protein expression (D) and immunoreactive (IR) intensity (E) in croaker liver tissues. Representative micrographs of eNOS protein in liver sections from fish after the various treatments (D). Scale bar= 250 μm. Each value represents the mean±SEM (N= 8 for eNOS protein levels; and N=25-35

for eNOS immunoreactive intensity). Results were analyzed by ANOVA followed by Fisher's PLSD test for multiple comparisons. Different letters indicate significant differences in the Fisher's PLSD test (P<0.05). Asterisks indicates significant differences by Student's t-test (P<0.05) for unpaired means. CTL, control. PM, protein marker.

FIGURE 5. Interactive effects of hypoxia and PCB 77 on PC contents and IL-1β mRNA levels in croaker liver. Effect of 4 weeks' hypoxia (HYP, 1.7 mg DO/L) and PCB 77 (PCB) (low dose, LD: 2 μg/g body weight, BW; high dose, HD: 8 μg/g BW) exposure on PC contents (A) and IL-1β mRNA levels (B) in croaker liver. Each value represents the mean±SEM (N= 8 for PC contents and mRNA levels). Results were analyzed by ANOVA followed by Fisher's PLSD test for multiple comparisons. Different letters indicate significant differences in the Fisher's PLSD test (P<0.05). Asterisk indicates significant difference by Student's t-test (P<0.05) for unpaired means. CTL, control.

Figure 1

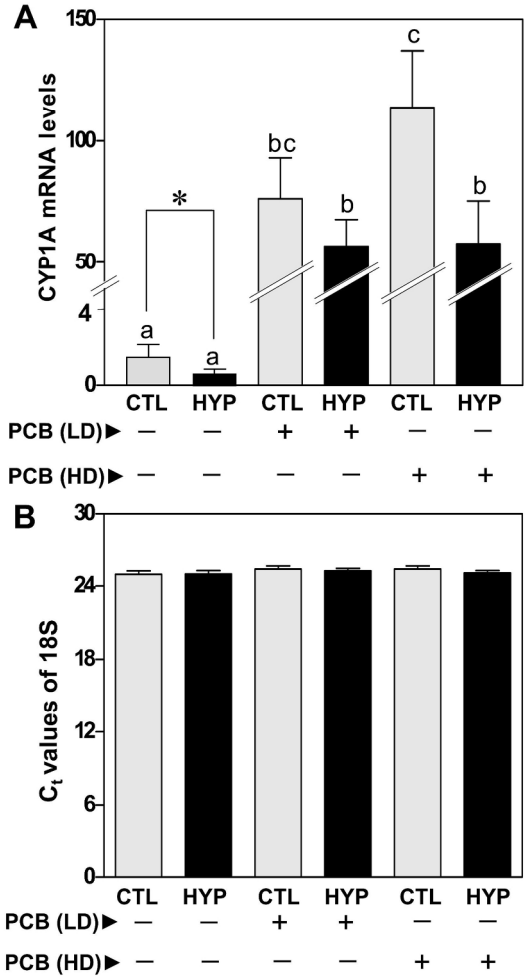

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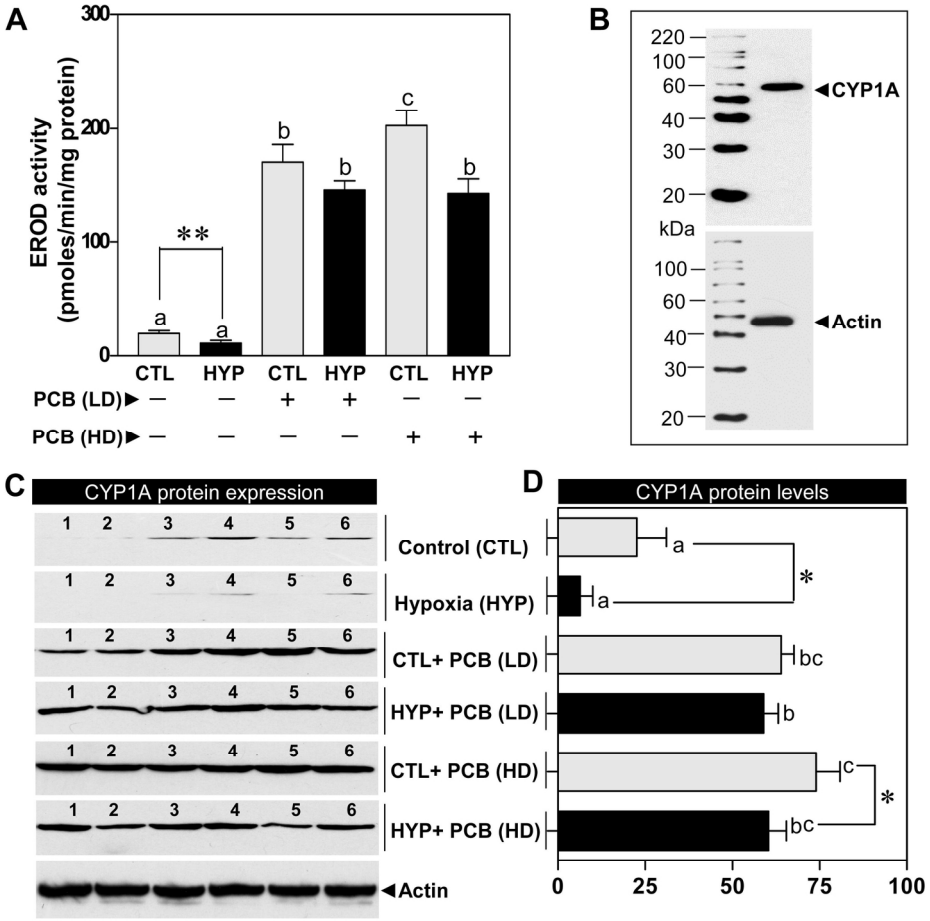
Figure 2



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163x193mm (300 x 300 DPI)

Figure 3

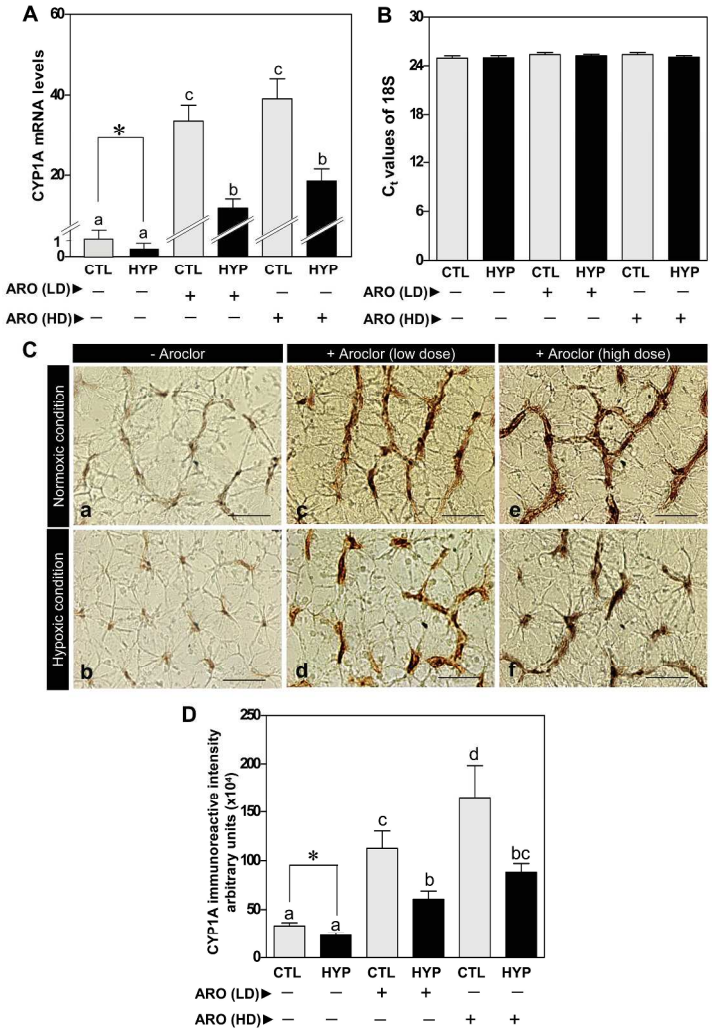

FIGURE 3. Interactive effects of hypoxia and Aroclor 1254 on CYP1A mRNA levels and protein expression in croaker liver. Effect of 4 weeks' hypoxia (HYP, 1.7 mg DO/L) and Aroclor 1254 (ARO) (low dose, LD: 0.5 µg/g body weight, BW; high dose, HD: 1 µg/g BW) on CYP1A mRNA (A), Ct values of 18S (B), and CYP1A protein expression (C) and immunoreactive intensity (D) in croaker liver. Representative micrographs of CYP1A protein in liver sections from fish after the various treatments (C). Scale bar= 250 µm. Each value represents the mean±SEM (N= 10-18). Different letters indicate significant differences (Fisher's PLSD test, $P<0.05$). Asterisk indicates significant difference (Student's t-test, *$P<0.05$). CTL, control.

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Figure 4

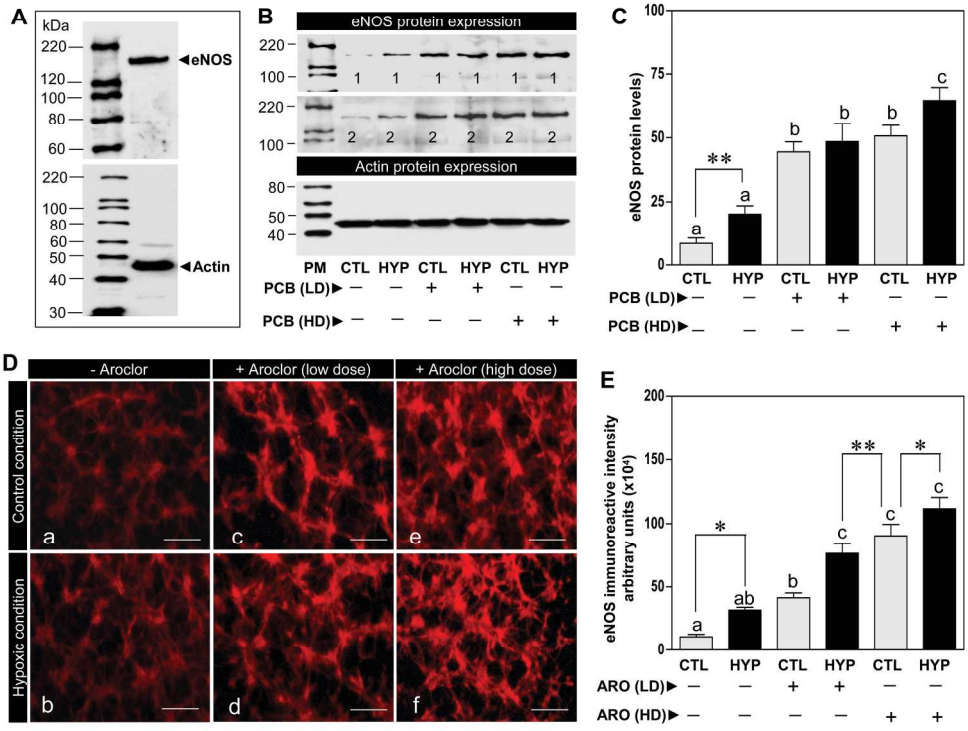

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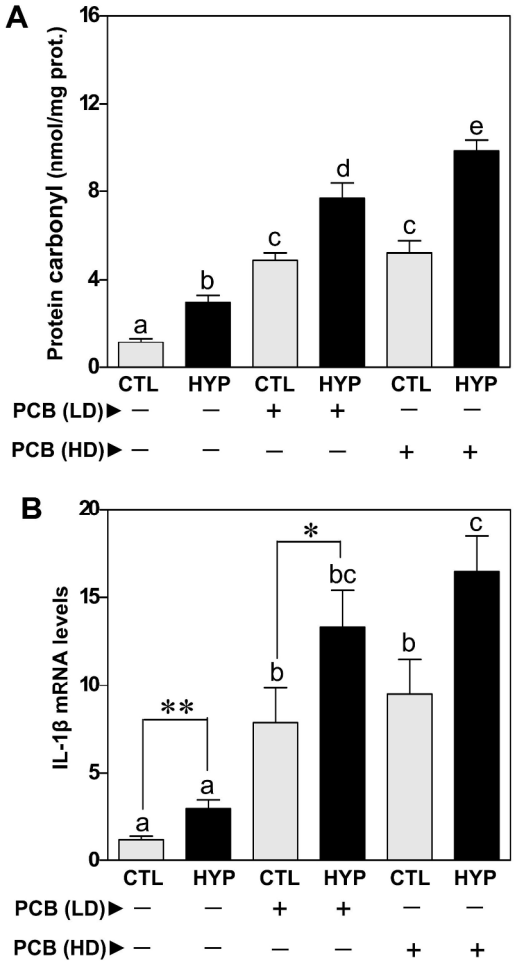
Figure 5



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162x339mm (300 x 300 DPI)