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Molecular cloning and characterization of two ARNT (ARNT-1 and ARNT-2) genes in Atlantic croaker and their expression during co-exposure to hypoxia and PCB77

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Abstract

Aryl hydrocarbon receptor nuclear translocator (ARNT) is an important transcription factor that binds/co-activates drug-metabolizing genes in vertebrates. In this study, we report the cloning and characterization of two ARNT (ARNT-1 and ARNT-2) genes and their mRNA and protein expression in liver tissues of Atlantic croaker after co-exposure to hypoxia and 3,3′,4,4′-tetrachlorobiphenyl (PCB77). The full-length croaker ARNT-1 and ARNT-2 genes encode proteins of 537 and 530 amino acids, respectively, and are highly homologous to ARNT-1 and ARNT-2 genes of other vertebrates. ARNT mRNAs are ubiquitously expressed in all tissues. Hypoxia (dissolved oxygen: 1.7 mg/L) exposure (1-4 weeks) did not affect hepatic ARNTs mRNA levels. Dietary PCB77 treatment (2 and 8 µg/g body weight/day for 4 weeks) caused marked increases in ARNTs mRNA and protein levels in normoxic fish. However, co-exposure to hypoxia and PCB77 for 4 weeks significantly blunted the increase in ARNTs mRNA and protein levels in response to PCB77 exposure. These results suggest that ARNT activity and functions induced by PCB aryl hydrocarbon receptor (AHR) agonist exposure could be compromised in croaker inhabiting hypoxic coastal regions.

KEYWORDS: Atlantic croaker, hypoxia, PCB77, ARNT

1 INTRODUCTION

Aryl hydrocarbon receptor nuclear translocator (ARNT, also referred to hypoxia-inducible factor-ß, HIF-ß) is an important transcription factor that belongs to the basic helix-loop-helix (bHLH)-Per-ARNT-Sim (PAS) superfamily protein.[1,2] The bHLH-PAS superfamily also contains another important transcription factor, HIF-α.[3,4] The regulation of HIF-α and HIF-ß (ARNT) transcripts differs during exposure to environmental stresses such as hypoxia and pollutants.[5-7] The HIF-α transcript is regulated in an oxygen-dependent manner.[4,8,9] In normoxic conditions, the HIF-α proteins are rapidly degraded through hydroxylation of proline residues in the oxygen-dependent degradation domain by prolyl hydroxylase (PHD) enzyme.[4] In hypoxic conditions, on the other hand, the HIF-α proteins are stabilized due to inhibition of the PHD enzyme, and consequently are able to form a dimer with the ARNT protein.[9]

The roles of ARNT are not limited to binding with the HIF-α protein. ARNT also dimerizes with other bHLH-PAS superfamily members including the xenobiotic ligand-

activated transcription factor, aryl hydrocarbon receptor (AHR), that activates transcription of several genes upon association with xenobiotics such as polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).[10-13] After dimerization, the ARNT/AHR binds with specific DNA elements in the promoter sequences of target genes which alters their rates of transcription.[12,13] Since ARNT serves as a common binding partner with HIF-α and AHR transcripts, it is likely that there is a competition between HIF-α and AHR-dependent gene expression during exposure to hypoxia and environmental pollutants.

In normoxic conditions, ARNT is expressed constitutively in vertebrate tissues.[14-16] However, there are conflicting reports on the effects of hypoxia exposure on ARNT expression. Several *in vitro* studies in mammals have shown that hypoxia exposure markedly increases ARNT mRNA and protein expression in human Hep3B, prostate cancer, melanoma and HL-1 cells, and also in murine L929 and Hepa1 cells.[17-21] In addition, hypoxia leads to increased ARNT mRNA levels in rat brain, lung and kidney tissues.[22] In contrast, Catron et al. (2001)[23] demonstrated that hypoxia down-regulates ARNT mRNA expression in the hearts of chick embryos. Moreover, Chilov et al. (1999)[24] and Huang et al. (1996)[25] demonstrated that hypoxia exposure did not affect ARNT protein expression in human HeLa, Hep3B and LN229 cells *in vitro*. Aquatic organisms are often exposed to hypoxia in their natural environment. However, the

regulation and functions of ARNT during exposure to hypoxia stress remain poorly understood in teleost fishes.

In addition to hypoxia exposure, environmental pollutants such as PCBs and TCDD also affect ARNT expression in vertebrates. Mammalian *in vitro* studies have shown that treatment with TCDD significantly increases ARNT and AHR mRNA expression in human embryonic palates and embryonic mice in nonhypoxic conditions.[26,27] Recently, Vorrink et al. (2014)[28] demonstrated that hypoxia exposure significantly reduces AHR functions in human HepG2 and HaCat cells after treatment with PCB126 (a dioxin-like PCB), and that hypoxia exposure negatively affects ARNT functions. These results suggest that exposure to hypoxia attenuates the xenobiotic-induced increase in ARNT expression in mammalian tissues.

Currently, there is no available information on the combined effects of exposure to hypoxia and PCB AHR agonists on ARNT expression in teleost fishes. The aim of the present study was three-fold: (i) to clone and characterize two ARNT (ARNT-1 and ARNT-2) genes in Atlantic croaker liver, a vital organ of xenobiotic metabolism[29]; (ii) to examine ARNTs mRNA expression in response to different periods of hypoxia exposure; and (iii) to examine the ARNTs mRNA and protein expressions in response co-exposure to hypoxia and a PCB AHR agonist, PCB77.

2 MATERIALS AND METHODS

2.1 Reagents

3,3′,4,4′-tetrachlorobiphenyl (PCB77) and rapid amplification of cDNA ends (RACE) amplification kits were purchased from Chem Service (West Chester, PA, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Biochemical and molecular biology grade reagents were purchased from Promega (Madison, WI, USA), Sigma-Aldrich (St. Louis, MO, USA), Argent Chemical (Redmond, WA, USA) and Agilent Technologies (La Jolla, CA, USA). All other biochemical and analytical grade chemicals were obtained from Thermo Fisher Scientific (Waltham, MA, USA) unless noted otherwise.

2.2 Experimental animals

Atlantic croaker (young-of-the-year old, 10-11 cm total length, 12-18 g body weight, BW), were purchased in the summer from local commercial fishermen, transported to laboratory at the University of Texas Marine Science Institute, and treated with Paracide-F (Argent Chemical) for 1 h to remove external parasites. Fish were then housed in indoor seawater tanks (4,727 L capacity including biofilter and recirculating seawater systems, salinity 30-32 ppt) under ambient photoperiod (13D:11L) and room temperature (22 ± 1^{o}C). All fish were fed chopped frozen shrimp (3% BW) once a day and allowed to recover for at least 1 month prior to hypoxia and PCB77 exposure experiments.

2.3 Experiment 1: Effects of short- and long-term hypoxia exposure

After acclimation, fish (mixed-sex) were housed in six experimental tanks (2,025 L capacity including biofilter and recirculating seawater systems, 30 fish/tank) under controlled laboratory conditions. Three tanks were assigned to hypoxic conditions (dissolved oxygen, DO: 1.7 mg/L) and the other three tanks were maintained under normoxic conditions (DO: 6.5 mg/L). Details of the hypoxia exposure experimental set-up were described previously by Rahman and Thomas (2009)[30]. Briefly, the DO levels in the hypoxia exposure experimental tanks were reduced by lowering and adjusting the air flow gradually from 100-90 to 80, 60, 40, and 20% through the control aeration system (supplementary Figure 1). A YSI handheld multi-parameter instrument (YSI Incorporated, Yellow Springs, OH, USA) was used to measure DO, water temperature and pH during the experimental periods. Fish in each tank were fed chopped frozen shrimp at the rate of 3% BW/day and were sampled after continuous hypoxia (target DO: 1.7 mg/L for 1, 2 and 4 weeks) exposure. No mortality was observed in any of the hypoxia exposure experiments. Fish were deeply anesthetized using quinaldine sulphate (20 mg/L), patted dry, weighed and the spinal cord served following the guidelines and ethical rules approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin (IACUC protocol #08080401). The shortest possible anesthetic exposure time (approximately 1 min) was chosen to limit the potential degradation of tissue samples. Liver tissues were rapidly collected, snap frozen in liquid

nitrogen and stored at -80^{o}C until RNA extraction and measurement of ARNTs mRNA expression.

2.4 Experiment 2: Interactive effects of hypoxia and PCB77 co-exposure

Six tanks (2,025 L capacity including biofilter and recirculating seawater systems) were maintained under hypoxic conditions (DO: 1.7 mg/L) and the other six tanks were held at normoxic conditions (DO: 6.5 mg/L). The fish in each tank (30 mixed-sex fish/tank) were fed the same amount of chopped frozen shrimp (3% BW) once a day. Details of the hypoxia and PCB77 co-exposure experimental set-up were described previously by Rahman and Thomas (2018).[31] Briefly, two tanks of fish at each DO level (two tanks for normoxia and two tanks for hypoxia exposure) were fed a low dose of PCB77 (low dose: 2 µg/g BW/day), another four tanks (two for normoxia and two for hypoxia) were fed a high dose of PCB77 (high dose: 8 µg/g BW/day), and the remaining four tanks (two for normoxia and two for hypoxia) of fish were given control food (no PCB77 added in chopped frozen shrimp). The concentrations of PCB77 and experimental period used in this study were based on environmental levels of PCBs and previous publications in croaker[31-33], bluefish[34] and rare minnow.[35] Briefly, PCB77 was dissolved in 100% ethanol which was then mixed with vegetable oil and the ethanol subsequently evaporated at 40^{o}C. PCB77 in vegetable oil was thoroughly mixed with chopped shrimp and the mixture was then frozen at -20^{o}C. All the frozen chopped food was consumed by the fish

within a few minutes in all groups (normoxic control, hypoxia, control and PCB77, and hypoxia and PCB77 exposure groups). No fish mortality occurred during the experiments in any of the treatment groups and no alterations of swimming behavior were observed in the treatment groups, suggesting a lack of sublethal toxic effects on behavior. After 4 weeks of hypoxia and PCB77 co-exposure, fish were anesthetized using quinaldine sulphate and sacrificed following ethical rules approved by IACUC (protocol #08080401). Liver tissues were rapidly excised and stored at -80^{o}C for later measurement of mRNA expression. Liver tissues were also fixed in ice-cold paraformaldehyde solution and stored at 4^{o}C for immunohistochemical detection of protein expression.

2.5 Reverse transcription-polymerase chain reaction (RT-PCR), cloning and sequencing of ARNT cDNAs

Molecular protocols used for RNA extraction, RT-PCR amplification, DNA purification, ligation, cloning and sequencing were similar to those of Rahman and Thomas (2012).[36] Briefly, RNA was extracted from liver tissues using a single-step RNA isolation chemical, TRI reagent (Sigma-Aldrich), and treated with RNase-free DNase for 30 min to eliminate genomic DNA according to the manufacturer's protocol (Promega). The purity and integrity of total RNA were determined by NanoDrop (Thermo Scientific) at 260/280 nm and electrophoresis on ethidium-bromide stained agarose gel. One µg of total RNA was reverse-transcribed using Superscript III First-strand cDNA synthesis kit according

to the manufacturer's protocol (Invitrogen). Partial cDNAs of ARNTs were obtained by PCR amplification using GoTag Green MasterMix (Promega). Degenerate primers were designed to span highly conserved regions of known sequences of ARNT-1 and ARNT-2 genes in teleost fishes for amplification of partial cDNAs of croaker ARNTs (Table 1). The amplified PCR products were separated on an ethidium-bromide stained gel (1% agarose) in TBE (Tris/Borate/Ethylenediaminetetraacetic acid, EDTA) buffer, purified, ligated into pGEM-T easy vector and transformed into JM109 competent cells (Promega). The resulting plasmid DNA was purified, checked for integrity by NanoDrop and agarose gel electrophoresis, and sequenced by Sanger sequencing (Institute for Cellular and Molecular Biology Core Research DNA Sequencing Facility, University of Texas at Austin, USA) in both directions using T7 and Sp6 primers. The full-length sequences of croaker ARNT-1 and ARNT-2 cDNAs were obtained with 5′- and 3′-RACE amplification kits (Invitrogen) using croaker ARNTs gene-specific primers (Table 1).

The full-length sequences of croaker ARNT-1 and ARNT-2 genes were verified using the NCBI database (https://www.ncbi.nlm.nih.gov/) and submitted to GenBank. Sequences were aligned using the multiple sequence alignments with hierarchical clustering program, CLUSTAL W, according to Corpet (1988).[37] The multiple sequence alignments were adjusted and formatted manually to the regions corresponding to different functional domains of croaker ARNT cDNAs.

2.6 Phylogenetic analyses of ARNT genes

Phylogenetic analyses of ARNT genes were carried out using the neighbor-joining method according to Saitou and Nei (1987).[38] The consensus trees of ARNT genes were constructed according to Tamura et al. (2007) using the MEGA4 software package (http://megasoftware.net).[39] To assess the degree of groupings on the tree, a heuristics search was used with bootstrap analysis of 1,000 replicates. Only complete full-length ARNTs amino acid sequences were used for the phylogenetic analyses. The species names and GenBank accession numbers for the amino acid sequences of ARNT-1 and ARNT-2 genes are listed in Figure 2.

2.7 Tissue distribution of ARNTs

The RT-PCR analyses were performed to detect the expression pattern of ARNT-1 and ARNT-2 mRNAs in different tissues using gene-specific primers of croaker ARNT cDNAs (Table 1). For the RT reaction, 1 μg of RNA was transcribed into cDNA using SuperScript III First-Strand System and oligo(dT) primer according to manufacturer's protocol (Invitrogen). The cDNAs from different tissues were used for PCR reactions. The quality of each PCR reaction was examined with gene-specific primers for croaker 18S rRNA (forward primer: 5'-AGAAACGGCTACCACATCCA-3' and reversed primer: 5'-TCCCGAGATCCAACTACGAG-3'; GenBank accession no. AY866435).

Negative reaction (no template control, NTC) was also performed to confirm that there was no contamination in the PCR reaction.

2.8 Real-time quantitative RT-PCR (qRT-PCR) analysis

Total RNA was extracted from liver tissues using TRI reagent (Sigma) and digested with RNase-free DNase (Promega) for 1 hour to remove genomic DNA from RNA samples. qRT-PCR analysis was performed according to Rahman and Thomas (2012).[36] Briefly, the relative expression of croaker ARNT-1 and ARNT-2 mRNAs in liver tissues was determined using total RNA, 1 step of Brilliant SYBR green qRT-PCR master Mix (Agilent Technologies) and a set of gene-specific primers of croaker HIF-1α (forward primer: 5'-AGACCGAGGATGTGAAACCA-3' and reversed primer: 5'-GCCCGAGTGAACAGTTTGAT-3'; GenBank accession no. DQ363931) and ARNTs cDNAs (Table 1). Primer3 software package[40] and NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) were used to evaluate the specificity of croaker HIF-1α, ARNT-1 and ARNT-2 primers for qRT-PCR analyses. The qRT-PCR amplification was assayed in a 25-μl reaction mixture containing 12.5 μl 2× SYBR green-qRT-PCR master mix, 50 nM of gene specific primers, 0.063 μl StrataScript RT/RNase enzyme, and 250 ng of RNA by Eppendorf MasterCycler RealPlex System (Thermo Fisher Scientific). The qRT-PCR cycling profiles were set up as follows: initial cDNA synthesis at 50°C for 30 min and

denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s, and melting curve cycling profile at 95°C for 1 min, 50°C for 30 s, and 95°C for 30 s. The melting curve was analyzed to determine the specificity of amplification. Each transcript level was normalized on the basis of the amplification of croaker 18S rRNA. In a previous study, the cycle threshold (C*t*) values for croaker liver 18S rRNA were unaltered after exposure to hypoxia and PCB treatments, indicating it is a reliable control for normalizing the results (supplementary Figure 2; Rahman and Thomas, 2018)[31]. Beta-actin is another reliable housekeeping gene for these studies because C*t* values in croaker livers are not altered by hypoxia exposure (supplementary Figure 3). The relative expression of ARNTs and HIF-1α mRNA levels were calculated using the $2^{-\Delta\Delta C}$T equation according to Livak and Schmittgen (2001).[41]

2.9 Immunohistochemical analyses

The single immunohistochemical analyses were performed to detect the expression pattern of ARNT-1 and ARNT-2 proteins in croaker liver tissues according to Rahman and Thomas (2012).[36] Briefly, liver samples were fixed in 4% buffered paraformaldehyde (pH 7.4) overnight at 4^{o}C, dehydrated in a series of ethanol, embedded in paraffin and sectioned at 7 µm. Sections were then deparaffinized with xylene, rehydrated with

ethanol, and rinsed three times (10 min each) in phosphate-buffered saline (PBS, pH 7.4). Nonspecific binding was prevented by blocking with 1% bovine serum albumin (Fisher Scientific) for 1 h at room temperature. Sections were then rinsed in PBS and incubated with rabbit polyclonal ARNT-1 (NB100-110, Novus Biologicals, Littleton, CO, USA) or ARNT-2 (SC-5581, Santa Cruz Biotechnology, CA, USA) primary antibodies at a dilution of 1:100 overnight at 4^{o}C. These antibodies were generated against peptide sequences in the non-consensus regions of these ARNTs and have been shown to be specific for ARNT-1 and ARNT-2 in other vertebrates[42]. The negative control slides were incubated with PBS instead of ARNT-1 or ARNT-2 primary antibodies. The fluorescence signals of ARNT-1 and ARNT-2 were amplified by adding Alexa Fluor 488 goat anti-rabbit (dilution 1:100, Invitrogen) and Texas Red goat anti-rabbit (dilution 1:100, Invitrogen) secondary antibodies, respectively. The sections were rinsed three times in PBS, dehydrated in a series of increasing percent ethanol solutions (50, 75, 95, and 100%), immersed in xylene and mounted with Cytoseal XYL (Thermo Fisher Scientific). The image was captured by Cool-SNAP camera (Photometrics, Tucson, AZ) and the immunostaining intensity of ARNTs protein expressions were estimated using ImageJ software.[43]

The double-immunostaining analyses were performed to detect ARNT-1 and ARNT-2 proteins which employs two primary antibodies raised according to Kroeber et al. (1998)[44], and Rahman and Thomas (2015)[45]. The fluorescent-labeled of ARNT-1 and

ARNT-2 protein signals were examined using a Nikon Eclipse microscope (Nikon Eclipse E600, Nikon, Japan) with green and red filters, respectively, and signals were captured by Cool-SNAP camera (Photometrics).

2.10 Histological procedures

Ovary and liver tissues were fixed in paraformaldehyde, dehydrated in series of increasing percent of ethanol solutions (50, 75, 90, and 100%), and embedded in paraffin. The paraffin-embedded tissues were sectioned at 7μm on a rotary microtome (Leica, Buffalo Grove, IL. Sections were then deparaffinized with xylene, rehydrated with a series of decreasing percent of ethanol solutions (100, 90, 75, and 50%), and stained with hematoxylin and eosin solutions using standard histological techniques according to Rahman et al. (2000).[46] The stained sections were examined under a microscope (Nikon Eclipse E600) and the histological pictures were taken by a Cool-SNAP camera (Photometrics).

2.11 Statistical analyses

The differences between experimental groups of their relative mRNA levels and protein expression were analyzed using Student's *t*-test for unpaired comparisons or one-way ANOVA with Fisher's Protected Least Significant Difference (PLSD) test for multiple comparisons. Values of $P<0.05$ were considered statistically significant for Student's *t*-

and Fisher PLSD-test. All statistical analyses were performed using Statview (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism (GraphPad, San Diego, CA, USA) statistical software packages on Apple Mac and PC compatible computers, respectively, and all graphs were also generated with GraphPad Prism software.

3 RESULTS

3.1 Molecular characterization of croaker ARNT-1 and ARNT-2 cDNAs

The full-length croaker ARNT-1 cDNA consists of 1843 bp nucleotides which contains a 5′-untranslated region (5′-UTR) of 62 bp, an open reading frame (ORF) of 1611 bp, and a 3′-untranslated region (3′-UTR) of 170 bp including a polyA signal sequence (GenBank accession no. DQ376248). Conceptual translation of croaker ARNT-1 cDNA predicts a protein of 537 amino acids, composed of bHLH, PAS-A and PAS-B domains (Figure 1A,B). The deduced amino acid sequence of croaker ARNT-1 shows high identity with those of the rainbow trout (74.2%), zebrafish (73.5%), mouse (64.6%), rat (63.7%) (supplementary Figure 4A), and also with that of chimpanzee (63.6%), cormorant (63.6%) and human (36.8%) ARNT-1s. Croaker ARNT-1 and zebrafish, rainbow trout and other vertebrate ARNT-1 proteins show relatively high homologies in the bHLH, PAS-A and PAS-B domains (supplementary Figure 4A).

The full-length 1737 bp nucleotide of croaker ARNT-2 cDNA consists of a 5′-UTR of 89 bp, an ORF of 1590 bp encoding a polypeptide of 530 amino acid residues

composed of bHLH, PAS-A and PAS-B domains (Fig. 1A,B), and a 3′-UTR of 58 bp (GenBank accession no. DQ376248). Comparison of the amino acid sequences of vertebrate ARNT-2 cDNAs reveals that the croaker ARNT-2 exhibits 93, 93.4, 92 and 85.4% sequence identity with killifish, grass carp, zebrafish, and human ARNT-2 proteins, respectively (supplementary Figure 4B). The bHLH, PAS-A and PAS-B domains show extensive sequence similarity in teleost fishes (supplementary Figure 4B).

3.2 Phylogenetic tree of the deduced amino acid sequences of ARNTs

A phylogenetic tree was constructed from deduced amino acid sequences of ARNTs to determine the evolutionary relationships of croaker ARNT-1 and ARNT-2 cDNAs to those of other vertebrate ARNT-1 and ARNT-2 cDNAs. The results show that croaker ARNT-1 and ARNT-2 phylogenies have high bootstrap values and are more closely related to the teleost ARNT-1 and ARNT-2 clades than to those of tetrapod ARNT clades (Figure 2).

3.3 Tissue-specific expression of croaker ARNTs mRNA and protein

The tissue distribution patterns of croaker ARNT-1 and ARNT-2 transcripts were examined by RT-PCR. The ARNT-1 transcript showed relatively high mRNA expression in all tissues (brain, eye, gill, heart, intestine, kidney, liver, muscle, testis and ovary) (Figure 3A-a). The ARNT-2 transcript showed a similar expression pattern with

relatively high mRNA expression in all of the tissues examined (Figure 3A-b). The same amounts of the cDNA templates in the PCR reactions for the different tissues were assayed and confirmed with amplification of croaker 18S rRNA (Figure 3A-c). No amplification of PCR products was detected in the RT negative reactions (Figure 3A-a, -b, -c).

Immunohistochemical analysis using the ARNT-1 and ARNT-2 antibodies showed specific immunorective signals in croaker ovary and liver tissues (Figure 3B). The ARNT-1 and ARNT-2 proteins were highly expressed in the nuclei of oocytes (ARNT-1: Figure 3B-a, ARNT-2: Figure 3B-b) and liver hepatocytes (ARNT-1: Figure 3B-e, ARNT-2: Figure 3B-f). Immunohistochemical results showed that ARNT proteins were co-expressed in the nucleus of oocytes (Figure 3B-c) and hepatocytes (Figure 3B-g) as shown in the merged images. No immunoreactive signals were detected in the negative reactions with ovary (Figure 3B-d) and liver tissues (Figure 3B-h).

3.4 Effect of short- and long-term hypoxia exposure on ARNTs mRNA levels in croaker livers

The mRNA levels of ARNT-1 and ARNT-2 in croaker liver tissues were quantified by real-time qRT-PCR analysis. The effects of short-term (1.7 mg DO/L for 1- and 2-weeks) and long-term (1.7 mg DO/L for 4 weeks) hypoxia exposure on ARNT-1 and ARNT-2 mRNA levels in croaker liver tissues were investigated in controlled laboratory

experiments. There were no significant changes in the relative mRNA levels of both ARNT-1 and ARNT-2 in croaker livers in the hypoxic group compared to those in normoxic controls during the 4-week experimental period (Figure 4A-B).

3.5 Effects of hypoxia and PCB77 co-exposure on ARNTs and HIF-1α mRNA levels in croaker livers

Exposure to hypoxia alone (1.7 mg DO/L for 4-week) markedly increased hepatic HIF-1α mRNA levels (Figure 5A), whereas hypoxia exposure alone had no effect on ARNT-1 and ANRT-2 mRNA levels in croaker liver tissues (Figure 5B,C). Exposure to PCB77 (low dose: 2 μg/g body weight/day; high dose: 8 μg/g body weight/day; for 4 weeks) caused a marked elevations (~5.5-fold) of hepatic ARNT-1 and ARNT-2 mRNA levels compared to no treatment normoxic controls (Figure 5B,C), whereas PCB77 exposure had no effect on hepatic HIF-1α mRNA levels in normoxic fish (Figure 5A). Co-exposure to hypoxia and PCB77 significantly reduced both ARNT-1 and ARNT-2 mRNA levels around 50% compared to those in response to PCB77-treatment alone (Figure 5B,C), whereas co-exposure to hypoxia and PCB77 had no effect on hepatic HIF-1α mRNA levels in croaker (Figure 5A).

3.6 Effects of hypoxia and PCB77 co-exposure on ARNTs protein levels in croaker livers

Hypoxia exposure had no effect on ARNT-1 and ANRT-2 protein levels in croaker liver tissues (Figure 6A,B,D,E). Exposure to PCB77 (both low and high doses) caused a marked increases in hepatic ARNT-1 (Figure 6A,D) and ARNT-2 (Figure 6B,E) protein levels compared normoxic controls (no PCB77 added in food). Co-exposure to hypoxia and PCB77 significantly reduced both ARNT-1 and ARNT-2 protein levels compared to those in response to PCB77-treatment alone (Figure 6A-D). Exposure to PCB77 alone and/or combined exposure to hypoxia and PCB77 caused hypertrophy of some hepatocytes a common response to xenobiotic chemical exposure, and also caused condensation of the nuclei in a small proportion of the hepatocytes, probably indicating apoptosis in these cells (Figure 6C-c-f).

4 DISCUSSION

4.1 Sequence and structure of ARNT cDNAs

Aryl hydrocarbon receptor nuclear translocator (ARNT) is a member of the bHLH-PAS protein superfamily and consists of multiple subunits.[1] Each subunit contains three heterogenic domains, bHLH, PAS-A and PAS-B.[2] The bHLH, PAS-A and PAS-B domains are involved in diverse physiological functions including transcriptional co-activation in response to xenobiotics and hypoxia.[47] Therefore, ARNT is important in vertebrates for adaptation to these environmental stressors. Currently, two ARNT cDNAs have been characterized in a number of fishes: zebrafish (partial ARNT and full-length

ARNT-2),[48,49] rainbow trout (full-length ARNTa and ARNTb),[50] killifish (full-length ARNT-2),[16] sea bream (partial ARNT).[51] In this study, the full-length ARNT-1 and ARNT-2 cDNAs cloned from croaker have presumed bHLH, PAS-A and PAS-B domains which display a high degree of sequence conservation with other teleosts and tetrapods ARNT cDNAs. These results suggest that the two ARNT cDNAs cloned from croaker are members of the bHLH-PAS superfamily. Furthermore, phylogenetic analysis reveals that the deduced amino acid sequences of croaker ARNT-1 and ARNT-2 share more than 90% similarity with other teleost ARNT-1 and ARNT-2 proteins, indicating that they are highly conserved in teleost fishes.

4.2 Tissue expression pattern of ARNTs

The expression patterns of ARNT-1 and ARNT-2 mRNAs and protein were determined by RT-PCR and immunohistochemical analyses in different tissues of croaker maintained under normoxic conditions and not treated with PCB77. The results reveal that ARNT-1 and ARNT-2 mRNAs are constitutively expressed in all the tissues examined in croaker. Similar to findings in rainbow trout, ARNTa and ARNTb mRNAs expressions were consistent in all tissues, but the ARNTa mRNA appeared to have much lower expression than ARNTb.[16] In killifish, ARNT-2 mRNA is constitutively expressed in all peripheral tissues.[52] In human, ARNT-1 mRNA is highly expressed in all organs.[53] In addition, ARNT-1 and ARNT-2 mRNAs are expressed in most organs in rat.[54] Furthermore,

ARNT protein is highly expressed in the tubules and interstitial cells (nuclei of cells) of the human testis.[55] In the present study, we found that ARNT-1 and ARNT-2 proteins are strongly expressed in the nuclei of croaker oocytes and hepatocytes, which is consistent with its role as a dimerization partner for other proteins such as HIF-1α and AhR in this subcellular compartment. Collectively, these results indicate that ARNTs regulate the physiological functions of a wide variety of vertebrate tissues.

4.3 Effect of short- and long-term hypoxia exposure on ARNTs expression

Environmental stressors such as hypoxia (15% O_2 for 9-day) have been shown to reduce ARNT mRNA levels in avian cardiac tissues *in vivo*.[23] On the other hand, hypoxia (1% O_2 for 2-hr) markedly increases ARNT mRNA and protein expression in human Hep3B cells *in vitro*.[18] In the present study, we have shown that short- (1-2-week) and long-term (4-week) hypoxia (20% O_2-saturation) exposure had no effect of ARNT-1 and ARNT-2 mRNA levels in croaker livers. Similarly, hypoxia (21% O_2 for 5-60-hr) had no effect of ARNT protein expression in human HeLa and Hep3B cells *in vitro*.[24,25] Taken together, these results suggest that the differences in ARNT mRNAs and protein expression responses to hypoxia may be related to differences in cell/tissue type and hypoxia-exposure conditions.

4.4 Effects of hypoxia and PCB co-exposure on ARNTs expression

An important finding of this study is that chronic exposure to hypoxia causes marked attenuation of the PCB77-induced increases in ARNT-1 and ARNT-2 mRNA levels, but not HIF-1α mRNA levels in croaker livers. Recently, Vorrink et al. (2014) demonstrated that hypoxia drastically reduces PCB126-induced ARNT activity in human HepG2 and HaCat cells *in vitro*.[56] In addition, Fleming et al. (2009) showed that hypoxia significantly inhibits PCB126-induced AHR activity in topminnow hepatocarcinoma cells *in vitro* in an ARNT-dependent manner.[57] The toxicological significance of hypoxia-induced decreases in ARNT expression and AHR-dependent transcription requires further investigation in teleost fishes which are often exposed to hypoxic conditions. Future *in vivo* studies should also aim to describe the potential pathway of AHR-ARNT regulation during co-exposure to hypoxia and xenobiotics in teleost fishes.

5 CONCLUSION

We cloned and characterized two ARNT cDNAs, ARNT-1 and ARNT-2, from Atlantic croaker, a relatively hypoxia-tolerant marine teleost that inhabits coastal regions along the US Atlantic coast and also in the northern Gulf of Mexico that are often hypoxic during summer months.[58,59] We have shown that hypoxia did not affect ARNTs mRNA and protein levels in croaker liver. However, PCB77 caused marked increases in hepatic ARNTs mRNA and protein levels in normoxic fish. Co-exposure to hypoxia and PCB77 significantly reduced the increase in ARNT mRNA and protein levels in response to

PCB77 exposure. This is the first information on the combined effects of hypoxia and PCB77 exposure on ARNTs expression in the teleost liver, an important organ of energy storage and xenobiotic metabolism.[60,61]

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest with the contents of this manuscript.

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FIGURE LEGENDS

FIGURE 1 Schematic of ARNT-1 and ARNT-2 proteins showing conserved domains: basic helix-loop-helix (bHLH), Per-Arnt-Sim (PAS)-A and PAS-B in Atlantic croaker (A). (B) Alignment of the full-length amino acid sequences of Atlantic croaker (ac) ARNT-1 and ARNT-2. Dashes indicate gaps introduced to facilitate alignment. The alignment was generated using the MultAlign (http://multalin.toulouse.inra.fr/multalin/) according to Corpet (1988)[37]. The bHLH, PAS-A and PAS-B domains are underlined. The gray blocks represent the residues conserved in croaker ARNTs. GenBank accession numbers of acARNT-1: DQ376248 and acARNT-2: DQ376249.

FIGURE 2 Molecular phylogeny of ARNT proteins. GenBank accession numbers for ARNT: dog (XM_856155), seal (AB201467), rat (NM_012780), chicken (AF348088),

fowl (XM_423387), human (AY430083), cattle (NM_173993), rabbit (D45239), xenopus (AY036894); ARNT-1: chimpanzee (XP_003817335), mouse (NM_001037737), rat (U61184), cormorant (AB264539), human (NM_001178), rainbow trout (U73840); ARNT-2: rainbow trout (U73841), zebrafish (NM_001011712), grass carp (AY596922), killifish (AF079311), cormorant (AB264540), human (NM_014862), mouse (NM_007488), rat (NM_012781). GenBank accession numbers for Atlantic croaker ARNT-1 and ARNT-2 proteins in Figure 1.

FIGURE 3 ARNT-1 and ARNT-2 mRNA (A) and protein (B) expression in croaker tissues. (A) One µg of total RNA from each tissue was used for RT-PCR reaction in thirty-five cycles to detect ARNT-1, ARNT-2, and 18S mRNAs in croaker tissues. Negative control (no template control, NTC) was run to ensure that there was no cross contamination in PCR reaction. Due to gel size limitation, 25 µl of PCR products from different tissues were run at room temperature under the same conditions on two separate ethidium-bromide stained gels. BR, brain; EY, eye; GI, gill; HE, heart; IN, intestine; KI, kidney; LI, liver; MU, muscle; OV, ovary; SP, spleen; TE, testis; M, DNA marker. (B) Immunohistochemical co-expression of ARNT-1 and ARNT-2 proteins in croaker ovary (B-a: ARNT-1, B-b: ARNT-2 and B-c: co-expression of ARNTs) and liver tissues (B-e: ARNT-1, B-f: ARNT-2 and B-g: co-expression of ARNTs). Sections were incubated with the primary and secondary antibodies showing the presence of immunoreactive

signals (arrow indicates expression of ARNT-1, ARNT-2, and their co-expression in the nucleus of oocyte [B-a-c] and hepatocytes [B-e-g]), and incubated with only secondary antibody showing the absence of signal in ovary (B-d) and liver tissue (B-h). Arrows indicate nuclei.

FIGURE 4 Effects of 1, 2 and 4 weeks of hypoxia (HYP, dissolved oxygen, DO: 1.7 mg/L) exposure on ARNT-1 and ARNT-2 mRNA (A,B) levels in croaker liver tissues determined by real-time qRT-PCR using croaker 18S rRNA as an internal control and calculated as relative values of mRNA levels per 250 ng of total RNA. Results of ARNTs mRNA levels from both male and female sexes were combined because they were not significantly different. Note: here and in subsequent figures 5,6; exposure duration only refers to period that fish were exposed to target DO: 1.7 mg/L; fish were previously exposed to declining DO for additional 2-day adjustment period. Each bar represents mean ± standard error of the mean (N=8). CTL: normoxic control.

FIGURE 5 Interactive effects of hypoxia and PCB77 on HIF-1α and ARNT mRNAs expression in croaker liver tissues. Effect of 4 weeks exposure to hypoxia (HYP, dissolved oxygen, DO: 1.7 mg/L) and PCB77 (PCB, LD: low dose, 2 µg/g body weight/day; HD: high dose, 8 µg/g body weight/day) on HIF-1α (A), ARNT-1 (B) and ARNT-2 (C) mRNA levels in croaker liver. Results of HIF-1α and ARNT mRNA levels from both male and female sexes were combined because they were no significantly

different. Each value represents the mean ± standard error of the mean (N=7-8). 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.

FIGURE 6 Immunohistochemical analysis of interactive effects of hypoxia and PCB77 on ARNT-1 (A, D) and ARNT-2 (B, E) protein expression and levels in croaker liver tissues. Effect of 4 weeks exposure to hypoxia (HYP, dissolved oxygen, DO: 1.7 mg/L) and PCB77 (PCB, LD: low dose, 2 µg/g body weight/day; HD: high dose, 8 µg/g body weight/day) on immunohistochemical expression and protein levels of ARNT-1 (A-a-f, D) and ARNT-2 (C-a-f, E), and histological observation (C-a-f) in croaker liver tissues. Arrows indicate enlarged hepatocytes with condensed nuclei in liver sections. Representative micrographs of ARNTs protein in liver sections from fish after the various treatments are shown (A,B). Each value (D,E) represents the mean ± standard error of the mean (N=20-30 for ARNTs immunoreactive intensity). Different letters indicate significant differences (Fisher's PLSD test, $p<0.05$). Asterisk indicates significant difference (Student's *t*-test, $**p<0.01$). CTL, control. Scale bar = 20 µm.