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Interactions between hypoxia and sewage-derived contaminants on gene expression in fish embryos

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ABSTRACT

Fish embryos were used to evaluate the interaction among common environmental and chemical stressors found in urban coastal environments, namely hypoxia, aryl hydrocarbon receptor (AhR) agonists, and estrogenic compounds. At the molecular level, the systems responding to these stressors share common response factors, and evidence exists for cross-talk between them. Biomarkers of exposure to these stressors, cytochrome P4501a (*Cyp1a*), estrogen receptor alpha (*ERα*), brain cytochrome P450 aromatase (*Cyp19a2* or *AromB*), and hypoxia inducible factor 1 alpha (*Hif-1α*) mRNA expression were examined using qRT-PCR simultaneously in embryos of two well studied species, the Atlantic killifish, *Fundulus heteroclitus*, and the zebrafish *Danio rerio*. Embryos of both species were exposed to the model *Cyp1a* inducer β-naphthoflavone (BNF) or 17-β estradiol (E2) under either normoxic or hypoxic (5% oxygen atmosphere) conditions and harvested prior to hatch at 9 days post fertilization (dpf) for the killifish, and 48 h post fertilization (hpf) for the zebrafish. BNF significantly induced *Cyp1a* expression in embryos of both species with killifish embryos being more responsive (700-fold > control) than zebrafish embryos (7-100-fold > control). *AromB* was also significantly influenced by treatment, but to a lesser extent, with mean expression levels increased by less than two-fold over control values in response to E2, and in one case upregulated by BNF. *ERα* and *Hif-1α* were constitutively expressed in embryos of both species, but expression was unaffected by exposure to either BNF or E2. Hypoxic conditions downregulated *AromB* expression strongly in killifish but not in zebrafish embryos. The impact of hypoxia on expression of other genes in either species was inconsistent, although an interactive effect between hypoxia and BNF on several of the genes evaluated was observed. These data are the first to examine expression patterns of these important environmental response genes together in embryos of two important model fish species. The results support the use of *Cyp1a* expression as a biomarker of AhR agonists in fish embryos, and indicate that *AromB* may be more responsive than *ERα* to estrogenic chemicals at this stage in development. *Hif-1α* expression was not found to be a good biomarker of hypoxic exposure in either killifish or zebrafish embryos. The interaction observed between BNF and co-exposure to hypoxia warrants further investigation. Finally killifish embryos are generally more sensitive than zebrafish embryos at this stage of development supporting their use in environmental assessments.

1. Introduction

Aquatic organisms in urban estuaries are exposed to myriad stressors, many of which have significant sources from sewage effluent and/or urban run-off. Probably the most well studied of these are polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), natural and synthetic estrogens, and estrogenic mimics such as the nonylphenol ethoxylate metabolites. Exposure to these contaminants has been shown to impair the health of resident organisms, and negatively affect reproduction and embryonic development (Barron et al., 2004; Cheek, 2006; Soares et al., 2008). In addition to exposure to chemical contaminants, aquatic species in coastal environmental are often exposed to seasonal or persistent hypoxia. Hypoxia often results from excess nutrient additions associated with sewage inputs, has been increasing in coastal waters world-wide (Diaz, 2001), and is well known to have negative effects on a variety of biological endpoints in aquatic organisms (USEPA, 2000). Mechanistic evaluation of the systems by which organisms respond to chemical stressors or hypoxia has demonstrated they share some common molecular targets and that substances that affect one

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system sometimes have affects on others. This so-called cross-talk has the potential to enhance or ameliorate toxicity associated with stressors acting alone, and confounds interpretation of molecular biomarkers of exposure and responses which are employed in environmental assessments.

Cytochrome P4501a (*Cyp1a*) is one of the mostly widely used biomarkers of exposure, responding to a wide range of organic contaminants known to act via the aryl hydrocarbon receptor (AhR). Upon binding to the AhR, the substrate receptor complex enters the nucleus, where dimerization with the aryl hydrocarbon nuclear translocator (Arnt) is required before it can bind to the xenobiotic response element on DNA and initiate transcription (Hahn, 1998). Halogenated aromatic hydrocarbons such as 2,3,7,8-dibenzodioxin (TCDD) and PCBs, and PAHs are known AhR agonists. The AhR transcription pathway is linked to the hypoxia sensing pathway through Arnt, which is also known as the hypoxia inducible factor 1β (*Hif-1β*). Hypoxia inducible transcription factor 1 (Hif-1) plays a central role in short-term response to oxygen status. The alpha subunit of Hif-1 is an oxygen sensor. Although genes for both factors are constitutively expressed, under normoxic conditions Hif-1α is degraded rapidly. Under hypoxic conditions it dimerizes with Hif-1β (Arnt) prior to binding to the hypoxia response element on DNA and initiating transcription of a variety of enzymes associated with blood supply, such as erythropoietin (EPO), and vascular endothelial growth factor (VEGF), and energy metabolism such as lactate dehydrogenase (LDH) (Gu et al., 2000). Reciprocal cross-talk between the AhR and Hif systems has been demonstrated, although the results of these studies are not always in agreement. Interference between AhR agonists and hypoxia has been demonstrated in a number of systems, including fish, where hypoxic conditions decreased the response of some but not all AhR agonists (Prasch et al., 2004; Maston et al., 2008). Conversely the AhR agonist benzo(*a*)pyrene (BaP) has been shown to enhance hypoxic stimulation of VEGF, EPO, and LDH through reactive oxygen species interactions (Yu et al., 2008).

The primary molecular responses involved with exposure to environmental estrogens in oviparous organisms, such as fish, are mediated by the estrogen receptor (ER) where binding leads to induction of more ER, increased production of estrogen and subsequent synthesis of the egg yolk precursor protein vitellogenin (Vtg) (Arcand-Hoy et al., 1998; Jobling et al., 1998). Induction of Vtg is a widely accepted biomarker of exposure to estrogenic compounds (Denslow et al., 1999). Although most environmental estrogens act via the ER, some, including 17β-estradiol (E2), the synthetic estrogen diethylstilbestrol (DES) and nonylphenol (NP) also act via stimulation of Cyp19a, aromatase, which catalyses to conversion of testosterone to E2. The form of aromatase found in the brain, AromB (Cyp19a2), is particularly sensitive to induction by NP (Meucci and Arukwe, 2006). Both the ER and aromatase are expressed early in development. *AromB* expression has also been shown to be inhibited by hypoxia leading to masculinization (Shang et al., 2006). Links also exist between AhR and ER receptor signaling. Several AhR agonists such as TCDD are known antiestrogens, and have been shown to have complex interactions with the ER signaling pathways (Safe et al., 1991, 2000), including direct interference with DNA binding and competition for transcriptional co-activators (Khan and Thomas, 2006). Although most of the data supporting these linkages are from mammalian breast cancer models, the AhR-ER interactions have more recently been documented in zebrafish (Cheshenko et al., 2007). Other studies have demonstrated that AhR agonists can also stimulate ER-dependent responses through a mechanism known as hijacking, where the AhR/Arnt complex binds to the ER (Mortensen and Arukwe, 2008). Xenobiotic response elements have been shown to exist in the promoter region of *Cyp19*, thus providing another mechanistic link between these systems (Kazeto et al., 2004). The ramifications of environmental chemicals and hypoxia interfering with these molecular signaling systems can be significant at the individual and population level, causing teratogenesis, altered sex ratios, pour reproductive condition, and even reproductive impairment (Shang and Wu, 2004; Kuhl et al., 2005; Thomas and Rahman, 2009; Bugel et al., 2010).

The goals of this study were to evaluate the response of AhR, ER, and hypoxia responsive genes to model inducers under normoxic and hypoxic conditions in fish embryos. We chose to evaluate two commonly used model organisms, one marine, the killifish, and one freshwater, the zebrafish, and focused on expression during embryonic development because these genes are known to be expressed at this time, and embryos are known to be a sensitive life stage. The results of this work provide valuable comparative data on the relative response of these two important model fish species to the potentially interactive effects of common stressors, and help guide interpretation of these biomarkers in environmental assessment.

2. Methods

2.1. Brood stock source and embryo collection

Adult Atlantic killifish, *Fundulus heteroclitus* (hereafter referred to as killifish), were collected using minnow traps from tidal creeks associated with the Flax Pond Marine Reserve in Old Field, NY. Ripe adults were held overnight in the laboratory in tanks receiving aerated and charcoal filtered seawater on the day prior to new or full moons. Pooled groups of embryos were created from at least five female and five male fish by strip spawning embryos into a dry glass Petri dish, and water-hardened in 23 ppt artificial seawater (ASW, Instant Ocean, Aquarium Systems, Mentor, OH). Embryos were randomly allocated from the pool to replicate treatments beginning at two dpf at 10 embryos per replicate.

Adult zebrafish, *Danio rerio* (Strain AB) (hereafter referred to as zebrafish) were purchased from the Zebrafish International Resource Center at Oregon State University, (Corvalis, OR) and maintained at 27–28.5 °C in a recirculating system with activated carbon and UV/treatment containing 60 mg/L (0.06 ppt) Instant Ocean. Zebrafish were fed a diet of Zeigler Aquatox flake food (Aquatic Ecosystems, Apopka, FL) and newly hatched *Artemia* brine shrimp nauplii. Male and female zebrafish were kept in separate tanks and mixed breeding pools were combined in a static nuptial tank with a false bottom the night prior to embryo collection. Fertilized developing embryos were randomly assigned to individual replicates at 24 hpf at a density of 10 embryos per treatment. Embryos were maintained in distilled water amended with 60 mg/L Instant Ocean. All fish used in this study were cared for in accordance with Stony Brook University IACUC standards under protocol (2010-1470).

2.2. Exposure conditions

Experiments with killifish embryos began at two dpf, to ensure that all embryos used were developing normally prior to exposure, and continued for seven days. Exposures were conducted in 20 ml scintillation vials each containing 10 embryos and 20 ml of Instant Ocean at 27 ppt amended with either β-naphthoflavone (BNF) or E2 at 1 μg/L, 10 μg/L, and 100 μg/L. All compounds were dosed using DMSO as a carrier with a final treatment concentration of 0.1% which was also added to the DMSO control treatment. Embryo containers were protected from light by a foil hood throughout the experiment to minimize production of photo-oxidation products. All treatments with killifish were carried out in quadruplicate.

Zebrafish embryos were treated in a similar manner except that exposures began at 24 hpf and were concluded 24 h later at 48 hpf. Embryos were held in distilled water augmented with 60 mg/L