Aquatic Toxicology 167 (2015) 157–171

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Regulation of Ahr signaling by Nrf2 during development: Effects of Nrf2a deficiency on PCB126 embryotoxicity in zebrafish (*Danio rerio*)

Michelle E. Rousseau[a], Karilyn E. Sant[a], Linnea R. Borden[a], Diana G. Franks[b], Mark E. Hahn[b], Alicia R. Timme-Laragy[a,b,*]

[a] Department of Environmental Health Sciences, School of Public Health and Health Sciences, University of Massachusetts Amherst, Amherst, MA 01003, United States
[b] Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, United States

ARTICLE INFO

Article history:
Received 8 June 2015
Received in revised form 5 August 2015
Accepted 7 August 2015
Available online 13 August 2015

Keywords:
Transcription factor
Dioxin-like compound
Gene expression
Embryonic development
Swim bladder
Yolk

ABSTRACT

The embryotoxicity of co-planar PCBs is regulated by the aryl hydrocarbon receptor (Ahr), and has been reported to involve oxidative stress. Ahr participates in crosstalk with another transcription factor, Nfe2l2, or Nrf2. Nrf2 binds to antioxidant response elements to regulate the adaptive response to oxidative stress. To explore aspects of the crosstalk between Nrf2 and Ahr and its impact on development, we used zebrafish (*Danio rerio*) with a mutated DNA binding domain in Nrf2a (*nrf2a*$^{fh318/fh318}$), rendering these embryos more sensitive to oxidative stress. Embryos were exposed to 2 nM or 5 nM PCB126 at 24 h post fertilization (prim-5 stage of pharyngula) and examined for gene expression and morphology at 4 days post fertilization (dpf; protruding – mouth stage). Nrf2a mutant eleutheroembryos were more sensitive to PCB126 toxicity at 4 dpf, and in the absence of treatment also displayed some subtle developmental differences from wildtype embryos, including delayed inflation of the swim bladder and smaller yolk sacs. We used qPCR to measure changes in expression of the *nrf* gene family, *keap1a*, *keap1b*, the *ahr* gene family, and known target genes. *cyp1a* induction by PCB126 was enhanced in the Nrf2a mutants (156-fold in wildtypes vs. 228-fold in mutants exposed to 5 nM). Decreased expression of heme oxygenase (decycling) 1 (*hmox1*) in the Nrf2a mutants was accompanied by increased *nrf2b* expression. Target genes of Nrf2a and AhR2, NAD(P)H:quinone oxidoreductase 1 (*nqo1*) and glutathione S-transferase, alpha-like (*gsta1*), showed a 2–5-fold increase in expression in the Nrf2a mutants as compared to wildtype. This study elucidates the interaction between two important transcription factor pathways in the developmental toxicity of co-planar PCBs.

1. Introduction

PCBs are carcinogenic and teratogenic persistent organic pollutants. Along with other dioxin-like-compounds, co-planar PCBs are potent, chlorinated ligands for the aryl hydrocarbon receptor (Ahr), which has been shown to mediate toxicity in numerous mammalian and fish species (e.g. rev. in Bock (2013), Denison et al. (2011), Hahn et al. (2006), King-Heiden et al. (2012)). These ligands for the Ahr are also associated with oxidative stress, such as via uncoupling of the cytochrome P450 catalytic cycle and the release of superoxide and/or hydrogen peroxide (Dalton et al., 2002). The adaptive response to oxidative stress is largely regulated by the redox-sensitive transcription factor nuclear factor erythroid related factor 2-like-2 (Nfe2l2 or Nrf2[1]). Nrf2 regulates

Abbreviations: Ahr, aryl hydrocarbon receptor; Ahrr, aryl hydrocarbon receptor repressor; ARE, antioxidant response element; Cyp1a, cytochrome P540 1a; DLC, dioxin-like compound; DMSO, dimethyl sulfoxide; dpf, days post fertilization; hpf, hours post fertilization; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid related factor 2-like-2; PAH, polycyclic aromatic hydrocarbon; PCB126, 3,3′,4,4′,5-pentachlorobiphenyl; ROS, reactive oxygen species; tBOOH, *tert* butylhydroperoxide; tBHQ, *tert* butylhydroquinone; TCDD, 2,3,7,8-tetracholordibenzo-p-dioxin; XRE, xenobiotic response element, aka dioxin response element.

* Corresponding author at: 686N. Pleasant St., Goessmann 149C, Amherst, MA 01003, United States.
E-mail addresses: merousse@umass.edu (M.E. Rousseau), ksant@umass.edu (K.E. Sant), lborden@umass.edu (L.R. Borden), dfranks@whoi.edu (D.G. Franks), mhahn@whoi.edu (M.E. Hahn), aliciat@umass.edu (A.R. Timme-Laragy).

[1] Nomenclature: *nrf* is a commonly used notation for NF-E2-related factor genes, which are officially designated as *nfe2l* (NF-E2-like). For example, *nrf2a* is officially designated as *nfe2l2a*. Throughout the paper, we use the more common *nrf* designation. Otherwise, we utilize the approved format for designating genes and proteins (see the ZFIN Zebrafish Nomenclature Web site). Human genes and proteins are designated using all capitals (*NRF2* and NRF2, respectively), rodent genes and proteins as *Nrf2* and NRF2 respectively, whereas zebrafish genes are designated *nrf2* and

http://dx.doi.org/10.1016/j.aquatox.2015.08.002
0166-445X/

transcription of numerous antioxidant and cytoprotective genes, and also participates in crosstalk with the Ahr to regulate transcription of genes, many of which encode Phase I and Phase II enzymes (Wakabayashi et al., 2010).

The Ahr is a member of the basic helix-loop-helix Per-Arnt-Sim (bHLH/PAS) family of transcription factors. Briefly, it is a constitutively expressed, cytosolic protein. In the canonical Ahr activation pathway, Ahr translocates to the nucleus upon ligand binding, where it forms a heterodimer with the aryl hydrocarbon nuclear translocator (Arnt). This heterodimer binds to the xenobiotic response element (XRE) in promoter regions and initiates the transcription of a variety of genes. These genes are mostly comprised of Phase I and Phase II enzymes which are often referred to as the "classical Ahr gene battery," but also include target genes involved in numerous other functions including liver development, cell cycle progression, cellular differentiation, inflammation, and cancer (Bock, 2013; Denison et al., 2011).

Zebrafish have three Ahr genes (*ahr*1*a*, *ahr*1*b*, *ahr*2), whereas most mammals have only one (*Ahr*). Ahr2 has been shown to mediate the effects of 3,3′,4,4′,5-pentachlorobiphenyl (PCB126), 2,3,7,8-tetracholordibenzo-p-dioxin (TCDD; dioxin), and some high molecular-weight PAHs; the roles of Ahr1a and Ahr1b are not yet fully understood (Andreasen et al., 2002; Garner et al., 2013; Goodale et al., 2012; Jonsson et al., 2007; Karchner et al., 2005; Prasch et al., 2003). PCB126 exposure in zebrafish embryos has been shown to induce expression of *cyp*1*a*, *cyp*1*b*1, *cyp*1*c*1, and *cyp*1*c*2 in an Ahr2-mediated manner (Billiard et al., 2006; Jonsson et al., 2007). *cyp*1*a* has been shown to be the most responsive of the *cyp*1 genes, and either expression of this gene or enzyme activity (ethoxyresorufin O-deethylase; EROD) is often used as a biomarker of Ahr ligand activation. Numerous studies of zebrafish embryos exposed to PCB126 or TCDD during development have demonstrated a suite of morphological deformities collectively referred to as "blue sac disease," which includes pericardial edema, craniofacial and heart malformations, and reduced swim bladder inflation; these deformities have been shown to be primarily regulated by Ahr2 (Garner et al., 2013; Jonsson et al., 2007, 2012; Lanham et al., 2014; Prasch et al., 2003), although the importance of other signaling pathways has also been demonstrated, including interactions with Wnt, Cox-2, Sox9 (Yoshioka et al., 2011).

In addition to activating Ahr2, exposure to co-planar PCBs and dioxins has also resulted in the generation of reactive oxygen species (ROS) and/or oxidative stress in mammals, frogs, birds, and fish, via several mechanisms including generation of reactive metabolites, an increase in mitochondrial respiration and hydrogen peroxide, uncoupling of the cytochrome P450 catalytic cycle, glutathione depletion, and inflammation (Dalton et al., 2002; Gillardin et al., 2009; Lu et al., 2011; Nebert et al., 2000; Schlezinger et al., 2000, 2006; Senft et al., 2002). Oxidative stress is defined as a loss of redox signaling and control (Jones, 2006), and the oxidative stress response is defined as the resulting changes in gene expression that serve to mitigate the oxidative challenge (Hahn et al., 2014). Despite the many studies that demonstrate an oxidative stress response to these co-planar PCBs and dioxins, there have also been studies that have failed to identify an oxidative stress response. In zebrafish for example, while *nrf*2*a* has been shown to be up-regulated by TCDD (Hahn et al., 2014), zebrafish embryos sampled immediately after a 6 h exposure to TCDD showed no evidence of altered expression of other genes typically found in the oxidative stress response at 4 dpf, and there was an increase in *gstp*1 expression only after 48 h of a TCDD exposure that began at 24 h post fertilization (hpf) (Hahn et al., 2014). An oxidative stress response was not observed in response to TCDD in at least two other studies (Alexeyenko et al., 2010; Wang et al., 2013). This may be due to many factors including differences in timing, dose, developmental stage, species sensitivity to Ahr ligand binding, or exposure duration, or it is possible that ROS generation may not always be sufficient to alter redox signaling or activate an oxidative stress response.

Nrf2 has been designated the "master regulator" of the adaptive response to oxidative stress (Ohtsuji et al., 2008). Nrf2 is a basic-region leucine zipper transcription factor that is constitutively and ubiquitously expressed and bound to Kelch-like ECH-associated protein 1 (Keap1) within the cytosol, targeting it for ubiquitination (Cullinan et al., 2004). There are several ways in which Nrf2 can be activated, such as protein kinase RNA-like endoplasmic reticulum kinase (PERK) signaling from the endoplasmic reticulum, phosphorylation, and electrophilic or ROS interactions (reviewed in Bryan et al. (2013), Niture et al. (2014)). After activation, Nrf2 accumulates in the nucleus where it dimerizes with small v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (Maf) proteins to upregulate transcription of numerous cytoprotective genes (Ma et al., 2004). Nrf2 mediates gene expression through the antioxidant response element (ARE). AREs are frequently located in promoter regions of genes involved in the oxidative stress response and Phase II detoxification, but also have more pleiotropic roles in functions such as cell cycle regulation and lipid metabolism (Malhotra et al., 2010). Disruption of NRF2 in mice has been linked to an increase in pulmonary inflammation, emphysema, neurodegeneration, and chemical carcinogenesis, thus indicating the importance of Nrf2's cytoprotective role (Kensler et al., 2007). NRF2 knockout mice have also been shown to have defects in liver growth and size (Beyer et al., 2008), reduced bile duct microbranching (Skoko et al., 2014), and retinal vasculature density (Wei et al., 2013); in some genetic backgrounds, NRF2−/− mice have a congenital defect resulting in an intrahepatic shunt (Skoko et al., 2014).

Zebrafish have partitioned the function of Nrf2 between two genes, *nrf*2*a* and *nrf*2*b* (reviewed in Hahn et al. (2015)). We have previously shown that Nrf2a is generally involved in activating gene expression, while Nrf2b is involved in repressing gene expression (Timme-Laragy et al., 2012). The *nrf*2*a*^{fh318} mutant is a recessive loss-of-function allele generated via the zebrafish Targeted Induced Local Lesions in Genomes (TILLING) mutagenesis project (Mukaigasa et al., 2012). The phenotype of *nrf*2*a*^{fh318} fish is similar to that of NRF2−/− mice (e.g. (Itoh et al., 1997)) in that they display normal development and reproduction and enhanced sensitivity to oxidants (Mukaigasa et al., 2012). *nrf*2*a*^{fh318} larvae are more sensitive to oxidative stress, but no morphologic or growth differences between Nrf2a mutant vs. wildtype larvae or adults have been identified (Mukaigasa et al., 2012).

Crosstalk between Ahr and Nrf2 has been demonstrated in mammalian systems and has been shown to be important in the transcriptional regulation of genes encoding many Phase I and Phase II detoxification enzymes (Anwar-Mohamed et al., 2011; Lo and Matthews, 2013; Miao et al., 2005; Tijet et al., 2006; Wang et al., 2013; Yeager et al., 2009). TCDD has been shown to increase both protein level and nuclear accumulation of NRF2 in mice (Yeager et al., 2009) and in Hepa1c1c7 cells (Wang et al., 2013), without evidence of oxidative stress; rather, Wang et al. provided evidence suggesting the AHR upregulated transcription of NRF2 and formed protein complexes with both NRF2 and KEAP1 that contributed to the stability of NRF2 and subsequent ARE-activation (Wang et al., 2013). However, the interactions of these pathways have not been thoroughly studied during embryonic development, which is a critical time for chemical sensitivity that is fundamentally different than adult physiology.

Zebrafish contain multiple paralogs of both Ahr and Nrf2, each of which demonstrates a seemingly different function. Here, we tested the hypothesis that Nrf2a plays a protective role in PCB126

Nrf2 for genes and proteins, respectively. When not referring to a specific species, we have used the zebrafish notation as a default format.