Toxicology and Applied Pharmacology 271 (2013) 266–275

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Comparative developmental toxicity of environmentally relevant oxygenated PAHs

Andrea L. Knecht [a], Britton C. Goodale [a], Lisa Truong [a], Michael T. Simonich [a], Annika J. Swanson [a], Melissa M. Matzke [b], Kim A. Anderson [a], Katrina M. Waters [b], Robert L. Tanguay [a,*]

[a] Department of Environmental and Molecular Toxicology, the Environmental Health Sciences Center, Oregon State University, Corvallis, OR, USA
[b] Computational Biology & Bioinformatics, Pacific Northwest National Laboratory, Richland, WA, USA

ARTICLE INFO

Article history:
Received 22 October 2012
Revised 3 May 2013
Accepted 4 May 2013
Available online 14 May 2013

Keywords:
Zebrafish
Development
Oxygenated
PAH
Malformation
Morpholino

ABSTRACT

Oxygenated polycyclic aromatic hydrocarbons (OPAHs) are byproducts of combustion and photo-oxidation of parent PAHs. OPAHs are widely present in the environment and pose an unknown hazard to human health. The developing zebrafish was used to evaluate a structurally diverse set of 38 OPAHs for malformation induction, gene expression changes and mitochondrial function. Zebrafish embryos were exposed from 6 to 120 h post fertilization (hpf) to a dilution series of 38 different OPAHs and evaluated for 22 developmental endpoints. AHR activation was determined via CYP1A immunohistochemistry. Phenanthrenequinone (9,10-PHEQ), 1,9-benz-10-anthrone (BEZO), xanthone (XAN), benz(a)anthracene-7,12-dione (7,12-B[a]AQ), and 9,10-anthraquinone (9,10-ANTQ) were evaluated for transcriptional responses at 48 hpf, prior to the onset of malformations. qRT-PCR was conducted for a number of oxidative stress genes, including the glutathione transferase(*gst*), glutathione peroxidase(*gpx*), and superoxide dismutase(*sod*) families. Bioenergetics was assayed to measure *in vivo* oxidative stress and mitochondrial function in 26 hpf embryos exposed to OPAHs. Hierarchical clustering of the structure-activity outcomes indicated that the most toxic of the OPAHs contained adjacent diones on 6-carbon moieties or terminal, para-diones on multi-ring structures. 5-carbon moieties with adjacent diones were among the least toxic OPAHs while the toxicity of multi-ring structures with more centralized para-diones varied considerably. 9,10-PHEQ, BEZO, 7,12-B[a]AQ, and XAN exposures increased expression of several oxidative stress related genes and decreased oxygen consumption rate (OCR), a measurement of mitochondrial respiration. Comprehensive *in vivo* characterization of 38 structurally diverse OPAHs indicated differential AHR dependency and a prominent role for oxidative stress in the toxicity mechanisms.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants in urban air, dust and in the soil of most industrial coal gassification, coal burning, coke production and wood preservation sites (Howsam and Jones, 1998). It is widely recognized that PAHs pose risks to human health, having been associated with increased risks of systemic inflammation (Delfino et al., 2010), cardiopulmonary mortality (Lewtas, 2007; Lee et al., 2011) and lung cancer mortality (Hoshuyama et al., 2006; Grant, 2009). The potential risks may be especially acute for the developing fetus and infant where PAH exposures have been linked to low birth weight, intrauterine growth retardation, *in-utero* mortality and lower intelligence (Perera et al., 1998; Dejmek et al., 1999; Perera et al., 1999; Dejmek et al., 2000; Perera et al., 2006; Perera et al., 2009; Wu et al., 2010). Despite the more than two decades of intensive study devoted to parent PAHs, they are only part of the hazard spectrum from PAH contamination.

Oxygenated PAHs (OPAHs) are transformation products of PAHs, toxic to humans and the environment and, until recently, a largely neglected class of contaminants at PAH contaminated sites (Lundstedt et al., 2007). OPAHs are ketone and quinone substituted PAHs deriving from the same sources of incomplete combustion and showing relatively high environmental mobility and persistence (Zielinska et al., 2004; Lundstedt et al., 2007; Simoneit et al., 2007; Medeiros and Simoneit, 2008; Layshock et al., 2010; Shen et al., 2011a; Shen et al., 2011b). It has been anticipated, though not yet clearly shown, that OPAH contamination may actually increase at sites remediated by methods that promote PAH degradation (Lundstedt et al., 2007) making them a potentially greater health hazard than the parent contamination. OPAHs are also secondarily produced through photo-oxidation reactions of

* Corresponding author at: Department of Environmental and Molecular Toxicology, Oregon State University, 28645 East HWY 34. Corvallis, OR 97333, USA. Fax: +1 541 737 0497.
E-mail addresses: andrea.knecht@tanguaylab.com (A.L. Knecht), goodaleb@onid.orst.edu (B.C. Goodale), lisa.truong.888@gmail.com (L. Truong), mtsimonich@oregonstate.edu (M.T. Simonich), swansoan@onid.orst.edu (A.J. Swanson), melissa.matzke@pnl.gov (M.M. Matzke), kim.anderson@oregonstate.edu (K.A. Anderson), katrina.waters@pnl.gov (K.M. Waters), robert.tanguay@oregonstate.edu (R.L. Tanguay).

0041-008X/$ – see front matter. Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.taap.2013.05.006

PAHs with atmospheric oxidants, including ozone and nitrogen oxides (Yu, 2002; Vione et al., 2004; Lundstedt et al., 2007; Wang et al., 2007).

OPAHs are found on diesel soot particles, wood smoke particles and gasoline engine soot (Rogge et al., 1993; Rogge et al., 1997; Mazurek, 2002; Layshock et al., 2010; Ding et al., 2012) and show an overall affinity for fine $PM_{2.5}$ particle-association, raising their hazard potential because of the proclivity of $PM_{2.5}$ to travel deep into the lung (Shen et al., 2011a). Diesel exhaust particles and associated PAH quinones and other oxygenated derivatives are involved in the formation of reactive oxygen species (ROS), which can result in inflammatory responses and are suspected to be a major driver of pulmonary oxidative stress and consequent cardiovascular disease in urban areas (Chung et al., 2006; Nemmar et al., 2011; Channell et al., 2012).

Airborne OPAH concentrations have been highly correlated with reactive oxygen species (ROS) formation, suggesting that oxidative stress is one of the toxicity mechanisms for aerosol-induced human health effects (Sklorz et al., 2007). PAHs and OPAHs in ambient particulate matter samples increased oxygen free radical formation, as measured by electron spin resonance, and some of these OPAHs were directly involved in ROS generation (Sklorz et al., 2007). Oxidative stress was a component of the developmental toxicity induced by the OPAHs α- and β-naphthoflavone in zebrafish (Timme-Laragy et al., 2009).

Some PAHs have demonstrated carcinogenic potential (Okona-Mensah et al., 2005) and contributed to the mutagenic activity of ambient aerosols (Pedersen et al., 2004; Pedersen et al., 2005; Avellaneda et al., 2011; Kim et al., 2011). The OPAH and nitro-PAH fractions of air samples from Beijing, China were shown to be twice as mutagenic as the parent PAH fraction, though no further specification of the fractions was made (Wang et al., 2011). OPAH derivatives have been reported as highly mutagenic compounds *in vitro* in a study of human-cell mutagens in respirable airborne particles from the northeastern United States (Pedersen et al., 2004). Numerous *in vitro* and *in vivo* toxic effects of PAH quinones have been described, but little is known about the developmental effects of OPAH exposure. Inference can be drawn from the known toxicology of napthoquinones, which bind to biomacromolecules; and quinones whose alkylating and redox cycling activities can create a variety of hazardous effects *in vivo*, including generation of ROS, acute cytotoxicity, immunotoxicity and carcinogenesis (Bolton et al., 2000). Characterization of these effects and their developmental outcomes *in vivo* would be a critically important step toward addressing questions about OPAH hazard to human health, especially *in utero* and infancy.

As a developmental model, the zebrafish is especially suited to the rapid and relatively inexpensive screening of large numbers of chemicals for developmental toxicity. Because zebrafish embryos develop externally and remain transparent throughout much of organogenesis, adverse effects of chemical exposure on development of the brain, notochord, heart, jaw, body segmentation and shape are easily observed in the living animal under low magnification. With early zebrafish development well-characterized and the annotated genome sequence readily available, mechanisms of toxicity and the genes involved can be elucidated.

Using the developing zebrafish platform, we undertook a systematic, concentration-response approach to hierarchically rank the toxicity of a library of commercially available OPAHs in their pure form. Rank was based on the severity and incidence of a battery of 22 primarily morphological endpoints. Some, but not all, PAHs induce toxicity in an AHR-dependent manner, and we sought to characterize the activation of AHR by each OPAH with immunohistochemical analysis of the downstream target, CYP1A. Lastly, we examined the contribution of oxidative stress to OPAH toxicity by quantifying the expression of a battery of known redox affected genes, and by the direct measurement of mitochondrial respiration rate in the intact animal. The body of data we present from 38 OPAH structures is the most comprehensive *in vivo* characterization of the developmental toxicity of an important class of environmental pollutants.

2. Methods

2.1. Fish care. Adult zebrafish were maintained with a water temperature of 28° ± 1 °C on a recirculating system with a 14 h light:10 h dark photoperiod at the Sinnhuber Aquatic Research Laboratory. All experiments were conducted with wild type 5D strain (*ahr2*$^{+}$) or AHR2-null (*ahr2*^{hu3335}) zebrafish (Goodale et al., 2012). Adult care and reproductive techniques were conducted according to Institutional Animal Care and Use Committee protocols at Oregon State University. All embryos used in exposures were collected following group spawning of adult zebrafish as described previously (Reimers et al., 2006).

2.2. Chemicals and developmental exposures. Analytical grade standards were obtained from several vendors including: 12-hydroxybenzo(a)pyrene, 10-hydroxybenzo(a)pyrene, and 9-hydroxybenzo(a)pyrene from MRI Chemical Carcinogen Respository; 1,2-dihydroxyanthraquinone, 1-hydroxyanthraquinone, 2,6-dihydroxyanthraquinone, and 2-hydroxyanthraquinone from TCI (Tokyo Chemical Industry Co. LTD.); 4H-cyclopenta(def)phenanthrene-4-one, benzo(c)phenanthrene(1,4) quinone, phenanthrene-1,4-quinone, and 6H-benzo(c-d)pyren-6-one from Chiron; 9-fluorenone, 9,10-anthraquinone (9,10-ANTQ), and 1,9-benz-10-anthrone (BEZO) from Fluka (part of Sigma-Aldrich, St. Louis, MO); 2-hydroxy-9-fluorenone and 1-hydroxy-9-fluorenone from Acros Organics; 1,4-anthraquinone from Alfa Aesar; xanthone (XAN), aceanthrenequinone, 1,3-dihydroxynaphthalene, chromone, 1,7-dihydroxynaphthalene, 1,6-dihydroxynaphthalene, 2,6-dihydroxynaphthalene, 5,12-naphthacenequinone, 2,3-dihydroxynaphthalene, 1,8-dihydroxyanthraquinone, benz(a)anthracene-7,12-dione (7,12-B[a]AQ), perinaphthenone, 1,4-dihydroxyanthraquinone, 9,10-phenanthrenequinone (9,10-PHEQ), 1,4-benzoquinone, 1,4-naphthoquinone, 1,5-dihydroxynaphthalene, benzo(a)fluorenone, 1,2-naphthoquinone, pyrene-4,5-dione, and acenaphthenequinone from Sigma Aldrich. In total, thirty eight different oxygenated polycyclic aromatic hydrocarbons (OPAHs) were obtained (Fig. S1) and dissolved in DMSO to make 50, 10, 2, 0.4, and 0.08 mM stock solutions. For static exposure to zebrafish, solutions were made at a 1:100 dilution in E2 embryo medium with a 1% DMSO final concentration. Embryos were enzymatically dechorionated at 4 h post fertilization (hpf) (Mandrell et al., 2012) and exposed to the OPAH from 6 to 120 hpf in duplicate 96 well plates. One embryo per well was placed into 100 µl of each solution. Serial dilutions of 0.8, 4, 20, 100, and 500 µM or 1% DMSO vehicle control were used for all 38 OPAHs. Zebrafish embryos were evaluated for developmental progress, somite and notochord malformations, and mortality at 24hpf and for total mortality and a suite of morphological endpoints at 120 hpf. Following morphological assessment, embryos were fixed overnight at 4 °C in 4% paraformaldehyde, rinsed in PBS and stored at 4 °C in PBS-NaAzide for IHC analysis.

For RNA samples, dechorionated embryos were exposed from 6 to 48 hpf or 6–120 hpf to a single concentration of selected OPAHs: 1 or 2 µM phenanthrenequinone (9,10-PHEQ), 5 or 10 µM 1,9-benz-10-anthrone (BEZO), 20 µM xanthone (XAN), 5 µM benz(a)anthracene-7,12-dione (7,12-B[a]AQ) and 20 µM 9,10-anthraquinone (9,10-ANTQ). Embryos were rinsed in fishwater and three samples of 24 embryos each were collected on ice in snap-safe Eppendorf tubes with 0.5 mm zirconiumoxide beads. 500 µl of RNAzol was added and samples were homogenized with a Bullet Blender (Next Advance) for 3 min at speed 2, and then placed at −80 °C until RNA isolation.

For the gene expression studies, total RNA was extracted via RNAzol/isopropanol precipitation. RNA was quantified using a SynergyMxmicroplate reader (Biotek) with the Gen5 Take3 module to calculate 260/280 O.D. ratios. Superscript III First-Strand Synthesis (Invitrogen) was performed with 5 µg of RNA and oligo(dT) primers to reverse transcribe cDNA from total RNA.