Environmental Pollution 178 (2013) 72–79

Contents lists available at SciVerse ScienceDirect

Environmental Pollution

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

Developmental effects of aerosols and coal burning particles in zebrafish embryos

Alba Olivares [a], Barend L. van Drooge [a], Marta Casado [a], Eva Prats [b], Montserrat Serra [c], Leo T. van der Ven [d], Jorke H. Kamstra [e], Timo Hamers [e], Sanne Hermsen [e], Joan O. Grimalt [a], Benjamin Piña [a,*]

[a] Institute of Environmental Assessment and Water Research (IDÆA-CSIC), Jordi Girona 18, 08034 Barcelona, Catalonia, Spain
[b] Centre dInvestigació i Desenvolupament (CID-CSIC), Jordi Girona 18, 08034 Barcelona, Spain
[c] Department de Ciències Bàsiques, Facultat de Medicina i Ciències de la Salut Universitat Internacional de Catalunya C/ Josep Trueta, s/n (Hospital General de Catalunya), 08195 Sant Cugat del Vallès, Barcelona, Spain
[d] Laboratory for Health Protection Research, National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands
[e] Institute for Environmental Studies (IVM), VU University Amsterdam, 1081 HV Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 10 September 2012
Received in revised form
15 February 2013
Accepted 21 February 2013

Keywords:
Zebrafish embryos
Quantitative RT PCR
Recombinant Yeast Assay (AhR-RYA)
Dioxin-like pollutants
PAH
Malformation
CYP1A
CALUX

ABSTRACT

Embryo toxicity of particles generated by combustion processes is of special concern for human health. A significant part of these toxic effects is linked to the binding of some pollutants (like polycyclic aromatic hydrocarbons or PAHs) to the Aryl hydrocarbon Receptor (AhR) and the activation of target genes, like the cytochrome P4501A. This activity was analyzed for ambient air and coal-combustion particle extracts in zebrafish embryos (the cyp1aDarT assay) and in two single-cell bioassays: the yeast-based YCM-RYA and the DR-luc (rat cells) assay. Observed AhR ligand activity of samples generally correlated to the predicted toxic effect according to their PAH composition, except for one of the coal combustion samples with an anomalously high activity in the cyp1aDarT assay. This sample induced deformities in zebrafish embryos. We concluded that the combination of morphological and molecular assays may detect embryonic toxic effects that cannot be predicted from chemical analyses or single-cell bioassays.

1. Introduction

Regular exposure to airborne pollution increases the risk of cardiovascular and respiratory diseases and lung cancer (Perez et al., 2009). Mortality rates in urban environments are directly linked to their pollution loads, and improvements in urban air quality result in reduction of mortality by respiratory and cardiovascular illnesses (Clancy et al., 2002; WHO, 2004). Subchronic and chronic exposure to ambient air pollutants has been adversely associated with adverse effects on birth and infant health and development (Brauer et al., 2008; Edwards et al., 2010; Karr et al., 2009).

PAHs contribute substantially to the overall toxicity of ambient air particles, due to their carcinogenic and mutagenic properties (Bostrom et al., 2002; Wenger et al., 2009). They originate from a range of incomplete combustion processes, such as fossil fuel combustion in vehicles, coal combustion or biomass combustion (Schauer et al., 2001, 2002). Air quality standards have been implemented to safeguard human health against the adverse effects of PAH and, specifically, benzo[a]pyrene (EC, 2004; Schoeny and Poirier, 1993).

A fundamental aspect determining PAHs' toxicity is their capacity to bind to the aryl hydrocarbon receptor (AhR), a key regulator of phase I and II metabolic enzymes (Gonzalez and Fernández-Salguero, 1998; Hankinson, 1995; Nebert et al., 1993; Shimizu et al., 2000). The ectopic activation of AhR-controlled genes is linked to the so-called dioxin-like activity, as dioxins are the most powerful known AhR agonists, with affinity constants in the picomolar range (Misaki et al., 2007). Therefore, assays to identify activation and subsequent signal transduction of AhR are useful for pollution load monitoring in environmental samples (Fent, 2003). A variety of aryl hydrocarbon-responsive reporter assays based on mammalian cell lines (Garrison et al., 1996; Hamers et al., 2000; Schoeters et al.,

* Corresponding author.
E-mail address: bpcbmc@cid.csic.es (B. Piña).

0269-7491/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.envpol.2013.02.026

2004) or genetically modified yeast strains (Misaki et al., 2007; Murahashi et al., 2007; Noguerol et al., 2006b; Olivares et al., 2011) have become common tools to detect AhR-binding activities in different samples and matrices. Bioassays do not provide a chemical identification of the AhR ligands but their low cost and easiness of handling makes them a first choice when testing large numbers of samples or compounds.

Zebrafish (*Danio rerio*) is becoming a preferred model for the analysis of sublethal effects of toxicants in vertebrates (Scholz and Mayer, 2008; Stegeman et al., 2010). This species is easy to maintain, it has a short life cycle, and readily produces relatively large quantities of transparent embryos. Its development has been extensively studied and can be observed using a variety of optical methods. Zebrafish embryos are becoming an important tool in toxicology, as the easiness of their handling allows running multiple tests on a routine basis. For example, the acute embryo toxicity test DarT (Nagel, 2002) is becoming increasingly implemented as a general fish toxicity assay, and many efforts are devoted to use zebrafish embryos as model to understand toxic mechanisms at sublethal levels and to predict possible adverse and long-term effects (McGrath and Li, 2008; Scholz et al., 2008). Zebrafish provides a unique opportunity to analyze survival, morphological alterations and specific gene expression in a single assay. Given the similarity of the embryo development in vertebrates, zebrafish embryos are considered an excellent model for the analysis of congenic human diseases and for detecting hazards for the developing foetus (Goldsmith, 2004; Xu and Zon, 2010). In addition, assays performed before day 5 post-fertilization (dpf) comply with the 3R principles of relative replacement of animal tests, which constitute a target for many international regulatory bodies (EC, 2010; Embry et al., 2010).

Cytochrome P4501A is an established marker of exposure to toxicants in many vertebrate species (Goksoyr and Forlin, 1992; McClain et al., 2003; Whyte et al., 2000). The zebrafish corresponding gene, *cyp1a*, is known to increase its expression in zebrafish embryos upon exposure to AhR ligands, which can be monitored by qRT-PCR methods (Voelker et al., 2007). Finally, exposure to dioxins, PAHs and other strong AhR inducers results in cardiac toxicity in zebrafish embryos, a phenotype linked to the binding of these compounds to the AhR (Antkiewicz et al., 2006; Scott et al., 2011).

In the present paper, we evaluate the embryo toxicity associated with ambient air particles and compare their results from those from condensates originated in spontaneous combustion of coal waste. Both type of samples have in common a very high content on PAHs, but differ widely on their chemical profiles. Air particle analyses are based on PM_{10} filter samples from the Po valley, south of the Alps (north Italy). The Po valley has large atmospheric daily and seasonal gradients of PAH concentrations that are related to wood combustion and traffic emissions (Van Drooge and Perez Ballesta, 2009). Condensates of spontaneous coal fires were sampled from two coal mining waste gobs in the Datong district (Shanxi Province, China), both extremely rich in PAHs, but with very different congener compositions (Querol et al., 2011). Results from the different samples were interpreted according their chemical composition and their AhR-ligand activity, assessed by both vertebrate and yeast cell lines. Our goal is to establish a set of bioassays to study airborne particle toxicity associated with organic compounds, and to characterize these toxic effects at molecular, cellular and systemic levels.

2. Materials and methods

2.1. Compound and sample description

2.1.1. Compounds

Benzo[a]pyrene (B[a]Pyr; ≥96%), Benzo[k]fluoranthene (B[k]Flu; 98%) and β-Naphthoflavone (BNF, 90–95%) were purchased from Sigma–Aldrich (St. Louis, MO). 3,3′,4,4′,5-Pentachlorobiphenyl (PCB126) (99.1%) was obtained from Promochem, Wesel, Germany. DMSO (99.5% GC) was obtained from Sigma–Aldrich. MeOH (≥99.9%) was obtained from Carlo-Erba Quimivita (Spain).

2.1.2. Field samples

24-h air particulate matter (PM_{10}) filter samples were collected in 2008 in a semi-rural area from the Po valley, northern Italy. They were obtained on 02–03/10/2008 (A1), 21–22/10/2008 (A2), 17–18/11/2008 (A3) and 27–28/11/2008 (A4)) and provide a gradient of PAH concentration related to biomass combustion (Van Drooge and Perez Ballesta, 2009) as well as a range of dioxin-like activity determined by AhR-RYA (Olivares et al., 2011). Coal tar condensates were sampled from spontaneous combustions in two coal waste gobs in the Datong district (Shanxi Province, China) (Querol et al., 2011). DT2 is an active and highly compacted coal waste gob in which no reclamation measures have been applied (hence it has no soil cover) and the condensates of gaseous emissions give rise to widespread fire hotspots. DT3 is a flat and extensively compacted gob with a thin layer (0.3 m thick) of soil. In this site spontaneous fires concentrate in fractures at the top of slope e.g. close to the front of the dump. The condensates collected in DT2 and DT3 have elevated PAH concentrations but, due to the absence or presence of the soil layer, the distributions of these compounds exhibit important qualitative differences (Querol et al., 2011). Supplementary Table 1 lists the content in parental PAHs of the extracts used in this work, derived from the original data (Querol et al., 2011; Van Drooge and Perez Ballesta, 2009).

2.1.3. Sample treatment

Collection and extraction of samples for chemical and biological analyses were performed as described elsewhere (Olivares et al., 2011; Querol et al., 2008; van Drooge et al., 2010). All samples were extracted with a mixture of dichloromethane and methanol (2:1 v/v) for the assays. Sample extracts were concentrated and resuspended in MeOH (yeast assay) or DMSO (zebrafish embryos and mammalian cell assays.) All concentration are given as nominal; however, we checked that non-volatile PAHs are stable in fish water during the time of the assay (not shown).

2.2. Biological assays

2.2.1. Aryl hydrocarbon receptor system (AhR-RYA)

Strain YCM4 was provided by Dr. Charles A. Miller from the University of New Orleans. This strain is a derivative of W303a (MAT**a**, *ade2-1*, *can1-100*, *his3-11*, *15*, *leu2-3*, *112*, *trp1-1*, *ura3-1*), which harbours a chromosomally integrated construct that co-expresses human aryl hydrocarbon receptor and ARNT genes under the Gal1-10 promoter. The strain also harbours a second construct is the pDRE23-Z reporter, encompassing three XRE5 sequence and the CYC1-LacZ fusion (Miller, 1997). The RYA assay was performed as described (Noguerol et al., 2006a). Yeast cells were grown overnight in minimal medium (6.7 g/L yeast nitrogen base without amino acids, DIFCO, Basel, Switzerland, supplemented with 0.1 g/L of prototrophic markers as required) plus 20 g/L galactose to express both AhR and ARNT. The final culture was adjusted to an optical density (OD) of 0.1 and split into 50 μL aliquots (see below) in 96-well polypropylene microtiter plates (NUNC™, Roskilde, Denmark; ref. 40) previously silylated by overnight exposure to an atmosphere of dimethylsilane. A serial dilution scheme was performed based on 1:3 dilution steps for environmental samples and 1:2 for standard compounds. Plates were incubated for 6 h at 30 °C under mild shaking. After incubation, 50 μL YPER™ (PIERCE™, Rockford, IL, USA) were added to each well and further incubated at 30 °C for 30 min. Afterwards, 50 μL of assay buffer supplemented with 0.1% 2-mercaptoethanol and 0.5% of the 4-methylumbelliferone ß-D-galactopyranoside (MuGal) solution (both from the FluorAce™ beta-galactosidase Reporter Assay Kit, Bio-Rad Laboratories, Hercules, CA, USA) were added to the lysed cells. After brief centrifugation, plates were read in a Synergy 2 spectrofluorometer (BioTek, USA), at 355 nm excitation and 460 nm emission wavelengths. Fluorescence was recorded for 15 min (about one measurement per min); β-galactosidase activity values were calculated as rates of the increment of arbitrary fluorescence units with time, using standard linear regression methods. Samples were tested in triplicate.

2.2.2. DR-luc assay

Rat hepatoma cells (H4IIE) stably transformed with pGudluc1.1 plasmid (Garrison et al., 1996) were provided by Prof Michael Denison (UC Davis). Cells were grown in culture medium (minimal essential medium (a-MEM, Gibco) with 10% heat-inactivated foetal calf serum (FCS, Gibco) and penicillin/streptomycin) at a 37 °C and 5% CO_2. DR-LUC experiments were performed in 96-well cell culture plates (Greiner) in culture medium. H4IIE.Luc cells were seeded in 100 μl growth medium. After 24 h incubation the cell layer was 80–90% confluent, and 100 μl of fresh culture medium containing the test compound in maximal 0.4% DMSO was added to each well. After a further 24 h incubation, medium from each well was discarded and 50 μl lysis buffer (10 mM Tris, 2 mM dithioltreitol (DTT), 2 mM 1,2,-diaminocyclohexane-N,N,N′,N′-tetra-acetic acid, 10% glycerol, 1% Triton-X100, pH 7.8) was added to each well. Plates were shaken at 700 rpm on a plate shaker for 10 min. For luciferase measurement, the microtiter plate was inserted in a Luminometer (Lucy2, Anthos) and 100 μl of glow mix was added. The light output was measured