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Methylated and unsubstituted polycyclic aromatic hydrocarbons in street dust from Vietnam and India: Occurrence, distribution and *in vitro* toxicity evaluation

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ABSTRACT

Methylated polycyclic aromatic hydrocarbons (MePAHs), unsubstituted PAHs and AhR-mediated activities were determined in street dust collected from Vietnam and India using a combined approach of chemical analysis and *in vitro* reporter gene assay. MePAHs and PAHs diagnostic ratios indicated that the main sources of MePAHs in Vietnam were pyrogenic emissions, whereas in India there were mixed sources of pyrogenic and petrogenic emissions. AhR-mediated activities determined by using DR-CALUX assay were observed in urban street dust at mean 40, 29 and 20 ng CALUX-TEQ/g dw for Hanoi, Bangalore and New Delhi, respectively. MePAHs and PAHs contributed only 5% or less to AhR-mediated activity in street dust, indicating the occurrence of unknown AhR agonists. The principal contributors to Theoretical-TEQs among target compounds were methyl benz[a]anthracene, benzo[b]- and benzo[k] fluoranthene. The present study indicates importance of MePAHs in evaluation of toxic risk related to AhR-mediated activity in urban polluted areas.

1. Introduction

Methylated polycyclic aromatic hydrocarbons (MePAHs), one of PAH derivative groups, are widely distributed in the environment as is the case with like unsubstituted PAHs. MePAHs such as methyl naphthalene (MeNap), methyl phenanthrene (MePhe), and methyl chrysen (MeChy) have been identified from industrial, petrogenic or incomplete combustion sources (Dimitriou-Christidis et al., 2003; Wang et al., 1999). Vietnam and India are two of the six countries in Asia reported as having the highest air pollution in the world (Kim Oanh et al., 2006). Well known as a major contributor of air pollution and potential source for the emission of PAHs (Tuyen et al., 2014), the number of motorcycles in Hanoi-Vietnam was reported to be more than 3.9 million in 2011 (Tran et al., 2012), auto vehicles in New Delhi and Bangalore-India was also shown to be high in India with an annual growth of the 10% for car and 9.5% for two-wheelers. The growth rate of the number of motorcycles was even higher than the growth rate of population (3.25%) (Lefèvre, 2009). The population of motorcycle was reported at 4.2 million units in New Delhi in 2004 (Goyal et al., 2010), reached to 6 million units in 2010 (Goyal et al., 2013) and for Bangalore the numbers are 2.2 million in 2005 (Lefèvre, 2009). Motor vehicles have been believed as major contributions of air pollution in urban cities of Asian developing countries (Agarwal, 2009; Kim Oanh et al., 2012; Goyal et al., 2013; Hien et al., 2014).

In the urban environment, street dust is known as a sink for complex mixtures of traffic related pollutants, contains PAHs and it is also believed to contain PAHs derivatives (Tuyen et al., 2014). We found significant levels of PAHs (1500 ng/g dry weight) and AhR-

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mediated activities (20 ng CALUX-TEQs/g dry weight) in the street dust with particle-size of less than 500 μm in a previous study. It is expected that the smaller particles may contain higher levels of vehicle exhaust contaminants as well have more toxic activity compared to larger particles. A previously report also showed that the highest levels of PAHs were in street dust particles less than 63 μm (Zhao et al., 2009). Therefore, the street dust particle-size less than 63 μm is preferred selection for chemicals' determination as well as toxic risk assessment because of their potential effects on human respiratory system (Saeedi et al., 2012) and adherence to human skin (Choate et al., 2006).

MePAHs known as generally more persistent and sometimes more toxic than their unsubstituted analogues (Sauer and P.B., 1991). The toxicities of MePAHs as well as unsubstituted PAHs are often involved in binding to the aryl hydrocarbon receptor (AhR), induction of AhR-related genes and subsequent transformation to toxic metabolites (Behnisch et al., 2001). Methyl benz[a]anthracene (MeBaA) has the ability to induce expression of cytochrome P450 1B1 or aldo-keto reductase 1C9 in rat liver epithelial cells similar to its parent. The AhR has been known to be involved in number of biological processes, such as development and detoxification of exogenous compounds (Denison and Whitlock, 1995) (Hahn et al., 2006). Using an *in vitro* reporter gene assay, DR-CALUX, aryl hydrocarbon receptor (AhR) mediated activities which had been known as genotoxic effects produced by many agonist compounds such as polyhalogenated aromatic hydrocarbons (PHAHs), PAHs or MePAHs have been determined. Some MePAHs/PAHs have been reported as potent stimulators of AhR activation (Trilecová et al., 2011). The model rat hepatoma H4IIE cells stably transfected with luciferase reporter under control of dioxin responsive element (DRE) have also been used to study the effect of MePAHs on toxic events associated with tumor promotion (Machala et al., 2008). Particularly MeBaAs also has ability to induce DNA adduct formation (Marvanová et al., 2008). Methyl anthrancenes (MeAnt) have been used for study of their inhibition effects on Gap Junctional Intercellular Communication (GJIC) in rat liver epithelial cells, in which MeAnt showed a potent role in tumor promotion and their biological effect on GJIC were depend on the position of methylated group (Upham et al., 1996). The MePAHs that have structural features with bay-, baylike region could be a potent inhibitor of GJIC such as MeBaAs (exception of 10-MeBaA), and 1-methyfluorene (MeFlu), then resulting in disruption of cell proliferation control, subsequently contributing to tumor promotion (Weis et al., 1998; Marvanová et al., 2008). Dimethylated PAHs were found to be the inducers of increased blue sac disease in Japanese medaka and also to affect its embryonic development (Rhodes et al., 2005).

So far, many studies have been carried out on the characterization of MePAHs as well as PAHs in the atmospheric environment (Fang et al., 2004; Lehndorff and Schwark, 2009; Lee and Dong, 2010; Di Filippo et al., 2010; Lee and Dong, 2011; Ha et al., 2012; Khairy and Lohmann, 2012, 2013), but there is no study on the evaluation of AhR-related toxicity for methylated PAHs in street dust and on identification of toxically relevant compounds. Integrated risk assessment for human health effect due to contaminants exposure in polluted areas requires data on contamination levels and sources. However, chemical data of specific groups of contaminants can not provide sufficient information on potential toxic effects of the complex mixtures of contaminants in the environment. Therefore, a combination of chemical analysis and bio assay based on specific mode of action could provide useful information on distribution and toxicity evaluation of contaminants. The aims of the present study are to determine not only PAHs but also their methylated analogues in street dust collected from two Asian developing countries, Vietnam and India and to measure AhR-mediated activities in street dust by using the dioxin responsive-chemically activated luciferase gene expression (DR-CALUX) bioassay, in order to evaluate the contamination status and to assess the toxic contribution of MePAHs and their parent compounds in overall toxicity.

2. Material and methods

2.1. Sample collection

Street dust samples were collected from the capital cities of Vietnam (Hanoi, $n = 16$) and India (New Delhi, $n = 7$) and a metropolitan city in India (Bangalore, $n = 7$) during 2012–2013 using straw brooms. The broom was washed with Milli-Q water before sample collection. A new straw broom was used for each sampling location. Duong Quang, a rural village in My Hao district, Hung Yen province, Vietnam, was chosen as a reference site (Fig. S1 and Table S1). Approximately 300 g of street dust was collected from an area of 50 m in length and 0.5 m in width and kept in an aluminum foil pocket washed with acetone and hexane and placed inside a zip-locked polyethylene bag. After collection, the samples were preserved at −25 °C until analysis.

2.2. Sample pre-treatment and extraction

Chemicals from one gram of each air-dried and 63 μm sieved street dust samples were extracted with a mixture of distilled acetone/hexane and then distilled toluene using a rapid solvent extractor (SE100, Mitsubishi Chemical Analytech) according to a previously reported method (Tue et al., 2010). A 0.1 g-equivalent portion of the crude extract was then concentrated, solvent-exchanged into 0.1 ml biochemical-grade dimethyl sulfoxide and stored at 4 °C for *in vitro* determination of AhR-mediated activities using the DR-CALUX assay. The remaining extract was used for chemical analysis of MePAHs and PAHs. Every set of seven samples was accompanied with a procedural blank.

2.3. Detection of the AhR-mediated activity

The AhR-mediated activities of all crude extract were determined individually using the DR-CALUX assay (expressed in CALUX-TEQ). This assay utilize the rat hepatoma cell line H4IIE (BioDetection Systems, The Netherlands) stably transfected with the firefly luciferase gene containing a multimerized DRE (dioxin response element) in front of a minimal promoter (Aarts et al., 1995; Garrison et al., 1996). All assays were performed following BioDetection Systems's protocol described elsewhere (Suzuki et al., 2004, 2006). Briefly, approximately 80,000 cells/well were seeded on 96-well plates. After 24 h of incubation at 37 °C and 5% CO_2, the cells were treated with conditioned medium (0.8% DMSO) containing either the 2,3,7,8-tetrachlorobenzo-*p*-dioxin (TCDD) as a standard reference in a series of concentrations (0–37.5 nM) or the samples (diluted by a factor of 1–1000). Each measurement was done in triplicates. Plates were incubated for 24 h at 37 °C, 5% CO_2. After exposure the cells were subjected to luminescence measurement. The AhR agonist activities were derived from the diluted samples with similar response to 1–3 pM TCDD (usually 300 to 1000 time dilution), and expressed in amounts of TCDD equivalent (CALUX-TEQ) per gram dry weight (dw). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to evaluate cell viability in these diluted samples (Suzuki et al., 2013).

2.4. Chemical analysis

Each of the remaining extract was spiked with deuterated PAH surrogate standards and then cleaned-up using 1.2% deactivated alumina chromatography and activated silica gel chromatography. The target compounds were eluted from alumina chromatography column with 150 ml acetone/hexane (v/v: 30/70), and concentrated to 1 ml. After, passing through the activated silica gel chromatography column the target compounds were eluted with 80 ml of 95:5 hexane:dichloromethane, and then spiked with chrysen-d_{12} (Chy-d_{12}) as internal standard. Finally, the extracts were concentrated in 1 ml of iso-octane before being subjected to gas chromatography-mass spectrometry analyses. All solvents were purchased from Wako Pure Chemical Ind., Osaka, Japan and distilled before use. Compounds analyzed in this study were parent PAHs including naphthalene (Nap), acenaphthylene (Acy), acenaphthene (Ace), fluorene (Flu), phenanthrene (Phe), anthracene (Ant), fluoranthene (Fluh), pyrene (Pyr), benzo[c]phenanthrene (BcPh), cyclopenta[c,d]pyrene (CPP), benz[a]anthracene (BaA), chrysene (Chy), benzo[b]-, benzo[k]-, and benzo[j]fluoranthene (BbF, BkF and BjF), benzo[e]- and benzo[a]pyrene (BeP and BaP), indeno[1,2,3-c,d]pyrene (IDP), dibenz[a,h]anthracene (DBA), benzo[g,h,i]perylene (BgP), dibenzo[a,h]-, dibenzo[a,i]-, dibenzo[a,l]pyrene (DBahP, DBaiP and DBalP); MePAHs including 1- and 2-MePhe, 2- and 9-MeAnt, 1-, 2-, 3- and 6-MeChy, 1-MePyr, 1-, 2- 3-, 4-, 5-, 6-, 7-, 9- and 10-MeBaA, 10-MeBaP) 3-methylcholanthrene (MCA), 3,6-Me_2Phe, 9,10-Me_2Ant, and 7,12-Me_2BaA. For surrogate standards, Nap-d_8, Acy-d_8, Phe-d_{10}, Ant-d_{10}, Flush-d_{10}, Pyr-d_{10}, BaA-d_{12}, BaP-d_{12} and BgP-d_{12} were used. All surrogate and internal standards were purchased from Sigma–Aldrich. The target compounds were identified and quantified by a gas chromatography mass spectrometry (GC–MS) following the method described previously (Tuyen et al., 2014) with slight modification. Briefly, we used Agilent model 7890A coupled with a mass spectrometer 5975C MSD and equipped a DB1 MS capitally GC column (30 m × 0.25 mm, 0.25 μm), for the determination of MePAHs and PAHs. Average