JA-244

RELATIVE POTENCIES OF INDIVIDUAL POLYCYCLIC AROMATIC HYDROCARBONS TO INDUCE DIOXIN-LIKE AND ESTROGENIC RESPONSES IN THREE DIFFERENT CELL LINES

Jong Seong Khim[1,2], Kurunthachalam Kannan[2], Daniel L.Villeneuve[2], Jisoo Kang[1,2]
Chul-Hwan Koh[1] and John P. Giesy[2]

[1]Department of Oceanography, Seoul National University, Seoul 151-742, Korea
[2]National Food Safety and Toxicology Center, Department of Zoology and Institute for Environmental Toxicology, Michigan State University, East Lansing, MI 48824, USA

Introduction

Recent studies have indicated that sediment extracts from Lake Shihwa, Masan Bay, and Ulsan Bay, Korea, elicit both dioxin-like and estrogenic responses *in vitro*[1,2,3]. Most of this activity has been associated with a moderately polar fraction known to contain polycyclic aromatic hydrocarbons (PAHs). There is also a growing body of evidence, which suggests that PAHs and their breakdown products may act through aryl hydrocarbon receptor (AhR)- and/or estrogen receptor (ER)- mediated mechanism of action. Thus, the objective of this study was to develop assay-specific relative potency (REP) estimates for PAHs including 16 priority parent PAHs, (EPA; method 8310), 7 methylated and 2 hydroxylated PAH compounds (Table 1). Dioxin-like and estrogenic potencies of individual PAHs mentioned above were characterized using three different cell bioassays. *In vitro* ethoxyresorufin *O*-deethylase (EROD) assay with PLHC-1 fish hepatoma cells and *in vitro* luciferase assay with H4IIE-luc recombinant rat hepatoma cells were used to characterize AhR-dependent, dioxin-like potency of PAHs. To evaluate the estrogenic potency of PAHs, *in vitro* luciferase assay with MVLN recombinant human breast carcinoma cells were utilized. Additionally, a mixture of 16 priority PAHs and acid treated mixture of these 16 PAHs were screened to test the hypothesis that breakdown products of PAHs may elicit dioxin-like responses *in vitro*. The REP estimates reported here will aid in determining the relative contribution of individual PAHs to the total dioxin-like and/or estrogenic activities associated with sediment extracts, as well as other environmental samples.

Methods and Materials

Twenty five individual PAHs were obtained from AccuStandard (New Haven, CT, USA) and prepared at appropriate concentrations with serial dilution. Concentrations tested in the bioassay varied and were limited by the mass of standard available. The maximum concentrations of 16 priority, 7 methylated, and 2 hydroxylated PAHs tested in *in vitro* bioassay were 400, 10, and 20 ug/ml, respectively. A mixture of 16 priority PAHs were treated with concentrated sulfuric acid for 1 and 10 h. The acid treated and non-treated PAH standards were examined in GC/MS and in *in vitro* bioassays. GC/MS analysis (SIM mode for PAHs) was performed to confirm that parent PAHs have been destroyed by acid treatment[2,3]. All standards were prepared in high purity acetonitrile and/or hexane (Burdick and Jackson, Muskegon, MI, USA) prior to dosing cells. PLHC-1 EROD, H4IIE-luc and MVLN luciferase assays have been described previously[4,5]. Appropriate cells for bioassay were plated into the 60 interior wells of 96-well culture plates (250 µl per well) and incubated overnight prior to dosing. Test and control wells were dosed with 2.5

μl of the appropriate standards or solvent. Blank wells received no dose. At least three replicate wells were analyzed for each sample dilution, control, and blank. Luciferase and protein assays were conducted after 72 h of exposure. Sample responses, expressed as mean pmol resorufin/min/mg protein or relative luminescence units over three replicate wells, were converted to relative response units, expressed as a percentage of the maximum response observed for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; %-TCDD-max.) standard curve generated on the same day. REPs of 25 PAHs to TCDD were estimated. REPs were expressed as a range of values calculated over multiple levels of response from 20-80%-standard-max. ($REP_{20\text{-}80}$-ranges) in order to account for potential uncertainty in the estimate due to deviations from parallelism to the standard curve[6].

Results and Discussion

Relative potency of individual PAHs

At the concentrations tested, seven of 16 priority PAH compounds caused significant dioxin-like activity both in PLHC-1 EROD assay and H4IIE luciferase assay (Table 1). Four to five ring aromatic hydrocarbons, exhibited REPs of around 10^{-4}-10^{-6} relative to 3130 pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard. Benzo(k)fluoranthene, with a PLHC-1 REP of 0.0005 and H4IIE-luc REP of 0.0001, was the most potent PAH compounds tested (Table 1). REPs for chrysene, indeno(1,2,3-cd)pyrene, and benzo(b)fluoranthene, were around 10^{-5}. REPs for the active compounds were similar to those for some polychlorinated biphenyls (PCBs) and polychlorinated naphthalenes (PCNs). However, REPs for seven active individual PAHs were generally one or two orders of magnitude less than that for the mixture of 16 PAHs, which were recently reported to be around 10^{-4} in the same cell bioassay system. Two substituted PAHs, 3, 6-dimethyl phenanthrene and 6-hydroxy chrysene, induced dioxin-like responses. Their REPs were 0.00025 and 0.00029, respectively, which are similar with those for coplanar PCBs. Among the 25 PAHs tested in MVLN cell bioassay, only two compounds, namely, benzo(a)anthracene (BaA) and dibenz(a,h)anthracene (DahA) were found to substantially induce estrogenic activity that was mediated through ER *in vitro*. Both compounds were estimated to be over a million times less potent than 17β-estradiol (5.7×10^{-6} for BaA, 8.8×10^{-6} for DahA). This is in contrast to a previous study that reported a REP value of 0.001 for BaA.

Dioxin-like activity of Acid Treated PAHs

A mixture of 16 priority PAHs was treated with concentrated sulfuric acid for 1 or 10 hours. After acid treatment standards were and/or were not washed with nanopure water. GC/MS analysis showed that most of the individual PAHs were destroyed by acid treatment, > 99 and 100% for 1 and 10 hours of acid treatment, respectively. However, significant dioxin-like activity was observed in acid treated PAHs for 1 hour acid treatment. The activities of water washed and non-washed PAHs were as great as 34 and 46%-TCDD-Max (Figure 1). This suggests that some breakdown products of PAHs may act through an AhR- dependent mechanism of action. None of the 10 hour acid treated PAHs were able to induce dioxin-like activity in H4IIE-luc cells. This indicates that most PAHs can be destroyed completely after 10 hour acid treatment and no dioxin-like breakdown products could be present. Overall, this result suggests that some breakdown products of PAHs following acid treatment might still have the ability to induce dioxin-like activity in *in vitro* bioassay. Further studies are needed in this aspect.

Table 1. Maximum Concentrations of Individual PAHs Compounds Tested in PLHC-1, H4IIE-luc, and MVLN *in vitro* Bioassay, and Relative Potency (REP) Estimates..

PAH Compounds	Max. Conc.	PLHC-1 EROD			H4IIE-luc		
	(ppb in well)	$REP_{20\text{-}80}$[a]		REP[b]	$REP_{20\text{-}80}$		REP
Acenapthene	4000			NA[c]			NA
Acenapthylene	4000			NA			NA
Antracene	4000			NA			NA
Benzo(a)anthracene	4000	1.2E-05	2.9E-08	6E-07	2.2E-06	1.7E-06	1.9E-06
Benzo(a)pyrene	4000	3.8E-06	4.6E-12	4.2E-09	2.4E-06	1.1E-06	1.6E-06
Benzo(b)fluoranthene	4000	0.00012	4.5E-05	7.3E-05	1.6E-05	1.6E-06	5.1E-06
Benzo(g,h,i)perylene	4000			NA			NA
Benzo(k)fluoranthen	4000	0.00107	0.00022	0.00049	0.00039	5.1E-05	0.00014
Chrysene	4000	7.5E-05	8.8E-06	2.6E-05	4.6E-06	1.2E-06	2.3E-06
Dibenz(a,h)anthracene	4000			NA	4.4E-05	4.8E-07	4.6E-06
Fluoranthene	4000			NA			NA
Fluorene	4000			NA			NA
Indeno(1,2,3-cd)pyrene	4000	0.00018	9.2E-06	4.1E-05	3.4E-05	6.4E-06	1.5E-05
Napthalene	4000			NA			NA
Phenanthrene	4000			NA			NA
Pyrene	4000			NA			NA
9-methyl Anthracene	100			NA			NA
9,10-dimethyl Anthracene	100			NA			NA
3,9-dimethyl benzo(a)anthracene	100			NA	6E-07	8.7E-14	2.3E-10 NA
1-methyl naphthalene	100			NA			NA
1,2-dimethyl naphthalene	100			NA			NA
3,6-dimethyl phenanthrene	100	0.00037	1.8E-06	2.6E-05			NA
2-methyl benzo(c)phenanthrene	100			NA			NA
6-hydroxy chrysene	200	0.00023	3.8E-06	2.9E-05			NA
1-hydroxy pyrene	200			NA			NA

[a] REPs reported as the range of REP estimates generated from multiple point estimates over a response range from 20-80%-TCDD-max. ($REP_{20\text{-}80}$-range).
[b] Refer to the dioxin equivalents generated from one point estimates made for response of 50%-TCDD-Max.
[c] NA: not available to calculate REP.

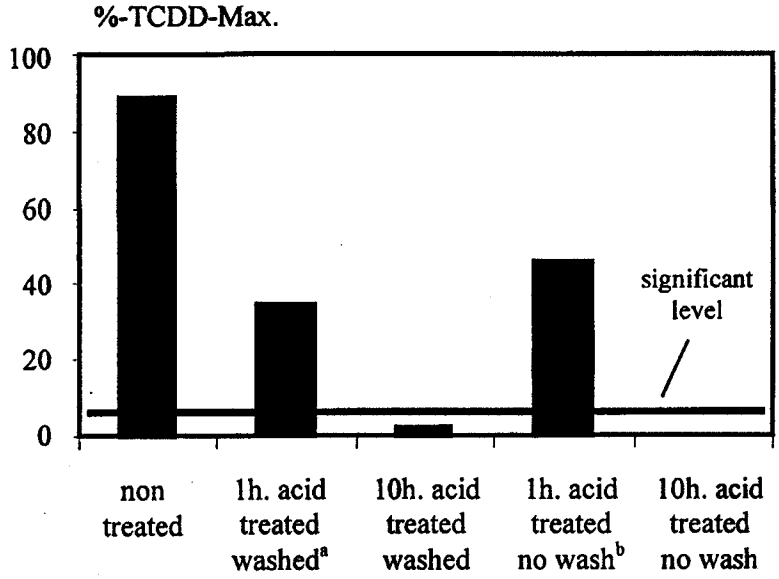

Figure 1. Luciferase induction in the H4IIE-luc cell bioassay elicited by acid treated and non treated PAH mixtures.
Response magnitude presented as percentage of the maximum response observed for a 3130 pM TCDD standard (%-TCDD-max.).
[a] washed means after acid treatment standards washed with nanopure water.
[b] no wash means after acid treatment standards were not washed with nanopure water.

Acknowledgements
We thank colleagues from the Benthos Lab at Seoul National University, Korea, and Aquatic Toxicology Laboratory at Michigan State University, USA.

References

1.Khim J.S., Villeneuve D.L., Kannan K., Lee K.T., Snyder S.A., Koh C.H., and Giesy J.P. (1999) Environ Toxicol Chem 18:2424-2432 .
2. Khim J.S., Villeneuve D.L., Kannan K., Koh C.H., and Giesy J.P. (1999) Environ Sci Technol 33:4199-4205.
3. Khim J.S., Lee K.T., Kannan K., Villeneuve D.L., Giesy J.P., Koh C.H. (2000) Arch Environ Contam Toxicol (submitted).
4. Khim J.S., Kannan K., Villeneuve D.L., Koh C.H., and Giesy J.P. (1999) Environ Sci Technol 33:4206-4211.
5. Villeneuve D.L., Khim J.S., Kannan K., Giesy J.P. (2000) Aquat Toxicol (submitted).
6. Villeneuve D.L., Blankenship A.L., Giesy J.P. (2000) Environ Toxicol Chem (in press).