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Aryl Hydrocarbon Receptor-Mediated Gene Expression by Chlorinated Polycyclic Aromatic Hydrocarbons and Cross-Talk with Estrogen Receptors

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Abstract—Chlorinated polycyclic aromatic hydrocarbons (ClPAHs) have been recently reported to occur widely in environmental matrices such as urban air, vehicle exhaust, snow, tap water and sediments. However, toxicological information on ClPAHs is currently very limited. Therefore, in the present study, we evaluated the influence of typical ClPAHs like chlorinated phenanthrenes (ClPhes), chlorinated pyrene (ClPy) and chlorinated benzo[*a*]pyrene (ClBaP), on the induction of aryl hydrocarbon receptor (AhR)-mediated cytochrome P-450 (CYP) 1A1 and also estrogen receptors (ERs)-mediated cathepsin D mRNA using MCF-7 human breast cancer cells. Expressions of CYP1A1 and catepsin D were measured upon treatment of 2 μM of each chemical, using quantitative RT-PCR system after incubation at 37°C for 12 hr. Phe did not induce any expression of CYP1A1 mRNA. On the other hand, mono-, di-, and tri-ClPhe increased these expressions with respect to the number of chlorine atoms on the Phe skeleton. ClBaP also induced CYP1A1 at the same levels as in the case of the parent chemical BaP. Py and ClPy did not induce any change. Other AhR activated ligands, such as 3-methylcholanthrene, have been reported to have AhR-ERs cross-talk activity. However, tri-ClPhe didn't stimulate this cross-talk pathway. These results indicate that estrogenic action mediated ERs signaling through AhR activation does not necessarily occur in all AhR avtivated lignads.

Keywords: chlorinated polycyclic aromatic hydrocarbons, aryl hydrocarbon receptor (AhR), cytochrom P-450 1A1, estrogen receptors (ERs), AhR-ERs cross-talk

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants, formed by the incomplete combustion of organic compounds (Mastral and Callen, 2000; Richter and Howard, 2000). Exposure to PAHs-containing substances increases the risk of cancer in humans to be mediated through aryl hydrocarbon receptor (AhR) activation (Menzie *et al.*, 1992; Bostrom *et al.*, 2002). The ligand-bound AhR, a cytosolic ligand-activated transcription factor complex activates the transcription of genes related to the drug-metabolizing enzymes such as cytochrome P-450 (CYP) 1A1. The most characteristic pathway involves translocation of the activated AhR to the nucleus, where it binds with the AhR nuclear translocator protein (Arnt) to form a heterodimer. Binding of the heterodimer leads to transcriptional modulation of genes that contain a xenobiotic responsive element (XRE) (Poland and Knutson, 1982). In addition to AhR activation, Ohtake *et al.* (2003) recently reported that AhR ligand such as dioxins stimulate the estrogen response elements (EREs)-mediated transcription without binding directly to estrogen receptors (ERs), and as a consequence exert estrogen related effects. Therefore, to promote PAHs activity, interaction with AhR and also estrogen related effects seem to be an essential mechanism.

Chlorinated forms of PAHs with 3-5 rings (ClPAHs, Fig. 1) have been recently reported to occur widely in environmental matrices such as urban air, vehicle exhaust, snow, tap water and sediments (Ohura, 2007). However, toxicological information on ClPAHs is limited, whereas mutagenic effects using the *Salmonella* assay have been to investigate some ClPAHs (Colmsjo *et al.*, 1984; Lofroth *et al.*, 1985). In this report, we therefore summarized our recent data on the effects of ClPAHs on AhR activation (CYP1A1 mRNA expression) and AhR-ERs cross-talk activation (cathepsin D mRNA expression) using human breast cancer MCF-7 cells.

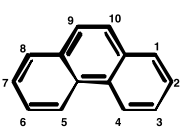
MATERIALS AND METHODS

Chemicals

The ClPAHs used in this study were mono-, di- and tri-ClPhe, mono-ClPy, and mono-ClBaP as illustrated in Fig. 1. These ClPAHs were individually obtained from the chlorination of parent compounds, Phe, Py, and BaP according to the method by Ohura *et al.* (2005). The purities of all these compounds were more than 95%. The ClPAHs and their parent compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 μM for use in the following experiments.

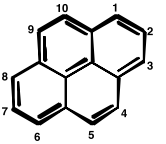
Cell culture

Human breast cancer MCF-7 cells kindly provided by Dr. H. Hagenmaier (University of Tuebingen, Germany) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 0.1 mg/ml kanamycin and 0.1 mg/ml ampicillin at 37°C under 5% CO_2.



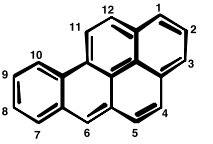
Phenanthrene (Phe)

	3	9	10
Phe	–H	–H	–H
Cl–Phe	–H	–Cl	–H
Cl_2–Phe	–H	–Cl	–Cl
Cl_3–Phe	–Cl	–Cl	–Cl



Pyrene (Py)

	1
Py	–H
Cl–Py	–Cl



Benzo[a]pyrene (BaP)

	6
BaP	–H
Cl–BaP	–Cl

Fig. 1. Structures of ClPAHs used in this study.

Table 1. Effects of ClPAHs on AhR and ERs activations in MCF-7 cells.

	AhR activation[1]	AhR-ERs cross-talk[2]
3-methylcholanthrene	+++	+++[3]
Phenanthrene (Phe)	—	—
9-chloro Phe	+	—
9, 10-dichloro Phe	++	—
3, 9, 10-trichloro Phe	+++	—
Pyren (Py)	—	n.i.
1-chloro Py	—	n.i.
Benzo[*a*]pyrene (BaP)	+++	n.i.
6-chloro BaP	+++	n.i.

Each activation was carried out using quantitative RT-PCR on [1]CYP 1A1 mRNA expression and [2]cathepsin D mRNA expression. The results were exhibited as "—", not effective; "+", significant increase compared with vehicle control. Increase in "+" signs indicate strong induction of gene expression.

n.i.: not investigated.

[3]AhR-ERs cross-talk activity of 3-methylcholanthrene was cited from the reports by Ohtake *et al*. (2003).

RNA isolation and cDNA synthesis

Subconfluent MCF-7 cells were exposed to 2 μM of ClPAHs or their parent chemicals. Same volume of DMSO was used as a vehicle control in the assay medium (phenol red-free DMEM supplemented with 10% charcoal-dextran-treated FBS). After incubation at 37°C for 12 hr, total RNA was isolated from collected cells using RNeasy plus Mini kit (QIAGEN GmbH, Hilden, Germany), and quantified by measuring absorbance at 260 and 280 nm. The RNA sample (600 ng) was added to 20 μL of reaction mixture containing random hexamers, MuLv Reverse Transcriptase, RNase inhibitors, 25 mM $MgCl_2$, 10 × PCR Buffer II (Applied Biosystems, Foster City, CA) and 10 mM dNTPmix (Promega Co.,

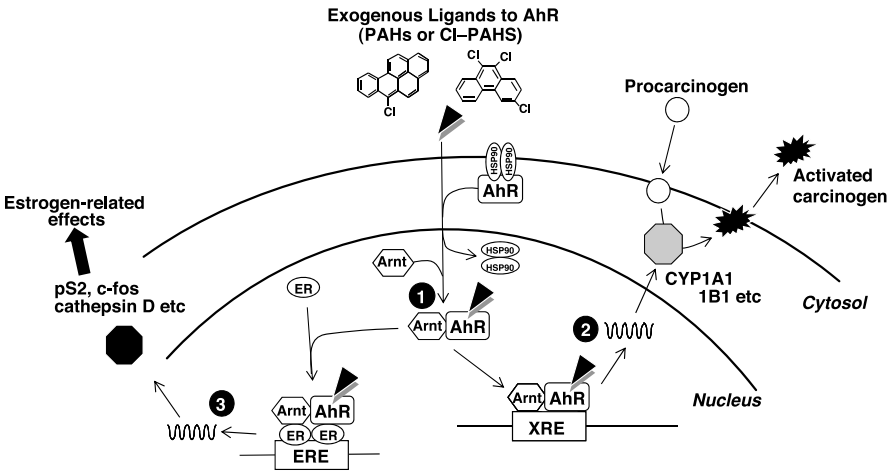


Fig. 2. Molecular mechanisms of AhR-mediated gene expression by ClPAHs. HSP90, heat shock protein 90; AhR, arylhydrocarbon receptor; Arnt, AhR nuclear translocator; XRE, xenobiotic responsive element; ER, estrogen receptor; ERE, ER receptor responsive element.

Madison, WI). Synthesis of cDNA was performed at 42°C for 60 min and the reverse transcription reaction was stopped by heating to 99°C for 5 min followed by chilling on ice.

Measurement of CYP1A1 and cathepsin D mRNA expression

Expression of CYP1A1 and cathepsin D mRNA were measured using quantitative RT-PCR system in this study. Briefly, a total of 1 μL of cDNA was added to 19 μL of PCR mixture containing 10 μL Taq Man Gene Expression Master Mix (Applied Biosystems, Foster City, CA), 7 μL distilled water DNase RNase Free (Invitrogen Corp., Carlsbad, CA), 1 μL housekeeping gene solution (glyceraldehydes-3-phosphate dehydrogenase; GAPDH), and 1 μL individual target gene expression reagents for human (CYP1A1, Assay ID: Hs00153120_m1; cathepsin D, Hs00157205_m1, Applied Bisystems). Measurements were done using the ABI 7500 Real-Time PCR System with denaturation for 10 min at 95°C followed by 40 PCR cycles of denaturation at 95°C for 15 sec and annealing or extension at 60°C for 1 min. The relative expression level of the target gene product was calculated by the comparative automatic threshold cycles method, using GAPDH as calibrator. The relative differences in expressions between groups were expressed using cycle time values and the relative differences between groups were expressed as relative increases, setting the control as 100%.

Statistical analysis

The statistical significance between control and treatments was evaluated by

Tukey using the Pharmaco Analyst Ver. 2.0 (Humanlife). The results were considered significant if the possibility of error was less than 5%.

RESULTS AND DISCUSSION

ClPAHs have been recently identified as ubiquitous environmental contaminants (Ohura, 2007). However, toxicological information on ClPAHs is limited. Therefore, it is important to evaluate the physiological effects of exposure to ClPAHs. Parent chemicals (2 μM), Phe and Py did not induce any change on the expression of CYP1A1 mRNA levels comparing with the vehicle control (Table 1). On the other hand, BaP significantly increased its expression. These results were found in the intestinal cell line Caco-2 by Lampen *et al.* (2004). Mono-, di-, and tri-ClPhes increased the CYP1A1 mRNA expression in accordance with the number of chlorine atoms on the Phe skelton. ClBaP also induced CYP1A1 mRNA to the same extent as in the case of the parent chemical BaP. These results were agreeable to our recent reports that evaluated whether some ligands, such as ClPhe, ClPy, and ClBaP could bind AhR, in lacZ reporter gene assays, using yeast YCM3 cells, which carried human AhR and Arnt genes (Ohura *et al*., 2007).

In addition to AhR activation, some xenobiotics such as dioxins and 3-methylcholanthrene have been recently reported to stimulate EREs-mediated transcription without binding directly to ERs or affecting expression levels of ERs, and consequently exert estrogen-related effects (Ohtake *et al*., 2003). These activations were found to be exerted *via* direct association of ligand-binding AhR/Arnt heterodimer with ERs, the so-called AhR-ERs cross-talk. Among the ClPAHs and PAHs that had AhR activity, treatment of tri-ClPhe didn't stimulate the gene expression of cathepsin D, which is one of the target genes expressed *via* ERs pathway (Table 1).

From our results it can be concluded that ClPhes and ClBaP activate AhR (Fig. 2-pathway 1), and exert induce CYP1A1 expression (Fig. 2-pathway 2). Therefore, these two ClPAHs have adverse effects on human health, for example *via* activation of procarcinogen. Moreover, typical AhR ligands, such as 3-methylcholanthrene, have been reported to stimulate the AhR-ERs cross-talk pathway (Fig. 2-pathway 3). On the other hand, tri-ClPhe, which has strong AhR activity, did not induce AhR-ERs cross-talk. These results suggest that estrogenic action mediated ERs signaling through AhR activation could not necessarily occur in all the AhR activated ligands. Such interesting differences on AhR ligands will be our future subject for elucidation.

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