CHLORINATED PHENANTHRENS INDUCE CYP1A1 THROUGH ARYL HYDROCARBON RECEPTOR ACTIVATION

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Abstract
Chlorinated polycyclic aromatic hydrocarbons (ClPAHs) have found in urban air, vehicle exhaust gas, snow, tap water and sediments. However, toxicological information on ClPAHs currently is very limited. In the present study, we evaluated the influence of chlorinated phenanthrenes (ClPhes), which is one of the major ClPAHs, on the induction of cytochrome P-450 (CYP) 1A1. Mono-, di- and tri-ClPhes showed aryl hydrocarbon receptor (AhR)-mediated activity in the yeast assay system, but not parent compound, phenanthrene. ClPhes activated EROD activity and also induced CYP1A1 mRNA levels with increasing the number of chlorine atoms on the phenanthrene skeleton in MCF-7 human breast cancer cells. These results indicate that ClPhes induce CYP1A1 through AhR activation as same as the effects of dioxin.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that are formed by the incomplete combustion of organic compounds.[1,2] Exposure to PAHs-containing substances increases the risk of cancer in humans to be mediated through aryl hydrocarbon receptor (AhR) activation.[3,4] 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). The most characterized 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).[5] Therefore, to promote PAHs activity, interaction with AhR seems to be an essential mechanism.

Phenanthrene (Phe), one of the major PAHs, exists in the environment at higher amount comparing with other PAHs, such as benzo[a]pyrene and pyrene.[6] Phe have basically no or weak effect to induce CYP.[7,8] On the other hand, chlorinated forms of PAHs (ClPAHs) have been recently reported to occur widely in the environment,[9-13] and have adverse biologic effects, although toxicological information on ClPAHs currently is very limited. Therefore, there is a possibility that chlorination changes the biological effects of Phe such as induction of CYP.

In the present study, we attracted the chlorinated phenanthrenes (ClPhes), and evaluated their effects for AhR activation using the yeast assay system, and also induction of CYP1A1 in MCF-7 cells.

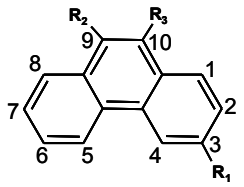
Materials and Methods

Chemicals. The ClPhes used in this study were mono-, di- and tri-ClPhe as illustrated in Fig 1. These three types of phenanthrenes were obtained from the chlorination of parent compound, Phe, according to the method by Ohura et al.[12] Phe and ClPhes were dissolved in dimethyl sulfoxide (DMSO) at the concentration of 10 μM before their use in the following experiments.

AhR Activity in Yeast Assay. Saccharomyces cerevisiae YCM3, which expresses human AhR and Arnt and carries a *lac* Z reporter plasmid containing XRE system, were kindly provided by Dr. Miller, CA III (Tulane University, New Orleans, LA). The assay procedure was essentially the same as described by Miller.[14] Briefly, YCM3 cells were grown overnight at 30°C in a synthetic complete medium containing 2% glucose and lacking tryptophan. The following day, test chemicals, 5 μl of the overnight culture, and 195 μl medium containing 2% galactose were mixed in a well of a 96-well microplate, with subsequent incubation at 30°C for 18 h. The optical densities of the wells were determined by reading the absorbance at a wavelength of 595 nm. A 30 μl aliquot of the suspension in each well was added to 120 μl Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM KCl, 2 mM dithiothreitol, and 0.2% sarcosyl, adjusted to pH 7), and the reaction was started by addition of

50 μl *o*-nitrophenol-*β*-galactopyranoside (4 mg/ml solution in Z-buffer), followed by incubation at 37°C for 60 min. The absorbance of *o*-nitrophenol was read at 405 nm. *β*-galactosidase activity (referred to as *lacZ* units) was calculated by use of the following formula: absorbance at 405 nm × 1000/(absorbance at 595 nm × ml of well suspension added × min of reaction time).

Cell Culture. MCF-7 cells were kindly provided by Dr. Hagenmaier H. (University of Tuebingen, Germany). MCF-7 cells 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 humidified 5% of CO_2.



	R_1	R_2	R_3
Phenanthrene (Phe)	**H**	**H**	**H**
mono-ClPhe	**H**	**Cl**	**H**
di-ClPhe	**H**	**Cl**	**Cl**
tri-ClPhe	**Cl**	**Cl**	**Cl**

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

EROD Activity Assay. A 7-ethoxyresorufin-*o*-deethylase (EROD) activity was determined by using the 24-well plate procedure. Subconfluent MCF-7 cells were exposed to 2 μM of Phe or ClPhes in the assay medium (phenol red-free DMEM supplemented with 10% charcoal-dextran-treated FBS) for 48 h. The media were replaced with 2 ml of medium containing 2.5 μM ethoxyresorufin and 10 μM dicumarol. After 20 min incubation at 37º C, fluorescence was determined at an excitation wavelength of 530 nm and an emission wavelength of 590 nm and was compared with a standard curve for resorufin prepared in the same medium.

RNA Isolation and cDNA Synthesis. Subconfluent MCF-7 cells were exposed to 2 μM of Phe or ClPhes in the assay medium (phenol red-free DMEM supplemented with 10% charcoal-dextran-treated FBS), and collected after incubation at 37°C for 12 h. Total RNA was isolated 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., 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.

CYP1A1 mRNA Expression. Expression of CYP1A1 mRNA was measured using the Gene Amp PCR System 2400 (Perkin Elmer Inc, Winter Street Waltham, MA) according to the method previously reported[15] with some modifications. Briefly, the cDNA of CYP1A1 was denatured at 95°C for 5 min and cycled immediately for 30 cycle: denatured 94°C for 30 s, annealed at 49°C for 30 s and extended at 72°C for 1 min. The CYP1A1 primers for sequencing were F: 5'-TCTTTCTCTTCCTGGCTATC-3' and R: 5'-CTGTCTCTTCCCTTCACTCT-3'. Amplified PCR products were electrophoresed in TAE buffer at 100 V for 30 min on 2% agarose containing ethidium bromide.

Statistical Analysis. The statistical significance between control and treatments was evaluated by Student's *t* test. The results were considered significant if the possibility of error was less than 5 or 0.1%.

Results and Discussion

We assessed the activities of AhR as an agonist of Phe and ClPhes in *lacZ* reporter gene assays using yeast YCM3 cells, which carried human *AhR* and *Arnt* genes. In this simple assay, compounds bind to expressed AhR, leading to transcription of the reporter gene, *β*-galactosidase. Three kinds of ClPhes, which were mono-, di- and tri-ClPhe as listed in Fig. 1, showed appreciable dose-dependent increases of *β*-galactosidase activity at the concentration of more than 0.1 μM, but not parent compound Phe (Fig. 2). In addition, their AhR mediated inducibility increased with the number of chlorine groups. These results indicate that ClPhes have an effect to activate AhR, although their parent Phe dose not has this effect under the concentration used in this study

(0.0001-100 μM).

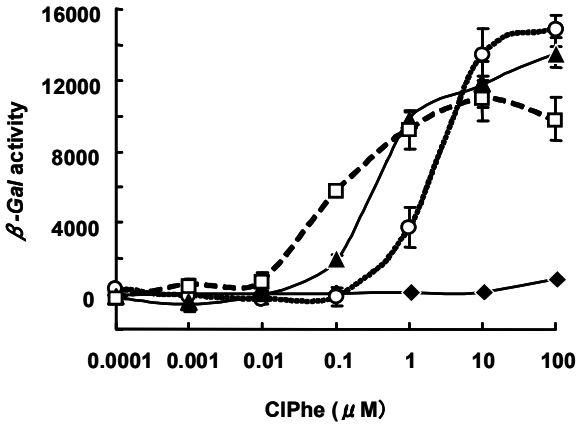


Fig. 2. Effect of ClPhes on AhR activation in yeast assay.
-♦-, Phe; -○-, mono-ClPhe; -▲-, di-ClPhe; -□-, tri-ClPhe

The ligand-bound AhR, a cytosolic ligand-activated transcription factor complex, activates the transcription of genes related to the drug-metabolizing such as CYP. In light of the effect of CYP1A induction on AhR ligand-derived toxicity, we measured EROD activity to examine the inducibility of CYP1A in MCF-7 cells exposed to Phe and ClPhes. Phe (2 μM) did not show any change for EROD activity comparing with vehicle control (Fig. 3), as the same result reported by Bols et al [7] using a trout liver cell line. On the other hand, ClPhes, particularly di- and tri-ClPhe, significantly increased the EROD activity. EROD assay is well known to detect the induction of CYP1A family, especially CYP1A1 isozyme.[8] In the present study, we also analyzed the influence of ClPhes on the expression of CYP1A1 in MCF-7 cells using the method of RT-PCR. Parent Phe did not result in the induction of CYP1A1 mRNA (Fig. 4), as same result in the intestinal cell line Caco-2 by Lampen et al.[16] However, in MCF-7 cells incubated with 2 μM of ClPhes, di- and tri-ClPhe, the levels of CYP1A1 mRNA increased about 2- and 3-folds compared with the vehicle control, respectively. Hence, these results indicated that CYP1A1 induction increased with the number of the chlorine atoms on Phe through AhR activation as same as the effects of dioxin. It is warranted to examine about CYP1A1 induction of other ClPAHs, such as chlorinated benzo[a]pyrene, chlorinated pyrene, and so on in the future experiments.

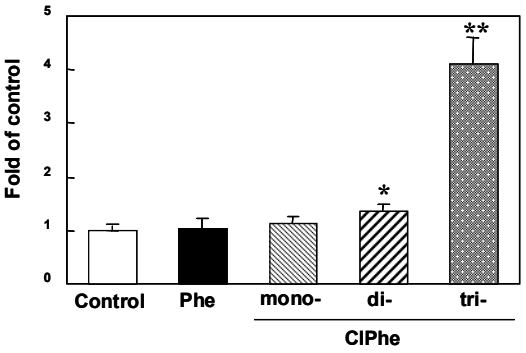


Fig. 3. Effect of ClPhes on EROD activity in MCF-7 cells.
MCF-7 cells were treated with 2 μM of Phe or ClPhes for 48 h. Data are presented as mean ± SD (n=3). *P<0.05, **P<0.001 vs Control.

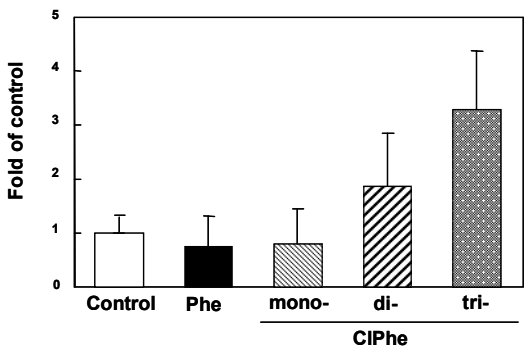


Fig. 4. Influence of ClPhes on the expression of CYP1A1 mRNA in MCF-7 cells.
MCF-7 cells were treated with 2 μM of Phe or ClPhes for 12 h. Data are obtained from three independent experiments.

Acknowledgements

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