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Bioassays of Polycyclic Aromatic Hydrocarbons Using *cyp1a1*-Luciferase Reporter Gene Expression System in Mouse Liver Hepa 1 Cells

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ABSTRACT : **Recent industrial society has human widely exposed to PAHs (polynuclear aromatic hydrocarbons) that are comming from the incomplete combustion of organic material as wider spread environmental contaminants. Biological activities of PAHs are not known although PAHs are considered as carcinogens. Our laboratory have been studied the effect of PAHs in the mouse liver hepa 1 cells. In this study, we examined the mouse liver hepa-1 cells as a new bioassay system to evaluate bioactivity of PAHs. We have selected 13 PAHs to examine bioassay using *cyp1a1*-luciferase reporter gene expression system where *cyp1a1* 1.6 Kb 5flanking region DNA was cloned in front of luciferase reporter gene and this plasmid was transfected into hepa 1 cells transiently. This cells then used for the study to observe the effect of PAHs. We demonstrated that PAHs induced the CYP1A1 promoter and 7-ethoxyresolufin O-deethylase (EROD) activities in a concentration-dependant manner. Some of PAHs showed stronger stimulatory effect on CYP1 gene expression than TCDD. Acenaphthene, anthracene, fluorine, naphthalene, pyrene, phenanthrene, carbazole were weak responders to cyp1a1 promoter activity stimulation and EROD induction in hepa 1 cells and these chemicals seemed to respond less to EROD than cyp1a1 promoter activity. Benz(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, chrysene, and dibenzo(a,h)anthracene showed strong response to cyplal promoter activity stimulation and also EROD induction in hepa 1cells. Results of dose response study suggested that four strong responding PAHs, such as benzo(a)anthracene benzo(k)fluoranthene, chrysene, and dibenzo(a, h)anthracene might be mediated through arylhydrocarbon receptor system in hepa 1 cells.**

Key words : **PAH, EROD, *Cyp1a1*, TCDD, luciferase, hepa 1 cells**

Introduction

Polycyclic aromatic hydrocarbons (PAH) are frequently detected in food, water, soil, and sediment (Menzie *et al.*, 1992), and are widespread environmental pollutants formed by the incomplete combustion of fossil fuels, woods and other organic matter. PAHs are considered to be probable human carcinogens. PAHs such as benzo(a)pyrene (B[a]P) undergo metabolic activation by cytochrome P450 and epoxide hydrolase to chemically reactive ultimate carcinogen diol epoxides (Chou *et al.*, 1986). Mutagenic and carcinogenic potency of many PAH metabolites has been demonstrated in vivo rodent assays and in vitro short-term assays (Hecht *et al.*, 1994 and Zaho and Ramos, 1998). The mechanisms of PAH bioactivation was also studied in human cDNA expressed CYP1A1 and purified CYP1A1, and results showed the enhancement of the genotoxicity of a proximate carcinogenic form of benzo[a]pyrene (shimada *et al.*, 1994). cDNA expressed CYP1A1 and CYP1A2 both catalyzed stereoselective epoxidation of a series of PAHs (shou *et al.*, 1996). Human CYP1B1 has also recently been demonstrated to be capable of bioactivating PAH carcinogens (Shimada *et al.*, 1996; Luch *et al.*, 1999). PAHs such as TCDD induce the expression of *CYP1* family, which consists of at least three enzymes, *CYP1A1, CYP1A2* and *CYP1B1* has been shown to be important in the metabolism of several xenobiotics such as PAH and heterocyclic amines. TCDD induction of *CYP1* transcription is mediated by the cytosolic AhR, which is known as a ligand-activated transcription factor. The activation of AhR involves ligand binding, dissociation of heat-shock protein-90, nuclear translocation, and dimerization with the Arnt followed by binding to dioxin responsive element (DRE, or XRE) enhancer elements in the 5'-noncoding region of the responsive gene (Carrier *et al.* 1992; Swanson *et al.*, 1993; Denison and Whitlock,

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1995). The mechanism of action of this compound is to activate the AhR to a form that binds to specific gene regulatory sequence elements, called XREs, through heterodimerization with Arnt (Dolwick *et al.*, 1993; Mason *et al.*, 1994; Ko *et al.*, 1996). AhR and Arnt have a similar overall structure and belong to the basic helix-loop-helix class of transcription factors (Pendurthi *et al.*, 1993; Poland *et al.*, 1994). Members in this class of factors are characterized by a bHLH motif contiguous with a region, PAS (Per-Arnt-Sim), which is conserved between the *Drosophila* neural cell developmental regulator Sim, the *Drosophila* circadian rhythm regulatory protein, Per, and Arnt (Littlewood and Evan, 1995). Upon binding XREs, the AhR-Arnt complex activates transcription of adjacent structural gene encode enzymes that are involved in the oxidative metabolism of these compounds (Whitelaw *et al.*, 1994; Whitlock *et al.*, 1996). In this study, we tried to develop the bioassay system of PAHs based on their ability to induce CYP1A1. We measure the reporter gene activity as a AhR activation in mouse liver hepa 1 and human breast cancer ZR-75-1 cells transfected with pCYP1A1-Luc to monitor the induction of cytochrome P4501A1 activity with PAHs treatment. Also we measured the increase in 7-ethoxyresorufin-O-dethylase (EROD) production as an indication of induction of CYP1A1 catalytic activity by PAHs treatments activation in mouse liver Hepa I cells.

Materials and Methods

Materials

2,3,7,8-tetrachloro-ρ-dioxin was kindly provided by Dr. K. Chae from NIEHS, (Research Triangle Park, NC, U.S.A.). Desferrioxamine (DFX), picolinic acid (PA), ferrous sulfate, and phenol were supplied by Sigma, cobalt chloride was purchased from Junsei chem. Co., agarose was purchased from FMC, LipofectAMINE and Hind III were ordered from by Gibco BRL, pGL3 basic vector and luciferase assay system were purchased from Promega.

Construction of *Cyp1a1*-Luc

Mouse *Cyp1a1* 5' flanking DNA (-1642~+53) was cloned into pGL3 vector at Hind III site.

Cell culture and transfection

Hepa I (Hepa 1c1c7) mouse liver cell lines were grown in Earle's Balanced Salt Solution (EBSS) supplemented with 10% (v/v) fetal bovine serum and 100 units penicillin-streptomycin/ml. For the transfection of pm*Cyp1a1*-Luc, 50,000 Hepa I cells were plated into each well of 24 well plate and maintained at 37°C with humidified 5% CO_2 for 24 hours. 150 ng of pm*Cyp1a1*-Luc and 1 μg of LipofecTAMINE were mixed in 50 μl of serum-free medium and incubated at room temperature for 45 minutes before adding it into Hepa I cells. And Hepa I cells were exposed to the DNA-lipid complexes in serum-free medium at least for 5 hours at 37°C in humidified 5% CO_2 incubator, before cells were maintained in normal minimum essential medium (MEM) containing 20% fetal bovine serum. The details were followed as supplier's manual.

Chemical treatment

Hepa I cells were rinsed with serum-free medium twice before the administration of various chemicals in serum free medium. Stock solutions of chemicals were made in DMSO as a vehicle and control cells were treated with 0.1% DMSO. 1~100 μM cobalt chloride or 1~100 μM desferrioxamine or 1~100 μM picolinic acid was administered in the presence or absence of 150 μM ferrous sulfate for 17 hours before the 1 nM TCDD treatment for 24 hours.

Luciferase reporter assay

Luciferase activity was determined in cell extracts containing 2 μg of total protein. 20 μl of cell extracts were mixed with 100 μl of reporter assay reagent at 20~25°C and luminescents were measured using Liquid scintillation counter. Protein assay of cell extracts was carried out using Micro BCA protein assay reagent kit and ELISA Reader. Luciferase activity data present as the fold induction of control cells that were treated with 0.1% DMSO when control luciferase activity is set at 1.

Results

Effects of 13 PAHs on the mouse *cyp1a1* promoter activity

1uM of 13 different PAHs and 1n M TCDD were administered into Hepa I cells transfected with expression plasmind that has mouse cyp1a1 5'flanking DNA 1.6 Kbs cloned in front of luciferase gene for 24 hrs, and the cyp1a1 promoter activity was monitored by measuring luciferase activity. As shown in Fig. 1, 1uM benzo(k) fluoranthene showed 65-fold induction of luciferase activity over control cells, and 1nM TCDD showed 48-

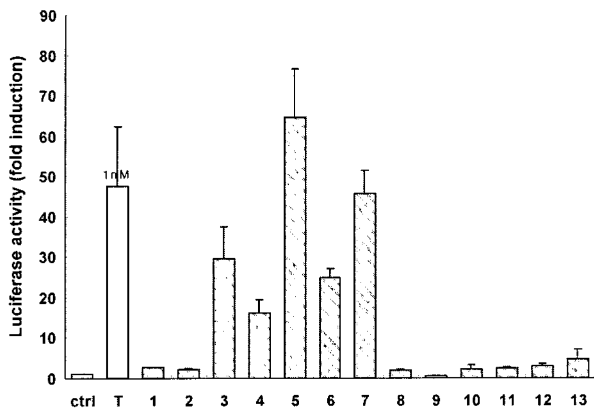

Fig. 1. The effect of PAHs on the luciferase activity in Hepa-I cells transfected with *pmCyp1a15'-Luc*. After transfection, cells were treated with 1 nM TCDD or 1 μM PAHs for 24 hours. The amount of luciferase transcription was normalized to the amount of protein. The data represent mean ± S.D. (n=3) C : Control, T : 2,3,7,8-TetraChloroDibenzoDioxin, 1: Acenaphthene, 2: Anthracene, 3: Benz(a)anthracene, 4: Benzo(b)fluoranthene, 5: Benzo(k)fluoranthene, 6: Chrysene, 7: Dibenzo(a,h)anthracene, 8: Fluorene, 9: Fluoranthene, 10: Naphthalene, 11: Pyrene, 12: Phenanthrene, 13: Carbazole.

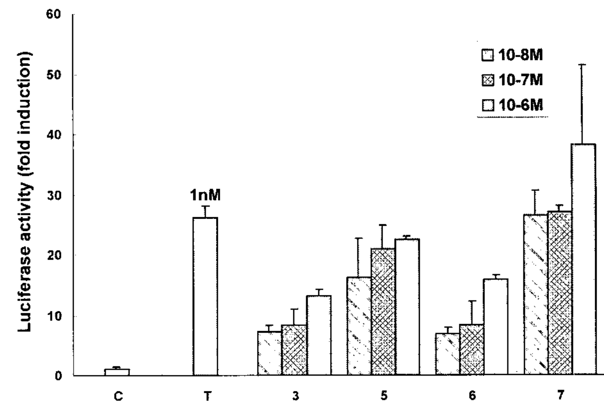

Fig. 2. The dose effect of PAHs on the luciferase activity in Hepa-I cells transfected with *pmCyp1a15'-Luc*. After transfection, cells were treated with 1nM TCDD, or 1 μM, 0.1 μM, 0.01 μM PAHs for 24 hours. The amount of luciferase transcription was normalized to the amount of protein. The data represent mean ± S.D. (n=3) C: Control, T: 2,3,7,8-TetraChloroDibenzo-Dioxin, 3: Benz(a)anthracene, 5: Benzo(k)fluoranthene, 6: Chrysene, 7: Dibenzo(a,h)anthracene.

fold induction of 1A1 promoter activity over control cells. 1uM dibenzo(a,h)anthracene treatment showed 46-fold induction, 1uM benz(a) anthracene treatment showed 30-fold induction, 1uM chrysene treatment showed 25-fold induction, 1uM benzo(b)fluoranthene treatment or 1uM Carbazole treatment showed 5-fold induction, 1uM phenanthrene or 1uM acenaphthene treatment showed 3.5-fold induction, 1uM anthracene treatment showed 3-fold induction, 1uM naphthalene, or 1uM pyrene or 1uM phenanthrene treatment showed 2.5-fold induction, 1uM fluoranthene treatment showed 2-fold induction over control, respectively. Among 13 different PAHs tested, 1 uM fluoranthene treatment did not induce luciferase activity over untreated control Hepa 1 cells (Fig. 1). The biological activities of selected 13 PAHs in terms of CYP1A1 promoter activity stimulation varied considerably. Fluoranthene showed no response, and acenaphthene, or anthracene, fluorine, or naphthalene, or pyrene, or phenanthrene, or carbazole showed weak response. Benz(a) anthracene, or benzo(b)fluoranthene, or benzo(k) fluoranthene, or chrysene, or dibenzo(a,h)anthracene showed strong response to cyp1a1 promoter activity stimulation.

Three different concentrations (0.01uM, 0.1uM, 1uM) of four strong responding PAHs, such as benzo(a) anthracene benzo(k)fluoranthene, chrysene, and dibenzo (a, h) anthracene in their potency to induce cyp1a1 promoter activity, were measured in the hepa 1 cells trnsfected with cyp1a1-Luc expression plasmid. All four tested PAHs showed concentration dependent stimulation of luciferase reporter gene expression (Fig. 2). These results suggested that four strong responding PAHs, such as benzo(a)anthracene benzo(k)fluoranthene, chrysene, and dibenzo(a, h)anthracene might be mediated through arylhydrocarbon receptor system.

Effects of 13 PAHs on the EROD activity in mouse Hepa 1 cells

The effects of 13 selected PAHs on EROD induction were studied in the hepa 1 mouse cell bioassay system. 13 selected PAHs and TCDD as a reference were measured in their capability to stimulate EROD activity at 1uM concentration for PAHs and 1nM for TCDD. As the results shown in figure 3, acenaphthene, anthracene, fluorene, fluoranthene, naphthalene, pyrene, phenanthrene, and carbazole showed no or a very weak response on EROD activity in mouse hepa 1 cells. Benzo(k) fluoranthene, chrysene, and dibenzo(a,h)anthracene showed strong response to stimulate EROD activity in mouse hepa 1 cells (Fig. 3). EROD inducing activity of three different concentrations (0.01uM, 0.1uM, 1uM) of four strong respnding PAHs such as, benz(a)anthracene, benz(k)fluoranthene, chrysene, and dibenzo(a,h)anthracene were measured in hepa 1 cells in vitro bioassay. As shown in Fig. 4, benz(a)anthracene, benz(k)fluoranthene, chrysene, and dibenzo(a,h)anthracene resulted in con-

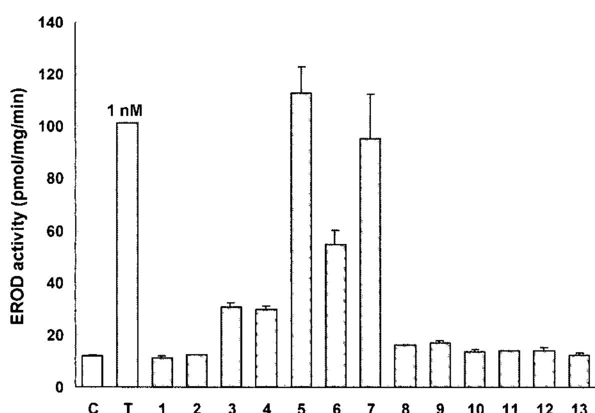

Fig. 3. The effect of PAHs on the EROD activity in Hepa-I cells. Cells were treated with 1 nM TCDD or 1 μM PAHs for 24 hours. EROD activity was determined as described in methods. The data represent mean ± S.D. (n=3) C : Control, T: 2,3,7,8-TetraChloroDibenzoDioxin, 1: Acenaphthene, 2: Anthracene, 3: Benz(a) anthracene, 4: Benzo(b)fluoranthene, 5: Benzo(k)fluoranthene, 6: Chrysene, 7: Dibenzo(a,h)anthracene, 8: Fluorene, 9: Fluoranthene, 10: Naphthalene, 11: Pyrene, 12: Phenanthrene, 13: Carbazole.

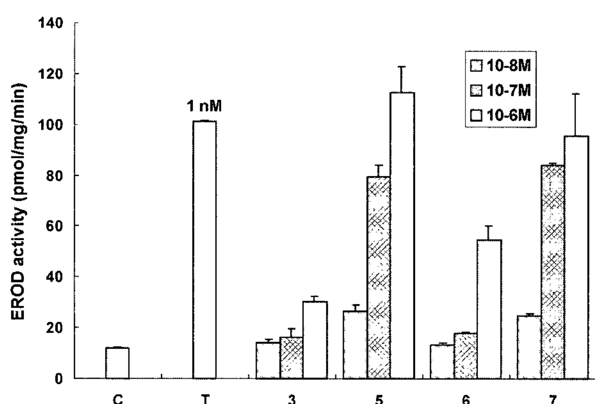

Fig. 4. The dose effect of PAHs on the EROD activity in Hepa-I cells. Cells were treated with 1 nM TCDD, or 1 μM, 0.1 μM, 0.01 μM PAHs for 24 hours. EROD activity was determined as described in methods. The data represent mean ± S.D. (n=3) C: Control, T: 2,3,7,8-TetraChloroDibenzoDioxin, 3: Benz(a)anthracene, 5: Benzo(k)fluoranthene, 6: Chrysene, 7: Dibenzo(a,h)anthracene.

centration dependent response to EROD induction. Benz(a) anthracene, benz(k)fluoranthene, chrysene, and dibenzo(a,h) anthracene responded strongly to EROD and cyp1a1 promoter activity. Acenaphthene, or anthracene, fluorine, or naphthalene, or pyrene, or phenanthrene, or carbazole seemed to respond less to EROD than cyp1a1 promoter activity.

Discussions

Our data showed that the inducibility of CYP1A1 promoter activity by TCDD was correlated to that of EROD in mouse hepa 1 cells. As shown in our study, PAHs with three aromatic rings are hardly capable to induce EROD activity in hepa 1 cells. The results suggest that PAH with three rings structure may not meet the structural requirements to bind to the Ah-receptor. Piskorska-Pliszczynska *et al* (1986) also suggested that low receptor binding affinities are of major importance to the non-responsiveness as generally observed for these compounds. When our results were compared to the results obtained in other test systems including rat hepatocyte (Till *et al.*, 1999), a rat hepatoma cell line (Willett *et al.*, 1997), rainbow trout liver cell line (Bols *et al.*, 1999), a fish cell line (Fent *et al.*, 2000) and the CALUX assay (Machala *et al.*, 2001), it was shown that anthracene, naphthalene, and phenanthrene did not show an effect in any of the difference test system and consequently could be regarded as non-responders (Willett *et al.*, 1997; Bola *et al.*, 1999, till *et al.*, 1999; fent *et al.*, 2000). In our experiment, anthracene, naphthalene and phenanthrene showed weak response based on CALUX assay in hepa 1 cells as well as EROD bioassay in hepa 1 cells (Fig. 2 and 4). Our data showed that both EROD bioassay and CALUX assay resulted in the same strong response with benzo(a)anthracene, benzo(k)fluoranthene, chrysene, and dibenzo(a,h)anthracene. However, with weak responding PAHs based on CALUX assay such as acenaphthene, anthracene, fluorine, pyrene no response by EROD bioassay in hepa 1 cells. This strongly suggested that CALUX assay might give more sensitive measurement of PAHs that EROD bioassay. This might indicated that CALUX assay might be suitable for high throughput screening bioassay system.

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