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Bioassays of Polycyclic Aromatic Hydrocarbons using *CYP1A1*-luciferase Reporter Gene Expression System in Human Breast Cancer MCF-7 Cells

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ABSTRACT : **Biological activities of PAHs are not known although PAHs are considered as carcinogens. 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. Our laboratory have been studied the effect of PAHs in the human breast cancer MCF-7 cells. In this study, we examined the human breast cancer MCF-7 cells as a new 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 MCF-7 cells transiently. This cells then used for the study to observe the effect of PAHs. We demonstrated that PAHs induced the CYP1A1 promoter, CYP1A1 mRNA and 7-ethoxyresolufin O-deethylase (EROD) activities in a concentration-dependant manner. None of PAHs that we have tested showed stronger stimulatory effect on CYP1 gene expression than TCDD. Benz(a)anthracene and benzo(b)fluoranthene were weak responders to *CYP1A1* promoter activity stimulation, CYP1A1 mRNA and EROD induction in MCF-7 cells and these chemicals seemed to respond less either CYP1A1 mRNA or EROD than *CYP1A1* promoter activity. Benzo(k)fluoranthene, chrysene, and dibenzo(a,h)anthracene showed strong response to *CYP1A1* promoter activity stimulation, CYP1A1 mRNA increase and also EROD induction in MCF-7 cells. Results of dose response study suggested that two strong responding PAHs, such as benzo(k)fluoranthene and dibenzo(a,h)anthracene might be mediated through Aryl hydrocarbon receptors system in MCF-7 cells.**

Key words : **PAH, EROD, TCDD, MCF-7**

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; Luchi *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.*,

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1993; Denison and Whitlock, 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 MCF-7 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 MCF-7 human breast cancer 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.). Agarose was purchased from FMC, Hind III were ordered from by Gibco BRL. Tfx™-50, pGL3 basic vector and luciferase assay system were purchased from Promega.

Construction of *CYP1A1*-Luc

Human *CYP1A1* 5'-flanking DNA was cloned into pGL3 vector at Hind III site.

Cell culture and transfection

MCF-7 human breast cancer cells were cultured in MEM media supplemented with 5% (v/v) fetal bovine serum and penicillin-streptomycin (100U/mL). For the transfection of *phCYP1A1-luc*, MCF-7 cells were seeded in 24-well plates. 400 ng of phCyp1A1-luc and 1.2 μl of Tfx™-50 (Promega) were mixed in 200 μl of serum-free medium and incubated at room temperature for 15 minutes before being added to each well. Cells were incubated for at least 1 hr before cells were maintained in normal MEM media containing 5% fetal bovine serum. The details were followed as supplier's manual

Chemical treatment

MCF-7 cells were rinsed with PBS three-times before the administration of various chemicals in serum-free medium. Stock solutions of chemicals were made in dimethyl sulfoxide (DMSO) as a vehicle and control cells were treated with 0.1% DMSO. Either 1 nM TCDD or PAHs (all 10^{-6}, 10^{-7}, 10^{-8} M) was administered for 24 hr. Final concentration of DMSO didnt exceed 0.1%.

Luciferase reporter assay

Luciferase assays were performed using the Luciferase Assay System (Promega). Briefly, the transfected cells were lysed with reporter lysis buffer. The lysates was incubated with luciferase substrate, and luciferase activity was determined with a luminometer. Protein assay of cell extracts was carried out using the Micro BCA protein assay reagent kit (Pierce) and an ELISA Reader (Bio-rad). Luciferase activity was normalized to protein content. The data are presented as the fold induction of control cells that were treated with 0.1% DMSO.

EROD assay

Ethoxyresorufin-O-dealkylase (EROD) activity was basically assayed as described by Kennedy S.W. with some modification. Cells were seeded in 48 well plates, allowed to attain 60% confluency, and adapted with serum free media for 24 hr. Cells were treated with TCDD and PAHs at the indicated concentration in legend. Sodium phosphate buffer was added to each well of 48 well plate. And then cells were pre-incubated with ethoxyresorufin (Sigma) at 37°C for 10 min. The reaction was started by adding β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH, Sigma). After incubation for 15 min in 37°C, the reaction was stopped by adding acetonitrile containing fluorescamine (Sigma). After 15 min the plates were scanned for resorufin (Sigma) with a 530 nm excitation and a 590 nm emission, and for proetin with a 360 nm excitation and a 460 nm emission. Fluorescence data were imported into Table Curve (Jandel scientific) for curve fitting.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNAs were exrtacted using Trizol reagent (Gibco-BRL) according to the manufacturer's instructions and cDNA were synthesized from 3 μg total RNA using reverse transcriptase (Gibco-BRL) in the presence of random primers in a 20 μl reaction volume at 37°C for 60 min.

1 μl of cDNA solution was amplified by Taq polymerase (Promega) in a volume of 10 μl. For mRNA detection of β-actin, GAPDH, mouse cyp1a1 and human CYP1A1 genes the PCR procedure was performed with 25 cycles of denaturation at 94°C for 0.5 min, annealing at 62°C for 0.5 min, and extention at 72°C for 1 min, with a predenaturing time of 5 min and a final extension time of 7 min. The primer sequences were GAPDH (150bp), 5'-ACATCGCTCAGACACCATGG-3' (sense) and 3'-GGGAAGTAACTGGAGTTGATG-5' (antisense); human CYP1A1 (146bp), 5'-TAGACACTGATCTGGCT GCAG-3' (sense) and 3'-GGGAAGGCTCCATCAGCATC-5' (antisense).

Following PCR amplification, the PCR products were run on a 2% agarose gel with ethidium bromide. The total band volumes of amplified products were calculated by alpha-image analyzer. All glassware and plastic ware was treated with diethyl pyrocarbonate (DEPC) and autoclaved.

Results

Effects of 13 PAHs on the human *CYP1A1* promoter activity

1 μM of 13 different PAHs and 1 nM TCDD were administered into MCF-7 cells transfected with expression plasmid that has human *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 Figure 1, 1 μM benzo (k)fluoranthene showed 44-fold induction of luciferase activity over control cells, and 1 nM TCDD showed 55-fold induction of *CYP1A1* promoter activity over control cells. 1 μM dibenzo(a,h)anthracene treatment showed 50-fold induction, 1 μM benz(a)anthracene treatment showed 10-fold induction, 1 μM chrysene treatment showed 9-fold induction over control, respectively. Among 13 different PAHs tested, 1 μM Carbazole or 1 μM phenanthrene or 1 μM naphthalene treatment did not induce luciferase activity over untreated control MCF-7 cells (Figure 1). The biological activities of selected 13

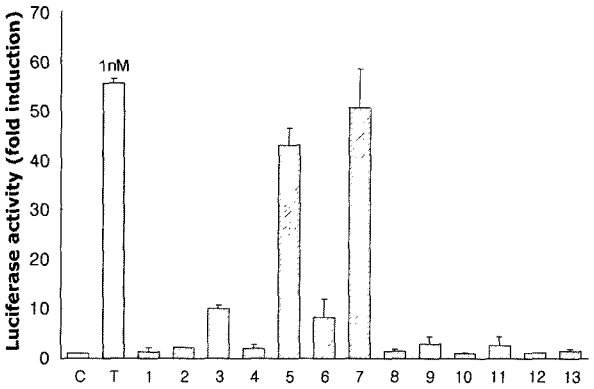

Fig. 1. The effect of PAHs on the luciferase activity in MCF-7 cells transfected with *phCYP1A15'-Luc*. After transfection, cells were treated with 1 nM TCDD or 1 μM PAHs for 24 hours. The amount of luciferase transcription was normalized to the amount of protein. The data represent mean ± S.D. (n=3) 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.

PAHs in terms of *CYP1A1* promoter activity stimulation varied considerably. Naphthalene or pyrene or phenanthrene, or carbazole showed no response, or Benz(a)anthracene, or chrysene showed weak response. Dibenzo(a,h)anthracene or benzo(k)fluoranthene showed strong response to *CYP1A1* promoter activity stimulation.

Three different concentrations (0.01 μM, 0.1 μM, 1 μM) of four 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 MCF-7 cells transfected with *CYP1A1*-Luc expression plasmid. All four tested PAHs showed concentration dependent stimulation of luciferase reporter gene expression (Figure 2). These results suggested that four 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 MCF-7

The effects of 13 selected PAHs on EROD induction were studied in the MCF-7 human breast cancer cell bioassay system. 13 selected PAHs and TCDD as a reference were measured in their capability to stimulate EROD activity at 1 μM concentration for PAHs and 1 nM for TCDD. As the results shown in Figure 3, acenaphthene, chrysene, anthracene, fluorene, fluoranthene,

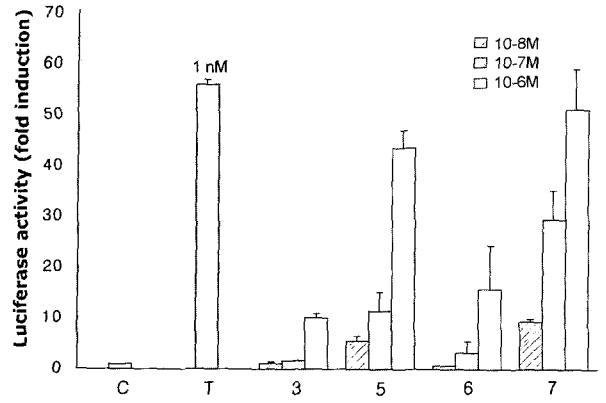

Fig. 2. The dose effect of PAHs on the luciferase activity in MCF-7 cells transfected with *phCYP1A15'-Luc*. After transfection, cells were treated with 1 nM TCDD, or 1 μM, 0.1 μM, 0.01 μM PAHs for 24 hours. The amount of luciferase transcription was normalized to the amount of protein. The data represent mean±S.D. (n=3) C: Control, T: 2,3,7,8-Tetrachlorodibenzodioxin, 3: Benz(a)anthracene, 5: Benzo(k)fluoranthene, 6: Chrysene, 7: Dibenzo(a,h)anthracene.

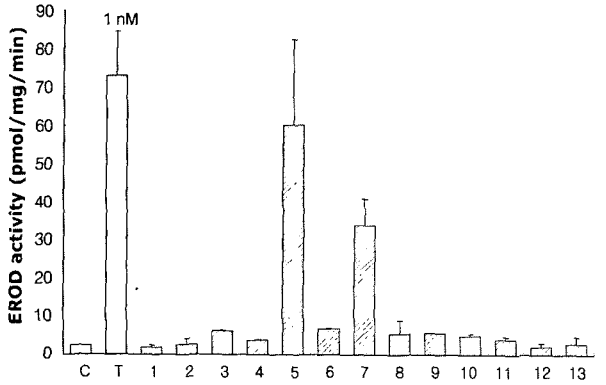

Fig. 3. The effect of PAHs on the EROD activity in MCF-7 cells. Cells were treated with 1 nM TCDD or 1 μM PAHs for 24 hours. EROD activity was determined as described in methods. The data represent mean ± S.D. (n=3) C: Control, T: 2,3,7,8-Tetrachlorodibenzodioxin, 1: Acenaphthene, 2: Anthracene, 3: Benz(a)anthracene, 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.

naphthalene, pyrene, phenanthrene, and carbazole showed no or a very weak response on EROD activity in human breast cancer cells. Benzo(k)fluoranthene, and dibenzo(a,h) anthracene showed strong response to stimulate EROD activity in MCF-7 human breast cancer cells (Figure 3). EROD inducing activity of three different concentrations (0.01 μM, 0.1 μM, 1 μM) of four responding PAHs such as, benz(a)anthracene, benzo(k)fluoranthene, chrysene, and dibenzo(a,h)anthracene were measured in MCF-7 cells in

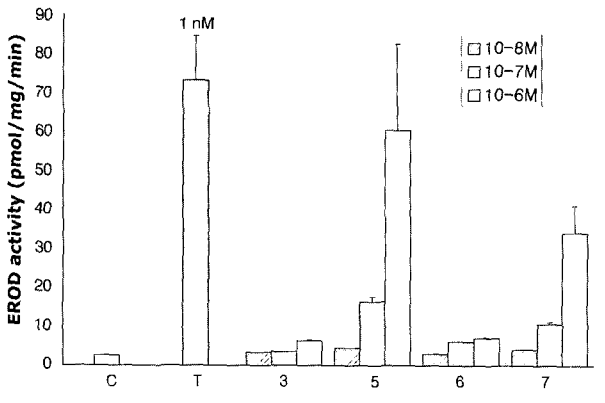

Fig. 4. The dose effect of PAHs on the EROD activity in MCF-7 cells. Cells were treated with 1 nM TCDD, orl μM, 0.1 μM, 0.01 μM PAHs for 24 hours. EROD activity was determined as described in methods. The data represent mean ± S.D. (n=3) C: Control, T: 2,3,7,8-Tetrachlorodibenzodioxin, 3: Benz(a)anthracene,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

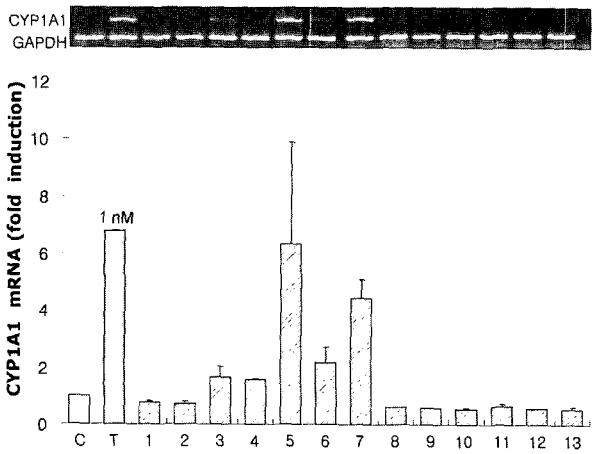

Fig. 5. The Effect PAHs on CYP1A1 mRNA in MCF-7 cells. Cells were treated with 1 nM TCDD or 1 μM PAHs for 24 hours and analysed by RT-PCR as described in methods. Each RT-PCR product was analysed by image analyser. The amount of CYP1A1 mRNA was normalized to the amount of GAPDH mRNA. The data represent 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.

vitro bioassay. As shown in Figure 4, benz(k)fluoranthene, and dibenzo(a,h)anthracene resulted in concentration dependent response to EROD induction. Benzo(k) fluoranthene, and dibenzo(a,h)anthracene responded strongly to EROD and *CYP1A1* promoter activity. Benz(a)anthracene and chrysene seemed to respond less to EROD than *CYP1A1* promoter activity.

Effect of 13 PAHs on the level of CYP1A1 mRNA

1 μM of different PAHs and 1 nM TCDD were administered into human breast cancer MCF-7 cells for 24 hours and the level of CYP1A1 mRNA were measured by RT-PCR as described in methods.

As shown in Figure 5, 1 μM benz(a)anthracene treatment showed 2-fold induction of CYP1A1 mRNA level, 1M benzo(k)fluoranthene treatment showed 6-fold induction, 1 μM chrysene treatment showed 2-fold induction, 1 μM dibenzo(a,h)anthracene treatment showed 2-fold induction, 1 μM benzo(b)fluoranthene treatment showed 2-fold of CYP1A1 mRNA level, respectively. Among 13 different PAHs tested, 1 μM fluoranthene or 1 μM naphthalene or 1 μM acenaphthene or 1 μM anthacene or 1 μM fluorene or 1 μM naphthalene or 1 μM phenanthrene or 1 μM carbazole treatment did not increase CYP1A1 mRNA level over untreated control cell.

Discussion

Our data from this study showed that MCF-7 human breast cancer cells responded to PAH as other hepatic cell line did in terms of *CYP1A1* promoter activity, EROD and CYP1A1 mRNA level increases. In other words, MCF-7 cells could be used for the bioassay of PAHs, although some PAHs were responded differently between breast cancer cell line and hepatic cell lines. Benz(a)anthracene, benzo(b)fluoranthene, chrysene were strong stimulators of *cyp1a1* promoter and EROD stimulators in use hepa 1 cells, however, these PAHs showed very weak activity in MCF-7 human breast cancer cells in term of stimulation of CYP1A1 promoter activity and EROD. 1 μM benzo(k)fluoranthene or 1ìM dibenzo(a,h)anthracene showed very strong stimulation of CYP1A1 mRNA level (Fig. 5), and this CYP1A1 mRNA increase was correlated to the EROD and CYP1A1 promoter activity stimulation.

Our data showed that the inducibility of CYP1A1 promoter activity by TCDD was correlated to that of CYP1A1 mRNA and EROD in MCF-7 cells. As shown in our study, PAHs with three aromatic rings are hardly capable to induce EROD activity in MCF-7 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, benzanthracene and chrysene showed weak response based on CALUX assay in MCF-7 cells as well as EROD bioassay in MCF-7 (Figure 2 and 4). Our data showed that both EROD bioassay and CALUX assay resulted in the same strong response with benzo(k)fluoranthene, and dibenzo(a,h) anthracene. However, with weak responding PAHs based on CALUX assay such as benzanthracene and chrycene showed no response by EROD bioassay in MCF-7 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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