

Antiestrogenicity of environmental polycyclic aromatic hydrocarbons in human breast cancer cells

Kathleen F. Arcaro^{a,b,*}, Patrick W. O'Keefe^{a,b}, Yi Yang^b, William Clayton^b,
John F. Gierthy^{a,b}

^a School of Public Health, State University of New York at Albany, Albany, NY 12222, USA

^b Wadsworth Center, New York State Department of Health, Albany, NY 12201, USA

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Abstract

The total concentration of 14 polycyclic aromatic hydrocarbons (PAHs) was determined to be 3400-fold greater in a sediment sample from an industrial site on the St. Lawrence River (SLR), NY, than in a sediment sample from a non-industrial site on the Kinderhook Creek (KC), NY. PAH fractions from extracts of the two environmental samples and two reconstituted mixtures as well as the 14 individual PAHs were examined for their toxic, estrogenic, and antiestrogenic activities using MCF-7 focus, recombinant human estrogen receptor (ER) binding, whole-cell ER binding, and 17 β -estradiol (E₂) metabolism assays. PAH fractions from the KC and SLR were antiestrogenic; they significantly inhibited the formation of foci elicited in MCF-7 breast cancer cells by 1 nM E₂. Eight of the 14 individual PAHs, and the reconstituted mixtures were also antiestrogenic. Results from the whole-cell ER binding assay and the radiometric analysis of E₂ metabolism indicate that the PAHs detected in the KC and the SLR environmental samples induce antiestrogenic responses in metabolically intact human breast cancer cells through at least two mechanisms: one involving competition for the ER by a PAH metabolite and the other involving depletion of E₂ through induction of metabolism. Published by Elsevier Science Ireland Ltd.

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Abbreviations: CYP, cytochrome P450; DMSO, dimethyl sulfoxide; E₂, 17 β -estradiol; KC, Kinderhook Creek; PAHs, polycyclic aromatic hydrocarbons; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzo dioxin; PCDF, polychlorinated dibenzo furan; rhER, recombinant human estrogen receptor; SLR, St. Lawrence River; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

* Corresponding author. Tel.: +1-518-4862553; fax: +1-518-4861505.

E-mail address: arcaro@wadsworth.org (K.F. Arcaro)

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic organic chemicals comprising hundreds of individual compounds. They are released into the environment primarily by oil spills, industrial processes, and the incomplete combustion of fossil fuels, wood, and other organic matter (Menzie et al., 1992). The principal risk currently associated with exposure to environmental PAHs is cancer, which is a consequence of the mutagenicity of the bioactivated forms of PAHs. Bioactivation of PAHs is mediated primarily by cytochrome P450 (CYP) mixed function oxidases and epoxide hydrolase.

A growing body of literature identifies PAHs as environmental endocrine disruptors (Santodonato, 1997). PAHs may act as antiestrogens by binding with the Ah receptor (Bigelow and Nebert, 1982; Piskorska-Pliszczynska et al., 1986; Chaloupka et al., 1992; Clemons et al., 1998), leading to induction of Ah-responsive genes that result in a broad spectrum of antiestrogenic responses, or PAHs may act as antiestrogens by antagonistically binding the estrogen receptor (ER) (Tran et al., 1996). PAHs also have been reported to act as weak estrogens by binding the ER (Schneider et al., 1976; Clemons et al., 1998).

Due to the concern that environmental pollutants may be disrupting the reproductive endocrine systems of wildlife and humans (Guillette et al., 1994; Jobling et al., 1995; Hileman, 1996; Kavlock et al., 1996; Skakkebaek and Meyer, 1996) we examined the estrogenic and antiestrogenic activity of PAHs detected in sediment extracts from two rivers in upstate New York: the Kinderhook Creek (KC) near Albany and the St Lawrence River (SLR) in the vicinity of Massena. The KC is a relatively undeveloped tributary of the Hudson River, whereas the sampling site on the SLR was close to the outfall from an aluminum plant that is known to have released high concentrations of PAHs into the aquatic environment (Wood et al., 1997). We monitored 14 PAHs (fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[e]pyrene, benzo[a]pyrene, indeno[1,2,3-cd]-

pyrene, dibenz[a,h]anthracene, and benzo[ghi]perylene). All 14 were detected in purified fractions from both extracts. The PAH fractions from the sample extracts, the individual PAHs, and the reconstituted mixtures were screened for their estrogenicity and antiestrogenicity in the MCF-7 focus assay, which measures estrogen-dependent postconfluent growth (Gierthy et al., 1991). Mixtures and individual PAHs were also examined for their ability to alter the metabolism of 17 β -estradiol (E₂), and to displace [³H]E₂ in the recombinant human estrogen receptor (rhER) and in the MCF-7 whole-cell ER binding assays.

2. Materials and methods

2.1. Chemicals

PAHs were obtained from Ultra Scientific (North Kingston, RI), and Accustandard (New Haven, CT) and 5 mM stock solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma). Recombinant human ER α and β were obtained from Panvera (Madison, WI.). [2,4,6,7,16,17-³H]E₂ (specific activity, 140–150 Ci/mmol) and [2,4,6,7-³H]E₂ (specific activity, 70 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA).

Culture medium (DC₅) consisted of Dulbecco's modified Eagle's medium (phenol red-free) supplemented with 5% bovine calf serum (Hyclone), insulin (10 ng/ml), L-glutamine (2 mM), non-essential amino acids, penicillin (100 U/ml), and streptomycin (100 μ g/ml). The complete medium was filter sterilized (0.2- μ m pore-size nalgene filter units).

2.2. Extraction

The St. Lawrence River sample was collected close to the outfall of an aluminum smelter, 3 miles east of Masena, NY. A hand-held coring device was used to collect approximately twenty 5 cm \times 20 cm sediment cores at a water depth of 6 ft. Before air-drying, the sample was passed through a 600 mesh sieve to remove stones, vege-

tation, etc. The Kinderhook Creek sample was collected close to the riverbank using a shovel. The sediment was sieved and air-dried as above. Complete details of the extraction and analysis of PAHs have been published previously (O'Keefe et al., 1997). Briefly, the PAHs were extracted from air-dried sediments (10 g) by Soxhlet extraction with toluene for 16 h. The polar organic contaminants and natural organic compounds such as humic acids were then removed from the extracts using a multiadsorbent column containing silica gel, KOH-treated silica gel, and silica gel bonded with benzenesulfonic acid. After solvent exchange to nonane, the extract was separated into a polychlorinated biphenyl (PCB)/polychlorinated dibenzo dioxin (PCDD)/polychlorinated dibenzo furan (PCDF) fraction and a PAH fraction using a chromatography column containing 13–24 µm LPS-1 silica gel (Whatman, Clifton, NJ). The PCB/PCDD/PCDF fraction was eluted with 50:50 dry/wet hexane and the PAH fraction was eluted with 20% methylene chloride/hexane. The dry hexane was prepared by passing hexane as received from the manufacturer through an alumina column and the wet hexane was prepared by equilibrating hexane with deionized distilled water in a separatory funnel.

Aliquots from the extracts were spiked with a mixture of 17 deuterated PAHs and analyzed by capillary gas chromatography/low-resolution

mass spectrometry using a DB5 column coupled to a Hewlett-Packard 5970 Mass Selective Detector (Hewlett-Packard Company, Wilmington, DE). The most prominent molecular ions for the 16 US Environmental Protection Agency (USEPA) Priority Pollutant PAHs (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene, benzo[ghi]perylene) and their deuterated analogs, plus benzo[e]pyrene, were monitored. Table 1 lists the 13 of the 16 USEPA Priority Pollutant PAHs examined in the present study plus benzo[e]pyrene. Data are not presented for three of the 16 USEPA Priority Pollutant PAHs (naphthalene, acenaphthalene, and acenaphthene) because they were recovered at low levels by the current methodology. Detection limits for the individual PAHs ranged from 50 to 150 pg/g sediment dry wt.

2.3. MCF-7 focus assay

The MCF-7 focus assay, in which human breast cancer cells respond to E₂ by producing multicellular nodules or foci on a confluent monolayer background, was conducted as previously described (Gierthy et al., 1991; Arcaro et al., 1998).

Table 1
PAH concentrations in sediment extracts

Compound	Kinderhook Creek (µg/g dry weight)	St. Lawrence River (µg/g dry weight)	Ratio SLR/KC
Fluorene	0.02	32	1600
Phenanthrene	0.05	220	4400
Anthracene	0.01	48	4800
Fluoranthene	0.14	300	2100
Pyrene	0.10	240	2400
Benzo[a]anthracene	0.05	120	2400
Chrysene	0.06	300	5000
Benzo[b]fluoranthene	0.08	300	3800
Benzo[k]fluoranthene	0.03	92	3100
Benzo[e]pyrene	0.05	200	4000
Benzo[a]pyrene	0.08	66	800
Indeno[1,2,3-cd]pyrene	0.03	68	2300
Dibenz[a,h]anthracene	0.01	24	2400
Benzo[ghi]perylene	0.04	88	2200

Briefly, MCF-7 cells were suspended in DC₅ after treatment with trypsin (0.25%), seeded into 24-well plastic tissue culture plates at a density of 1×10^5 cells/ml per well, and placed in a 37°C, humidified, CO₂ incubator. Cells were refed at 24 h and every 3–4 days thereafter with 2 ml of DC₅ containing various concentrations of the test compounds in DMSO ($\leq 0.1\%$). The test media for dose-response curves of the extracts, reconstituted mixtures, and individual PAHs were prepared using 1:10 serial dilutions from the highest concentration (usually 5 μ M). Cells in the 24-well plates were visually inspected for evidence of cytotoxic or cytostatic effects of the test compound. Cytotoxic effects were indicated by changes in cell morphology, e.g. pycnosis, lysis or detachment; cytostatic effects were indicated by a delay in reaching confluence as compared to the control wells. After 14 days the cultures were fixed with formalin and stained with 1% Rhodamine B. The stained foci were counted using a New Brunswick Biotran II automated colony counter (Edison, NJ). The extracts, reconstituted mixtures, and individual PAHs were tested for both their ability to induce foci and to inhibit the foci induced by 1.0 nM E₂, the minimum dose necessary to induce maximal response in the focus assay.

2.4. Competitive estrogen-receptor binding assay

The competitive ER binding assay was conducted as previously described (Arcaro et al., 1998). Briefly, 1.2 nM recombinant human estrogen receptor (rhER) α or β was incubated for 4 h at room temperature with E₂ or PAHs in the presence of [2,4,6,7-³H]E₂ (2.5 nM); 100 μ l of a 50% (v/v) hydroxyapatite slurry was then added to the reaction mixture. The hydroxyapatite-bound receptor–[³H]E₂ complex was separated by centrifugation at $200 \times g$ for 20 min and the radioactivity of the pellet was counted in a Beckman LS 2800 liquid scintillation counter (Irvine, CA). The amount of receptor-bound [³H]E₂ in the presence or absence of the test compounds was calculated after correcting for non-specific binding as measured by the amount of bound [³H]E₂ in the presence of 200-fold excess E₂. Reconstituted mixtures and individual PAHs were tested in tripli-

cate at each of four concentrations (5.0, 0.5, 0.05, and 0.005 μ M) in at least two separate experiments.

2.5. Whole-cell competitive estrogen-receptor binding assay

The whole-cell binding assay was similar to that previously reported (Gierthy et al., 1996). Briefly, MCF-7 cells suspended in DC₅ were seeded into 24-well plates at a density of 5×10^5 cells/ml per well. Twenty-four hours later, the seeding medium was changed to DC₅ containing 1.0 nM [2,4,6,7,16,17-³H]E₂ and varying concentrations of either E₂ or the test compounds (reconstituted mixtures or individual PAHs), and incubated at 37°C for 3 h. The cultures were then washed three times with PBS, and 300 μ l of ethanol was added to each well for 20 min to solubilize the bound [³H]E₂. The radioactivity in 200 μ l of the ethanol extract was determined. The amount of bound [³H]E₂ in the presence or absence of the test compounds was calculated after correcting for non-specific binding as measured by the amount of bound [³H]E₂ in the presence of 200-fold excess E₂. Data are expressed as the ratio of bound [³H]E₂ in the presence of a competitor to the bound [³H]E₂ in control (0.1% DMSO) medium, $\times 100$. All compounds were tested with four replicates at each of three concentrations (5.0, 1.0, and 0.5 μ M) in at least three separate experiments. To investigate the role of metabolism of the PAHs in the whole-cell binding, we varied both the temperature and incubation times of the assay. The assay was conducted at both 4 and 37°C with both 1- and 3-h incubations, and with an incubation of the test compound at 37°C for 3 h followed by the addition of [³H]E₂ and further incubation at 4°C for 1 h.

2.6. Radiometric analysis of E₂ metabolism

Radiometric analysis of the metabolism of E₂ by enzymes induced by reconstituted mixtures of PAHs or individual PAHs was conducted with MCF-7 cells. MCF-7 cells were seeded in 24-well plates at 5×10^5 cells/ml per well. After 24 h, cells were refed with either DC₅, 1.0 nM 2,3,7,8-tetra-

chlorodibenzo-*p*-dioxin (TCDD), or 5 μ M of the PAH mixture or individual PAHs. After 72 h of incubation, 2.0 ml of medium from each well was removed and mixed with 0.2 ml of 10 nM [2,4,6,7,16,17- 3 H] E_2 and then returned. Media were collected after 24 h, and the [3 H] E_2 was separated from the media by adsorption on charcoal. The tritium recovered in the media (present as tritiated water) was then measured in a Beckman liquid scintillation counter. The amount of dissociated tritium is an indication of the degree of E_2 metabolism at any or all of the [3 H]-substituted positions. The reconstituted mixtures and the 14 individual PAHs were tested at four concentrations with four replicates at each concentration. All compounds were tested in at least two separate experiments. Data from representative experiments are presented as the percent of metabolism as compared to that induced by TCDD, a strong inducer of E_2 metabolism. To determine whether the increased metabolism of E_2 could account for the decrease in specific binding of [3 H] E_2 observed in the whole-cell binding assay, the radiometric assay was also conducted with a 3-h incubation of the [2,4,6,7,16,17- 3 H] E_2 and the individual PAHs or the reconstituted mixtures.

2.7. Statistics

SigmaPlot[®] software (Jandel Scientific Software, San Rafael, CA) was used to analyze data and perform linear regressions from which the IC_{50} s were calculated from each curve. Comparisons were made with Student's *t*-tests.

3. Results

3.1. Environmental extracts and reconstituted mixtures

Sediment samples from the KC and SLR were analyzed for the presence of 14 PAHs (13 of the USEPA Priority Pollutant PAHs and benzo[*e*]pyrene). The PAHs detected in the samples are listed in Table 1 in order of increasing molecular weight. All 14 PAHs were detected in both

the KC- and the SLR-extracts. However, as expected, the concentrations of individual PAHs in the SLR-extract were much higher—generally 2000–5000 times greater than those detected in the KC-extract. The concentration of PAHs in the SLR-extract ranged from 24 μ g/g (dibenz[*a,h*]anthracene) to 300 μ g/g (fluoranthene, chrysene and benzo[*b*]fluoranthene). The concentration of the PAHs in the KC-extract ranged from 0.01 μ g/g (dibenz[*a,h*]anthracene) to 0.14 μ g/g (fluoranthene).

The purified PAH-fractions from the extracts were next examined for their estrogenic and anti-estrogenic activity in human breast cancer cells. Fractions from 2.0 g of sediment from the KC and from 0.1 g of sediment from the SLR were tested in the MCF-7 focus assay for their ability to elicit and inhibit the formation of foci. Neither the KC- nor the SLR-extracts were estrogenic, i.e. adding the PAH-extract to the medium of MCF-7 cells did not elicit the formation of foci as compared to the control medium, DC₅ (Fig. 1). Both the KC-extract and the SLR-extract were anti-estrogenic. As can be seen in Fig. 1, both extracts significantly inhibited the foci induced by 1.0 nM E_2 (KC IC_{50} = 30 nM; SLR IC_{50} = 50 nM).

It is possible that the PAH fractions contained compounds other than the extracted and identified PAHs, therefore we tested reconstituted mixtures. Reconstituted mixtures, comprising the individual 14 PAHs detected in the KC- and SLR-extracts (Table 1), were prepared in DMSO as 5 mM (total concentration) stock solutions and tested in the MCF-7 focus assay. Neither of the reconstituted mixtures were estrogenic at any of the concentrations tested (Figs. 2 and 3). This is consistent with the results from the sediment extracts. Both of the reconstituted mixtures were antiestrogenic, which is also consistent with the antiestrogenic results from the sediment extracts. The IC_{50} s for the reconstituted KC-mixture (IC_{50} , 110 nM; Fig. 2) and reconstituted SLR-mixture (IC_{50} , 300 nM; Fig. 3) were higher than the IC_{50} s for the extracts (see Discussion).

To determine whether the observed antiestrogenicity of the two PAH mixtures in the MCF-7 focus assay was due to cytostatic or cytotoxic

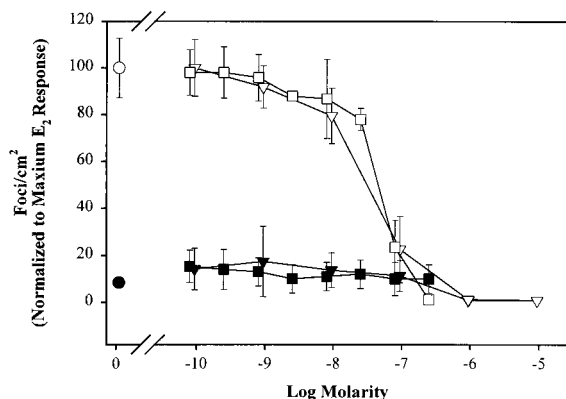


Fig. 1. Tests for estrogenicity (filled symbols) and antiestrogenicity (open symbols) in MCF-7 cell cultures by PAH-extracts from the KC (■□) and SLR (▼▽). To test for estrogenicity, the KC-extract (■) and the SLR-extract (▼) were added to the DC₅ medium of MCF-7 cell cultures and the percent of foci induced by the extracts was compared to the foci induced by 1 nM E₂ (○). To test for antiestrogenicity, the KC-extract (□) and the SLR-extract (▽) were added to the DC₅ medium of MCF-7 cell cultures containing 1 nM E₂ and inhibition of focus formation was calculated as a percent of focus formation induced by E₂. Means and standard deviations are shown; *n* = 4 at each concentration; ●, DC₅ control medium. Molarity refers to the sum of the molarities of the individual PAHs detected in the extracts.

effects of the PAHs, we examined the daily growth of cells in each well during a 14-day focus assay. Cells in all wells reached 90% confluency by day 4 and were confluent by day 5, indicating that inhibition of the development of foci in the presence of the PAH extract was not due to toxicity or retarded cell growth.

There are a number of mechanisms by which the PAH-mixtures could inhibit the foci induced by 1 nM E₂: the PAHs in the reconstituted mixtures may directly bind to the ER and inhibit estrogen-dependent responses, the PAHs may be metabolized in MCF-7 cells to compounds that bind the ER, or the PAHs may induce CYP enzymes that increase the metabolism of E₂. We investigated these three mechanisms.

To determine whether the PAHs in the mixtures bind directly to the ER, we tested the KC- and SLR-mixtures at four concentrations (5, 0.5, 0.05 and 0.005 μM) in competitive binding assays with rhERα and rhERβ. Neither of the mixtures sig-

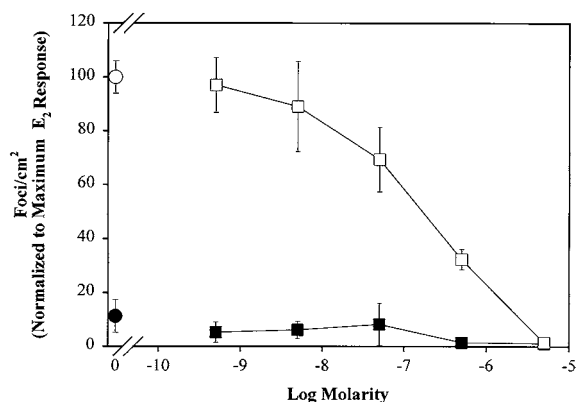


Fig. 2. Test for estrogenicity (■) and antiestrogenicity (□) by the reconstituted KC-mixture. A mixture was prepared containing the 14 PAHs detected in the KC-extract. The mixture was tested in the MCF-7 focus assay in the absence and presence of 1 nM E₂ as described in Section 2. Means and standard deviations are shown; *n* = 4 at each concentration for both curves. ●, DC₅ control medium; ○, 1 nM E₂.

nificantly inhibited the binding of [³H]E₂ to either rhERα or rhERβ (data not shown). In contrast, E₂ and LY156758, an ER antagonist, significantly displaced [³H]E₂ from ERα (IC₅₀s, 0.5 and 0.8 nM, respectively), and from ERβ (IC₅₀s, 0.9 and 1.4 nM, respectively). These data indicate that the PAHs do not directly bind the ER. Therefore we

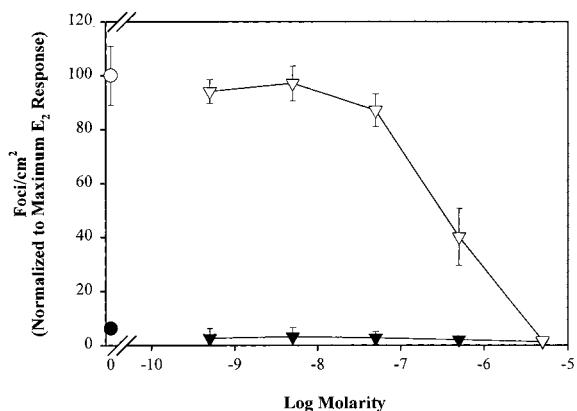


Fig. 3. Test for estrogenicity (▼) and antiestrogenicity (▽) by the reconstituted SLR-mixture. A mixture was prepared containing the 14 PAHs detected in the KC-extract. The mixture was tested in the MCF-7 focus assay in the absence and presence of 1 nM E₂ as described in Section 2. Means and standard deviations are shown; *n* = 4 at each concentration for both curves. ●, DC₅ control medium; ○, 1 nM E₂.

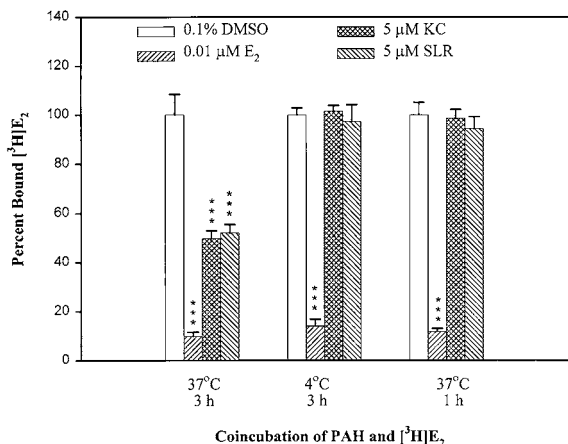


Fig. 4. Displacement of $[^3\text{H}]\text{E}_2$ in the MCF-7 whole-cell ER binding assay by 0.1% DMSO (vehicle control), 0.01 μM E_2 , 5 μM KC-mixture or 5 μM SLR-mixture at 37 and 4°C with a 3-h incubation and at 4°C with 1-h incubation. Means and standard deviations from a representative experiment are shown; $n = 4$ for each group; $***P < 0.001$ for t -tests comparing test group with the DMSO control.

examined the possibility that the PAHs are metabolized in MCF-7 cells to compounds that bind the ER.

The reconstituted KC- and SLR-mixtures were tested in an MCF-7 whole-cell ER binding assay. In this assay the cells are incubated with both the PAH-mixture and $[^3\text{H}]\text{E}_2$ for 3 h at 37°C , during which time the PAHs may be metabolized and compete with $[^3\text{H}]\text{E}_2$ for binding to the ER. As can be seen in Fig. 4, both the KC-mixture and the SLR-mixture (5 μM in the culture medium) significantly displaced $[^3\text{H}]\text{E}_2$ in the MCF-7 whole cell binding assay (50 and 52%, respectively). Neither of the PAH-mixtures displaced $[^3\text{H}]\text{E}_2$ when tested at either 4°C or in a 1-h incubation at 37°C . These results suggest that (1) 4°C reduces the metabolism of PAHs to compounds that bind the ER, and (2) more than 1 h at 37°C is necessary for substantial metabolism and receptor binding to occur.

It is also possible that the observed displacement of $[^3\text{H}]\text{E}_2$ at 37°C was due to a decrease in cellular $[^3\text{H}]\text{E}_2$ caused by an increase in the metabolism of $[^3\text{H}]\text{E}_2$, as opposed to competition by metabolites of PAHs for binding to the ER. To examine this further, we repeated the whole-

cell binding assay but changed the incubation parameters to allow for potential metabolism of the PAHs while inhibiting the metabolism of the $[^3\text{H}]\text{E}_2$. When the MCF-7 cell cultures were incubated with either of the PAH-mixtures for 3 h at 37°C and then cooled to 4°C before the addition of $[^3\text{H}]\text{E}_2$ for 1 h at 4°C , the PAH-mixtures still significantly displaced $[^3\text{H}]\text{E}_2$ as compared with the DMSO control (Fig. 5). Conducting the $[^3\text{H}]\text{E}_2$ incubation at 37°C rather than 4°C resulted in a significant increase in the amount of displaced $[^3\text{H}]\text{E}_2$ (Fig. 5), suggesting that either there was further metabolism of the PAHs that resulted in increased displacement of $[^3\text{H}]\text{E}_2$, or that the PAH-mixture induced CYP enzymes that increased the metabolism of E_2 .

To directly determine whether the mixtures of PAHs increased the metabolism of E_2 , we conducted a radiometric analysis of E_2 metabolism. In this assay E_2 metabolism causes the release of tritium, which is measured as the amount of tritiated water recovered in cell culture medium. MCF-7 cells were incubated with PAH-mixtures

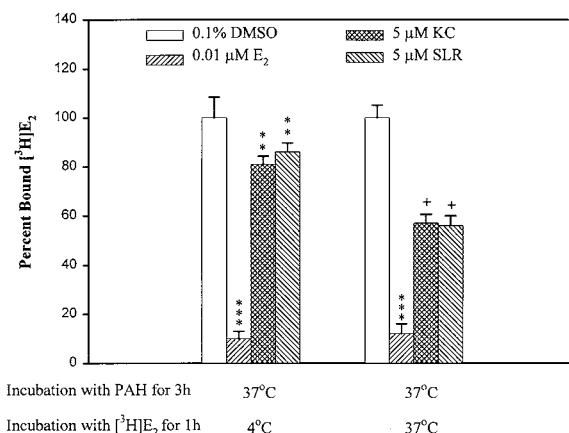


Fig. 5. Displacement of $[^3\text{H}]\text{E}_2$ in the MCF-7 whole-cell ER binding assay by 0.1% DMSO (vehicle control), 0.01 μM E_2 , 5 μM KC-mixture or 5 μM SLR-mixture. Cells were incubated with the PAH-mixture for 3 h at 37°C after which $[^3\text{H}]\text{E}_2$ was added and the cells were incubated for 1 h at either 4 or 37°C . Means and standard deviations from a representative experiment are shown; $n = 4$ for each group; $***P < 0.001$ and $**P < 0.01$ for t -tests comparing test compound with the DMSO control, $+P < 0.01$ for t -tests comparing the incubation temperatures of 4 versus 37°C for the 1-h $[^3\text{H}]\text{E}_2$ incubation for the KC and SLR mixtures.

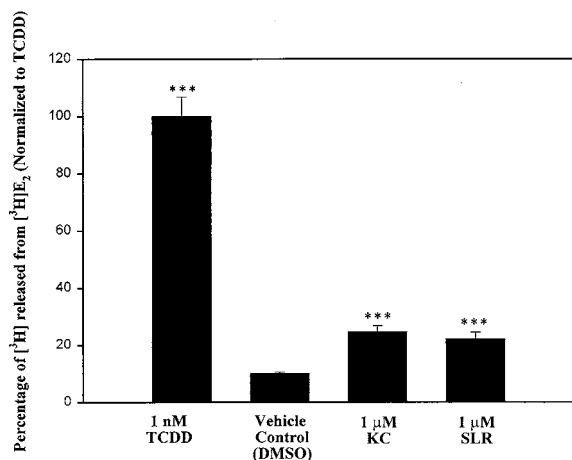


Fig. 6. Radiometric assay for metabolism of E₂ by reconstituted PAH-mixtures from the KC and SLR. Reconstituted PAH-mixtures were incubated for 72 h with MCF-7 cells prior to the addition of [³H]E₂ for an additional 24 h. Comparison with Student's *t*-test between DMSO control and test compounds, ****P* < 0.001, means and standard deviations are shown, *n* = 4 in each group.

for 72 h, [³H]E₂ was then added and the cells were incubated for an additional 24 h, and finally the tritium in the medium was measured. Fig. 6 shows that both the KC- and SLR-mixtures induced a significant increase in the metabolism of E₂ (twofold over background) as compared with DMSO control. Both of the PAH-mixtures increased significantly less metabolism of E₂ as compared with the positive control TCDD (Fig. 6).

3.2. Individual PAHs comprising the reconstituted mixtures

To determine which of the 14 PAHs comprising the mixtures contributed to the observed antiestrogenicity in the MCF-7 focus assay, we tested each of the individual PAHs. Eight (benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*e*]pyrene, benzo[*a*]pyrene, indeno[1,2,3-*cd*]pyrene and dibenz[*a,h*]anthracene) of the 14 PAHs were significantly antiestrogenic when tested in the MCF-7 focus assay. The IC₅₀s of these antiestrogenic PAHs ranged between 0.01 and 2.6

μM (Table 2). None of the 14 PAHs were estrogenic in the MCF-7 focus assay.

As with the reconstituted mixtures, none of the 14 PAHs bound rhERα or rhERβ in a competitive binding assay (Table 2). Results from the PAH-mixtures suggested that some of the PAHs were metabolized in MCF-7 cells to compounds that displaced [³H]E₂ in a whole-cell ER binding assay. To determine which of the PAHs may be metabolized to forms that displace [³H]E₂, we tested each of the 14 PAHs in the whole-cell binding assay at three concentrations (5.0, 1.0 and 0.5 μM). We initially conducted the assay with a single incubation including both the PAH and the [³H]E₂ at 37°C for 3 h. Under these conditions, all eight of the antiestrogenic PAHs (5 μM) significantly displaced [³H]E₂ (data not shown). To determine the extent that incubating the [³H]E₂ at 37°C for 3 h with the PAH could increase the metabolism of E₂, we repeated the whole-cell ER binding assay under conditions that suppressed the metabolism of [³H]E₂. MCF-7 cell cultures were preincubated with a PAH for 3 h at 37°C and then cooled to 4°C before the [³H]E₂ was added and incubation was continued for 1 h at 4°C. Under these conditions, seven of the eight antiestrogenic PAHs significantly displaced [³H]E₂ as compared with the DMSO control, and none of the non-antiestrogenic PAHs (fluorene, phenanthrene, anthracene, fluoranthene, and pyrene) displaced [³H]E₂ (Table 2). Dibenz[*a,h*]anthracene was the only antiestrogenic PAH that did not significantly displace [³H]E₂ in the whole-cell ER binding assay when conducted under conditions which eliminated metabolism of E₂. However, dibenz[*a,h*]anthracene did significantly displace [³H]E₂ when both the preincubation and the incubation with the [³H]E₂ were conducted at 37°C, suggesting that it may increase the metabolism of E₂.

To directly determine which of the PAHs increase the metabolism of E₂ we conducted the radiometric assay of E₂ metabolism with each of the 14 PAHs as we had previously done with the reconstituted mixtures. Four of the eight antiestrogenic PAHs (benz[*a*]anthracene, benzo[*k*]fluoranthene, indeno[1,2,3-*cd*]pyrene, and dibenz[*a,h*]anthracene) significantly increased the metabolism of E₂ (Table 2).

4. Discussion

Sixteen PAHs are included in the US Environmental Protection Agency's list of 106 priority pollutants (EPA, 1984). In the present study we detected 13 of these PAHs, and benzo[*e*]pyrene in sediment extracts from sites on two rivers (Table 1). Both extracts contained similar profiles of PAHs, however, the concentration of PAHs was 3400-fold greater in the extract from the more highly polluted site on the St. Lawrence River than from the less polluted site on the Kinderhook Creek. The total concentration of PAHs in the SLR-extract, 2098 µg/g, is greater than concentrations reported for a number of other polluted environments. For example Naes and Oug, (1998) reviewed results from four Norwegian fjords studied between 1983 and 1990 in which total PAHs ranged from of 0.3 to 784 µg/g of sediment.

There is concern that pollutants in the environment may disrupt reproductive systems by acting as estrogens or antiestrogens (Kavlock et al., 1996). Since PAHs are ubiquitous environmental pollutants and a few PAHs have been shown to exhibit weakly estrogenic and antiestrogenic responses in a variety of test systems (reviewed by Santodonato, 1997), we examined the PAH-fractions for their estrogenic and antiestrogenic activities in the MCF-7 focus assay, which measures post-confluent, estrogen-dependent cell growth. The PAH-fractions from the KC and the SLR inhibited the development of estrogen-dependent foci in MCF-7 cell cultures. A major question regarding this antiestrogenicity is the extent to which this activity was due to the PAHs detected in the fractionated extract as opposed to unidentified compounds. We addressed this question by testing reconstituted mixtures prepared to reflect the PAHs detected in the extracts. Qualitatively,

Table 2
Summary of results from the 14 PAHS identified in the extracts^a

Compound	Induce foci (EC ₅₀ µM)	Inhibit foci (IC ₅₀ µM)	Bind ER α or β (% inhibition)	Bind ER in MCF-7 cells (% inhibition ^b)	Metabolize E ₂ (% TCDD ^c)
DMSO control	ND	ND	0	0	11
TCDD control	ND	0.008	0	0	100
Fluorene	ND	ND	0	0	11
Phenanthrene	ND	ND	0	0	7
Anthracene	ND	ND	0	0	4
Fluoranthene	ND	ND	0	0	1
Pyrene	ND	ND	0	0	12
Benz[<i>a</i>]anthracene	ND	0.32	0	33*	19*
Chrysene	ND	0.03	0	13*	15
Benzo[<i>b</i>]fluoranthene	ND	0.07	0	17*	13
Benzo[<i>k</i>]fluoranthene	ND	0.01	0	27*	52*
Benzo[<i>e</i>]pyrene	ND	2.6	0	14*	12
Benzo[<i>a</i>]pyrene	ND	0.02	0	57*	15
Indeno[1,2,3- <i>cd</i>]pyrene	ND	0.04	0	20*	22*
Dibenz[<i>a,h</i>]anthracene	ND	0.01	0	5	35*
Benzo[<i>ghi</i>]perylene	ND	ND	0	0	10

^a ND, not detectable.

^b % displacement of ³[H]E₂ in MCF-7 cells incubated with 5 µM of the PAH at 37°C for 3 h and then incubated with ³[H]E₂ at 4°C for 1 h.

^c % metabolism of E₂ (% TCDD) in MCF-7 cells incubated with 1 µM of the PAH for 72 h and then incubated with ³[H]E₂ for 24 h (see Section 2 for details).

* Significantly different (*P* < 0.05) from DMSO control.

results from the focus assay for the reconstituted mixtures were similar to those obtained for the fractionated extracts. However, the IC_{50} s of the KC- and SLR-extracts were 3.7- and 6-fold lower, respectively, than those obtained with the reconstituted mixtures, suggesting that there are some unidentified antiestrogenic compound(s) in the fractionated extracts. The unidentified compound(s) is not a PCB, a PCDD, or a PCDF, because these compounds were separately fractionated from the sample extracts. In particular, the more potent compounds such as the 2,3,7,8-substituted PCDDs and PCDFs and coplanar PCBs were never detected in the PAH fraction during method development studies (O'Keefe et al., 1997). While we monitored for 14 parent PAHs, it is likely that any unidentified compounds in the fractionated extract are additional PAHs for which we did not monitor, including the alkylated PAHs. A recent report showed that the alkylated PAHs of 13 parent PAHs detected in sediment samples contributed an additional 60–330% more toxicity as measured by amphipod mortality (Ozretich et al., 1997). Possibly some of these alkylated PAHs are also antiestrogenic and were present in the extracts we tested.

The antiestrogenicity of PAHs generally has been interpreted to be a result of the PAH binding to the aryl hydrocarbon receptor (AhR), triggering the induction of Ah-responsive genes and resulting in a broad spectrum of antiestrogenic responses including an increase in the metabolism of E_2 and a decrease in the nuclear ER (Piskorska-Pliszczyńska et al., 1986; Chaloupka et al., 1992; Santodonato, 1997). A few papers have reported that PAHs or their metabolites bind to the ER resulting in estrogenic and antiestrogenic activity (Keightley and Okey, 1974; Morreal et al., 1979; Ebright et al., 1986; Clemons et al., 1998). Based on these reports, we examined three possible mechanisms for the observed antiestrogenicity: (1) direct binding to the ER, (2) binding by a metabolite of the PAH to the ER, and (3) an increase in the metabolism of E_2 .

None of the 14 PAHs bound the rhER α or β , which eliminates the first mechanism. Support for the second mechanism comes from the finding that the reconstituted PAH-mixtures displaced

[3H]E $_2$ in the whole-cell ER binding assay. To further characterize the role of PAH metabolites, we compared the [3H]E $_2$ displacement for 3 h at 37 and 4°C (Fig. 4). The results indicate a loss of [3H]E $_2$ displacement at 4°C, suggesting a role of metabolism. Furthermore, a 37°C treatment for only 1 h resulted in no displacement of [3H]E $_2$ in the whole cell binding assay, suggesting that the effect is not immediate but requires more than 1 h. This result is also consistent with a metabolic event involving a PAH-metabolite binding to the ER. It is possible that the observed displacement of [3H]E $_2$ in the whole-cell ER binding assay was due in part to increased metabolism of [3H]E $_2$, resulting in decreased cellular radioligand concentrations. We examined this possibility by conducting the whole-cell ER binding assay under conditions that permitted metabolism of PAHs but suppressed metabolism of E $_2$, i.e. PAH treatment at 37°C for 3 h followed by 1 h of [3H]E $_2$ exposure at 4°C. Under these conditions a significant displacement of [3H]E $_2$ still occurred (Fig. 5). The difference between the [3H]E $_2$ displacement shown in these studies suggests that both binding to the ER by a PAH metabolite and [3H]E $_2$ depletion by PAH-induced enzymes play a role in the antiestrogenicity induced by PAHs. The dual mechanisms of PAHs in estrogen modulation also support a recent study by Clemons et al. (1998), which examined PAHs in air-borne particulate material. They reported evidence for ER binding and estrogen-like activity as well as TCDD-like activity using short-term transfected reporter gene assays.

Results from whole-cell ER binding assays conducted with the individual PAHs suggest that seven of the eight antiestrogenic PAHs are metabolized in MCF-7 cells to species that bind the ER. It is known that one metabolite of benzo[a]pyrene, 2-hydroxybenzo[a]pyrene, binds the ER in cytosol preparations of the rat uterus and liver (Ebright et al., 1986). Further studies are needed to determine which of the other numerous metabolites bind the ER.

Further support for the third mechanism, increased metabolism of E $_2$, was obtained from the radiometric analyses. Some PAHs are associated with induction of the cytochrome P450 CYP1A

family of enzymes through an Ah receptor-mediated mechanism. TCDD is the prototypical inducer of these enzymes and is used in this study to provide a maximal response. TCDD induces CYP1A1, 1A2 and 1B1 in MCF-7 cells which results in E₂ metabolism predominantly at the 2 (CYP1A1, CYP1B1) and 4 (CYP1B1) positions (Spink et al., 1990, 1992, 1994), causing a depletion of E₂ and production of less potent metabolites (Spink et al., 1990, 1992, 1994). This metabolic depletion of E₂ is considered to have a role in the antiestrogenicity of TCDD (Gierthy et al., 1988; Spink et al., 1990, 1992, 1994). Induction of E₂ metabolism by the PAHs under study were examined by displacement of tritium from E₂ labeled in the 2 and 4 positions, as well as other positions, and compared to the metabolism induced by TCDD. Four of the 14 PAHs, benz[*a*]anthracene, benzo[*k*]fluoranthene, indeno[1,2,3-*cd*]pyrene, and dibenz[*a,h*]anthracene, and the reconstituted mixtures significantly increased the metabolism of E₂.

It is also possible that down-regulation of ER may play a role in the antiestrogenicity observed in the focus assay. One published report measured a decrease in nuclear ER in MCF-7 cells after 12 h of incubation with either 3-methylcholanthrene, benzo[*a*]pyrene, benz[*a*]anthracene or 7,12-dimethylbenz[*a*]anthracene (Chaloupka et al., 1992). Thus, at least two of the PAHs that were antiestrogenic in the MCF-7 focus assay, benzo[*a*]pyrene and benz[*a*]anthracene, could have caused a decrease in nuclear ER during the 14-day duration of the focus assay.

In the present study we examined 10 of the 21 PAHs previously characterized by Tran et al. (1996) for their estrogenic and antiestrogenic activity by using a yeast strain transfected with a human ER and a reporter gene. The results obtained with the two assays are similar for some PAHs and different for others. First, neither study detected estrogenic activity for any of the PAHs. Second, neither study detected antiestrogenic activity for fluorene, phenanthrene, anthracene, or fluoranthene. Third, both studies reported antiestrogenic activity for benzo[*a*]pyrene and dibenz[*a,h*]anthracene. However, Tran et al. (1996) observed weak binding by benzo[*a*]pyrene

with hER (produced in Sf9 insect cells using the baculovirus expression system and prepared as ammonium sulfate precipitates) whereas we did not observe this binding with rhER α or β . In addition four PAHs that were not antiestrogenic in the yeast system, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, chrysene, and indeno[1,2,3-*cd*]pyrene, were antiestrogenic in the MCF-7 focus assay. The most likely explanation for these differences is that these four PAHs were metabolized in the MCF-7 cells, and their metabolites were antiestrogenic. This difference points out an important benefit of assays in which relevant metabolism of the test compounds occurs.

Recently the issue of synergy among endocrine disrupting chemicals has received much attention (Bergeron et al., 1994; Ashby et al., 1997; Gaido et al., 1997; Wang et al., 1997; Arcaro et al., 1998). Although we did not look specifically for synergistic responses, the antiestrogenic potencies of the reconstituted mixtures in the focus assay were roughly an average of the antiestrogenic potencies of the individual PAHs, suggesting that no substantial overall synergy occurred.

The finding that seven PAHs competitively displace [³H]E₂ when added to cultures of human breast cancer cells may have consequences for human health. It has been suggested that by binding to the ER, carcinogens may accumulate in the nucleus and result in increased mutagenicity (Ebright et al., 1986). The PAHs examined in this study are common environmental pollutants that are metabolized in the body and thus could potentially bind the ER, resulting in both a suppression of estrogenic responses and a possible increase in mutations in specific target tissues.

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