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The Anti-estrogenic Activity of Selected Polynuclear Aromatic Hydrocarbons in Yeast Expressing Human Estrogen Receptor

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Polynuclear aromatic hydrocarbons (PAH) represent a large class of chemicals present in the environment. We have used yeast strain ER(wt) expressing human estrogen receptor (hER) and an estrogen-sensitive reporter to characterize the estrogenic or anti-estrogenic activities of 21 PAHs. The PAHs did not exhibit estrogenic activity in yeast strain ER(wt). Four of the PAHs, dibenz[*a,h*]anthracene, 6-hydroxy-chrysene, 2,3-benzofluorene, and benzo(*a*)pyrene, inhibited estradiol-dependent reporter activity in strain ER(wt). A mutant hER lacking the amino terminus expressed in yeast was inhibited by the four PAHs to a lesser extent than the full-length hER. 6-hydroxy-chrysene, 2,3-benzofluorene, and benzo(*a*)pyrene, but not dibenz[*a,h*]anthracene, weakly displaced [^{3}H]estradiol from the hER in a competition binding assay. A strong correlation between the inhibition of [^{3}H]estradiol-binding from the hER and the reduction of hER-mediated transactivation in yeast was not observed. These observations suggest that the PAHs dibenz[*a,h*]-anthracene, 6-hydroxy-chrysene, 2,3-benzofluorene, and benzo(*a*)pyrene may antagonize estradiol activity in yeast by binding to an anti-estrogen binding site on the hER or by mechanisms independent of the hER.

One of the largest classes of chemicals measured in some contaminated ecosystems are polyaromatic hydrocarbons (PAH). PAHs are produced and released into the environment by fossil fuel combustion, oil spills and industrial processes (1). PAHs have been demonstrated to produce some forms of cancer in laboratory animals (2). One of the best-studied examples is the induction of mammary cancer in rodents by dimethybenz[*a*]anthracene (DMBA) (3).

In addition to the carcinogenic properties of the PAHs these compounds may also possess (anti)-hormonal activity. It has been sugested that a structural similarity exists between polynuclear aromatic hydrocarbons and estrogens (4). The hydroxylated PAHs 3,9-dihydroxy-benz[*a*]-anthracene and 3,9-dihydroxy-7,12-dimethylbenz[*a*]anthracene were weakly estrogenic in rat bioassays and bound to the estrogen receptor (ER) *in vitro* (5). However, DMBA does not interact with the ER *in vitro* suggesting that the induction of mammary cancer is probably not caused by this chemical functioning as an estrogen (6). Several other classes of environmental chemicals have estrogenic activity using laboratory tests and whole animal models. For example, the insecticide 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT) and its metabolites and some polychlorinated biphenyls (PCB) possess estrogenic activity (7, 8). For the most part, the estrogenic activity of these chemicals is mediated by their interaction with the ER.

Besides the estrogenic activity of some environmental chemicals, other chemicals function as anti-estrogens. The chloro-*S*-triazine compounds exhibit anti-estrogenic activity in yeast

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Abbreviations: DMBA, dimethybenz[*a*]anthracene; DMSO, dimethylsulfoxide; hER, human estrogen receptor; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; PAH, polynuclear aromatic hydrocarbon; PCB, polychlorinated biphenyl.

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strain ER(wt) expressing hER and an estrogen-sensitive reporter (9). The activity of estradiol in strain ER179C containing a mutant ER lacking the amino terminus was not inhibited by the triazines. A previous report demonstrated that substituted benz[*a*]anthracene-3,9-diols inhibited estrus in rats suggesting that these compounds functioned as anti-estrogens (10).

We have characterized 21 PAHs for their estrogenic or anti-estrogenic activity in yeast strains ER(wt) and ER179C. The PAHs were selected based on their presence at measurable concentrations in Bayou Trepagnier in Louisiana (unpublished data). The PAHs did not exhibit estrogenic activity in yeast strain ER(wt). The four PAHs dibenz[*a,h*]anthracene, 6-hydroxy-chrysene, 2,3-benzofluorene and benzo(*a*)pyrene inhibited the activity of estradiol in strain ER(wt). 6-hydroxy-chrysene, 2,3-benzofluorene and benzo(*a*)pyrene, but not dibenz[*a,h*]-anthracene, weakly displaced [^{3}H]estradiol from binding recombinant hER in a competition binding assay. These results demonstrate that four PAHs have previously unrecognized anti-estrogenic activities with the hER in yeast.

MATERIALS AND METHODS

Materials. Estradiol-17β and the amino acids for culturing yeast were purchased from Sigma Chemical Co. (St. Louis, MO.). 17β-3,4,6,7[^{3}H](N) estradiol (99 Ci/mmol) was purchased from DuPont/NEN (Wilmington, DE). The PAHs at 99.0% purity were purchased from AccuStandard Inc. (New Haven, CT.).

Yeast assays. Yeast strains ER(wt) and ER179C as described (9) were grown overnight at 30 °C in synthetic medium-tryptophan, uracil. The next day, 25 μl of the overnight culture was diluted into 975 μl of fresh medium and grown overnight with dimethylsulfoxide (DMSO) or estradiol-17β in the presence or absence of the various PAHs. Estradiol-17β was prepared in DMSO and the concentration of DMSO in the assay was 0.1%. The same volume of solvent was used for each concentration of PAH and estradiol tested. None of the chemicals inhibited the growth of the yeast at the concentrations tested.

β-galactosidase assays. The yeast cells were collected by centrifugation, resuspended in 700 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM β-mercaptoethanol) and permeabilized by the addition of 6 μl of $CHCl_3$ and 4 μl 0.1% SDS followed by vortexing for 25 s. The reactions were equilibrated at 30 °C for 10 min, then 160 μl of ONPG (4 mg/ml in Z-buffer) was added and the reactions returned to 30 °C for between 5 and 60 min. The reactions were terminated by the addition of 400 μl 1M $NaCO_3$, the cell debris removed by centrifugation and the absorbance at 420 nM measured (A_{420}). The growth of the yeast strains was monitored by measuring the absorbance at 600 nM (A_{600}). Miller units were determined using the following formula: [A_{420}/(A_{600} of 1/10 dilution of cells $\times$ volume of culture $\times$ length of incubation)] $\times$ 1000. Statistics were performed by one-way ANOVA least significant difference test (Microsoft Excel). Significant differences were defined when $p<0.05$. The data are representative of three independent experiments with three replicates.

Binding assays. Recombinant hER was produced in Sf9 insect cells using the baculovirus expression system and prepared as ammonium sulfate precipitates (8, 9). The hER produced in Sf9 insect cells has approximately the same affinity for [^{3}H]estradiol-17β as the hER produced in yeast cells (unpublished data). In addition, the Sf9 extract containing hER had less proteolytic activity than an extract from strain ER(wt). Recombinant hER at a concentration of 0.4 nM was dissolved in the 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_4$, 10% glycerol, γ-globulin (10 mg/ml), 0.5 mM phenylmethylsulfonyl fluoride and 0.2 mM leupeptin for 1 h at 25 °C with 2 nM [^{3}H]estradiol-17β in the presence or absence of increasing concentration of radioinert PAHs or estradiol-17β. The same volume of solvent was used for each concentration of PAH tested. Non-specific binding of [^{3}H]estradiol-17β was assessed by the addition of 300-fold molar excess of radioinert estradiol-17β. Free [^{3}H]estradiol-17β was removed by incubation with Chardex (5% activated charcoal and 0.5% dextran dissolved in phosphate-buffered saline) for 10 min at 4 °C and centrifugation for 3 min at 15,000 $\times$ g. The bound [^{3}H]estradiol-17β was measured using liquid scintillation counting. The data are representative of three independent experiments with three replicates.

RESULTS AND DISCUSSION

To identify PAHs that have estrogenic activity, yeast strain ER(wt) was incubated with vehicle, 0.5 nM estradiol or 21 PAHs, and the β-galactosidase activity was measured. Estradiol increased β-galactosidase activity in strain ER(wt) compared to vehicle (Fig. 1). The PAHs, at 1 μM, did not increase β-galactosidase activity above vehicle. These results suggest that the PAHs have no estrogenic activity in strain ER(wt). The absence of estrogenic activity associated with the PAHs was not unexpected since only 2 of the 21 PAHs, 6-hydroxy-chrysene

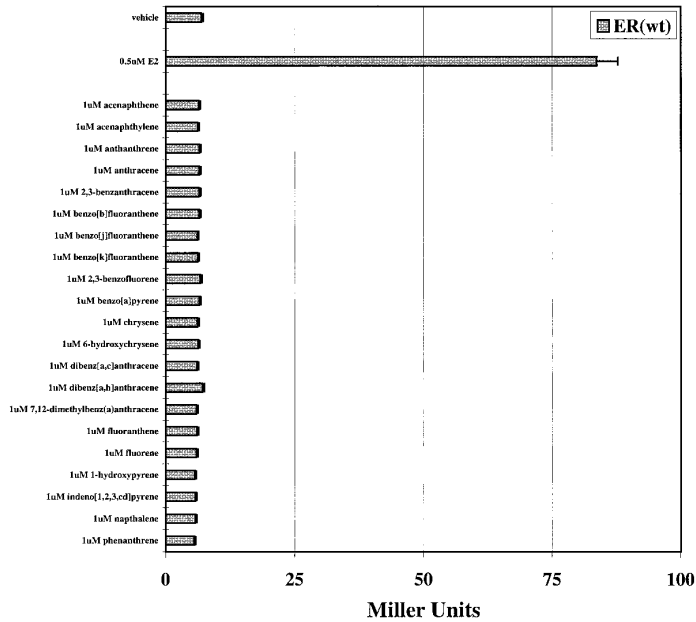

FIG. 1. Incubation of yeast strain ER(wt) with estradiol or various PAHs. The yeast strain ER(wt) was incubated in the presence of vehicle, 0.5 nM estradiol or the various PAHs at 1 μM. Strain ER(wt) was grown for 12 h and then β-galactosidase activity was measured. β-Galactosidase activity is expressed as Miller units as described under Materials and Methods.

and 1-hydoxy pyrene, contained hydroxyl groups. We and others have shown that the addition of a hydroxyl group increased the estrogenic activity of some chemicals (8, 11).

To identify PAHs with anti-estrogenic activity, strain ER(wt) was coincubated with 0.5 nM estradiol and 1 μM of the 21 PAHs. Four PAHs at 1 μM, dibenz[*a,h*]anthracene, 6-hydroxy-chrysene, 2,3-benzofluorene and benzo(*a*)pyrene, significantly reduced estradiol-dependent β-galactosidase activity (Fig. 2). To characterize the inhibitory activity of the four PAHs, the chemicals were used at varying concentrations in the presence of 0.5 nM estradiol in yeast strains ER(wt) and ER179C. The most effective PAH in strain ER(wt) was dibenz[*a,h*]-anthracene with an IC_{50} (the concentration of PAH necessary to reduce estradiol-dependent β-galactosidase by 50%) of approximately 1 μM (Fig. 3A). 6-hydroxy-chrysene had an IC_{50} of 10 μM. The IC_{50} values for 2,3-benzofluorene and benzo(*a*)pyrene were not determined since estradiol activity was not reduced by 50% at the concentrations tested.

The inhibitory activities of the four PAHs were also investigated in strain ER179C containing a mutant hER lacking the amino terminus. In this strain, only 10 μM dibenz[*a,h*]anthracene and 5 and 10 μM 6-hydroxy-chrysene significantly reduced estradiol-induced β-galactosidase activity (Fig. 3B). 2,3-benzofluorene and benzo(*a*)pyrene did not function as inhibitors of estradiol activity in strain ER179C. The four PAHs and the chloro-*S*-triazine compounds (9) had less inhibitory activity in strain ER179C than strain ER(wt). These results suggests that the amino terminal of the hER may contribute to the binding or inhibitory activity of these chemicals.

The binding of the four PAHs to the hER was determined using a competition binding assay. Recombinant hER was incubated with [^{3}H]estradiol in the presence or absence of varying concentrations of PAHs. The binding of [^{3}H]estradiol to the hER was reduced to 11% in the presence of a 300-fold excess of radioinert estradiol (Fig. 4). Among the PAHs, 6-hydroxy-

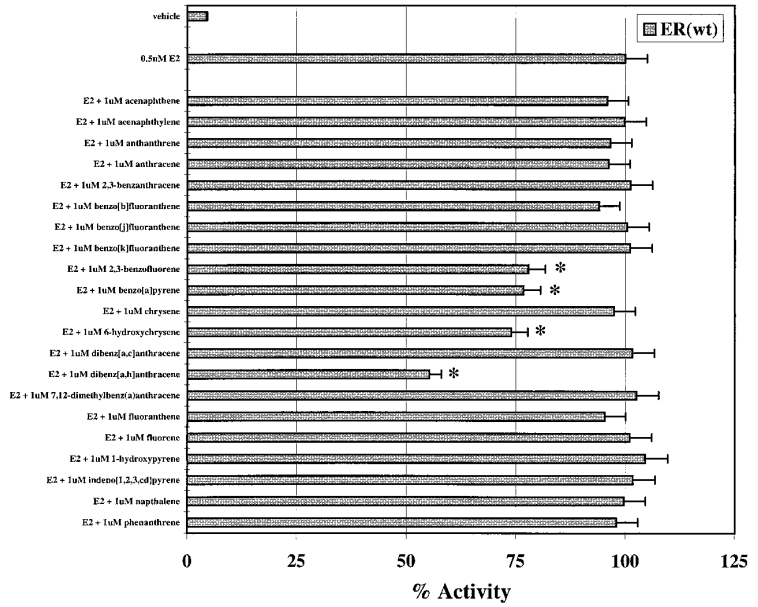

FIG. 2. Coincubation of yeast strain ER(wt) with estradiol and the various PAHs. The yeast strain ER(wt) was incubated with vehicle, 0.5 nM estradiol in the presence or absence of various PAHs at 1 μM. Strain ER(wt) was grown for 12 h and then β-galactosidase activity was measured. The % activity is defined as a percentage of β-galactosidase activity of estradiol in the absence of competitor. The * indicates % activity that is significantly different from 0.5 nM estradiol.

chrysene reduced [^{3}H]estradiol-binding to the greatest extent. At 10 μM, 6-hydroxy-chrysene decreased binding over 40%. 2,3-benzofluorene and benzo(*a*)pyrene reduced binding 23 and 32%, respectively, at 10 μM. Dibenz[*a,h*]anthracene did not show a significant reduction in binding to the hER.

The lack of correlation between chemicals binding to the recombinant hER *in vitro* and their anti-estrogenic activity in yeast has been observed previously. The concentration that the chloro-*S*-triazine compounds functioned as anti-estrogens in strain ER(wt) was approximately 10-fold lower than their binding to recombinant hER *in vitro* (9). A similar situation with the PAHs is presented in this paper. In fact, dibenz[*a,h*]anthracene did not appear to interact with the ER at the concentrations tested.

Overall, our results may be explained by the presence of an anti-estrogen binding site on the hER. This site has been suggested to mediate the anti-estrogenic activities of tamoxifen and may not recognize [^{3}H]estradiol (12). This observation would explain the lack or weak displacement of [^{3}H]estradiol from the hER with the PAHs. Another explanation for the discrepancy may be the conformation of the ER used in the *in vitro* binding and yeast assays. This idea suggests that the binding characteristics of the hER in yeast may be different than the recombinant hER. This hypothesis is currently under investigation in our laboratory. Alternatively, the anti-estrogenic activity of the PAHs may be derived by affecting the activity of conserved signaling pathways that participate in ER activity. For example, mitogen-activated protein kinase (MAPK) has been shown to participate in ER activity (13) and yeast contain kinases homologous to MAPK (14).

The experiments in this paper were performed because of an interest in understanding how the hormonal activity of environmental chemicals in Bayou Trepagnier may impact the reproduction and health of organisms in this ecosystem. To this end, we have employed an *in*

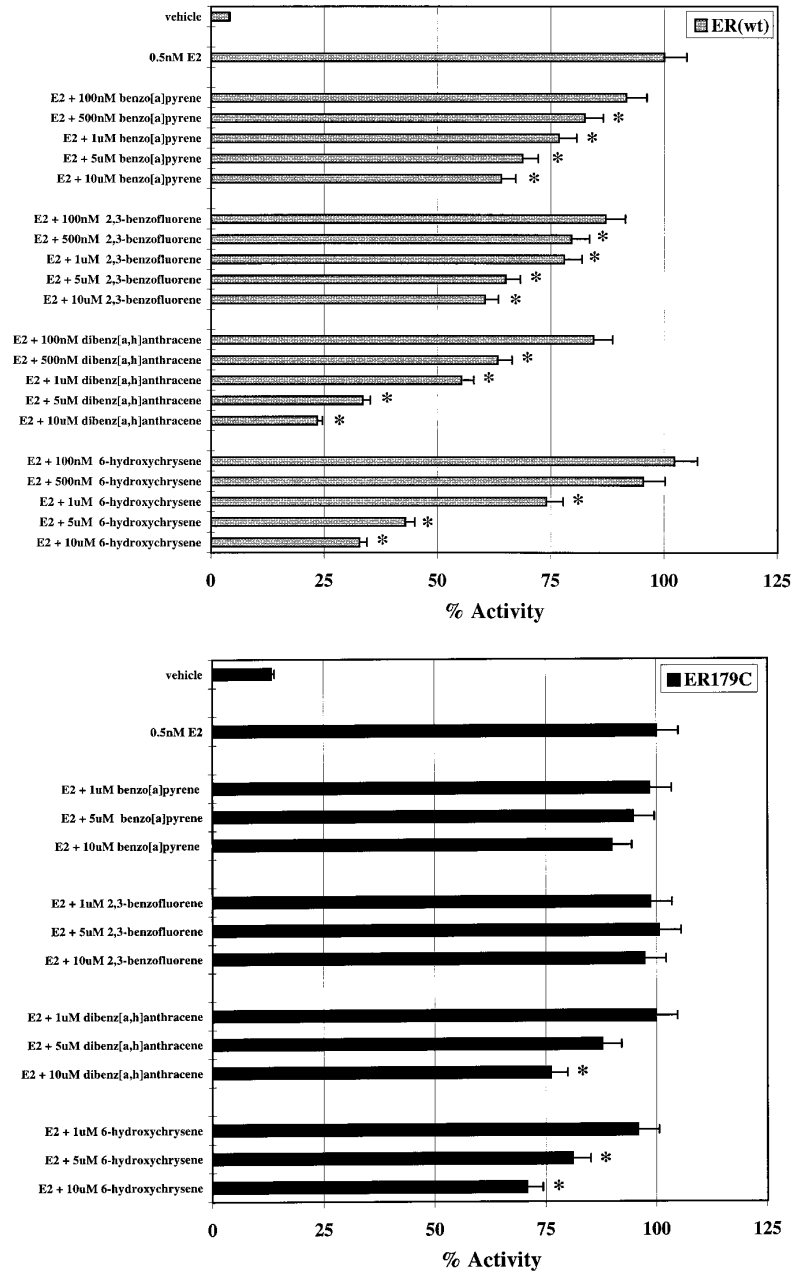

FIG. 3. The yeast strains ER(wt) (A) and ER179C (B) were incubated with vehicle, 0.5 nM estradiol in the presence or absence of benzo(*a*)pyrene, 2,3-benzofluorene, dibenz[*a,h*]anthracene and 6-hydroxy-chrysene at the indicated concentrations. The % of control is defined as a percentage of β-galactosidase activity of estradiol in the absence of competitor. Treatment of strain ER179C with 0.5 nM estradiol produced 20 Miller units. The * indicates % activity that is significantly different from 0.5 nM estradiol.

vitro system to characterize the (anti)-estrogenic activity of one class of chemicals found in the Bayou, the PAHs. The environmental significance of the characterization of hormonal activity of the PAHs using *in vitro* systems is unclear at the present time. However, the results

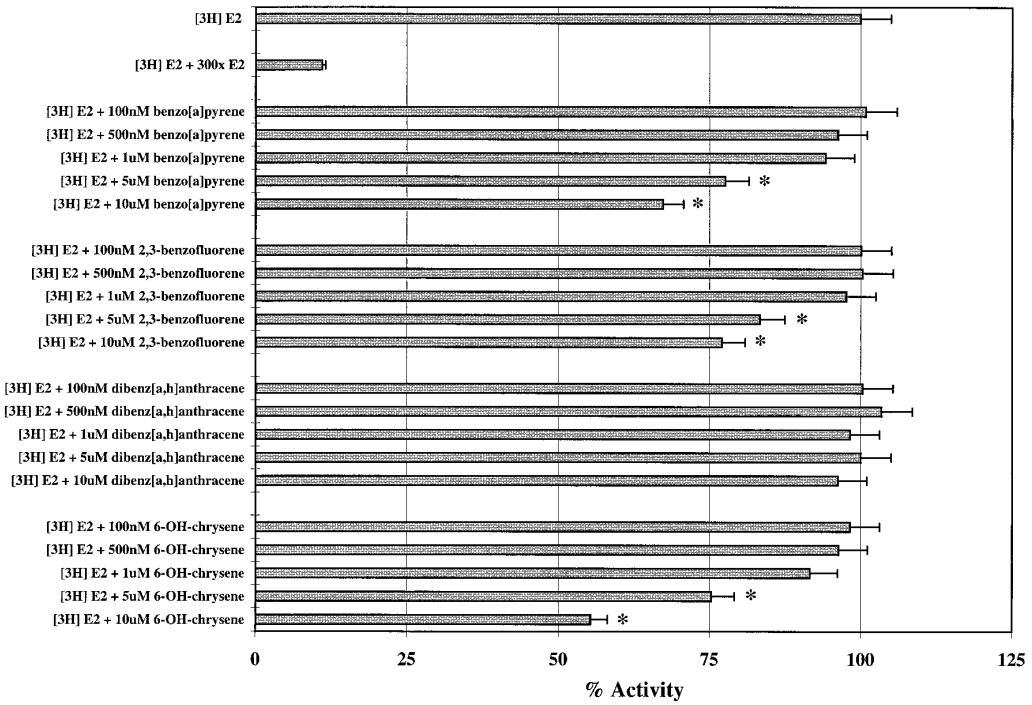

FIG. 4. Competition binding assay with recombinant hER and various PAHs. Recombinant hER was incubated with 2 nM [^{3}H]estradiol-17β in the presence or absence of radioinert estradiol (600 nM) or increasing concentrations of benzo(a)pyrene, 2,3-benzofluorene, dibenz[*a*,*h*]anthracene and 6-hydroxy-chrysene. The bound [^{3}H]estradiol-17β was measured by liquid scintillation counting and expressed as percentage of [^{3}H]estradiol-17β in the absence of competitor (5100 dpms). The * indicates % activity that is significantly different from 0.5 nM estradiol.

obtained using *in vitro* systems will allow us to rapidly test hypotheses concerning the effects of these chemicals at specific sites in the Bayou or in other laboratory systems.

We have demonstrated that 21 PAHs do not possess estrogenic activity in yeast strain ER(wt). Four PAHs dibenz[*a*,*h*]anthracene, 6-hydroxy-chrysene, 2,3-benzofluorene and benzo(*a*)pyrene inhibited the activity of estradiol in strain ER(wt). The inhibitory activity of these chemicals was substantially reduced in strain ER179C. The four PAHs displaced [^{3}H]estradiol from the hER to a limited extent in a competition binding assay. Our results reveal that some PAHs have anti-estrogenic activity in yeast strain ER(wt) through hER-dependent and -independent mechanisms.

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