In Vitro Synergistic Interaction of Alligator and Human Estrogen Receptors with Combinations of Environmental Chemicals

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The effect of mixtures of environmental chemicals with hormonal activity has not been well studied. To investigate this phenomenon, the estrogen receptor (ER) from the American alligator (aER) or human (hER) was incubated with [3H]17β-estradiol in the presence of selected environmental chemicals individually or in combination. The environmental chemicals included the insecticide chlordane, which has no estrogenic activity, and the pesticides dieldrin and toxaphene, which have very weak estrogenic activity. Chlordane, dieldrin, and toxaphene individually demonstrated no appreciable displacement of [3H]17β-estradiol from aER and hER at the concentration tested. A combination of these chemicals inhibited the binding of [3H]17β-estradiol by 20 to 40%. Alachlor, a chemical recently discovered to have weak estrogenic activity, also displaced [3H]17β-estradiol more effectively in combination with dieldrin than alone. These results indicate that combinations of some environmental chemicals inhibit [3H]17β-estradiol binding in a synergistic manner. This suggests that the ER may contain more than one site for binding environmental chemicals. The possibility that the ER binds multiple environmental chemicals adds another level of complexity to the interaction between the environment and the endocrine system. — Environ Health Perspect 105(Suppl 3):615–618 (1997)

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The presence of chemicals in the environment with hormonal or antihormonal activity has generated interest in understanding their potential for producing adverse health effects in wildlife and humans (1). To date, research concerning the capacity of individual chemicals to produce hormonal responses in cell culture or whole animal studies has been limited to concentrations that are higher than those generally measured in ecosystems. For example, the pesticides dieldrin and toxaphene stimulated the proliferation of human breast cancer cells in the E-SCREEN at 10 μM (2). This concentration was 10,000-fold greater than the concentration of estradiol required for proliferation, indicating that these chemicals have very weak activity. The insecticide chlordane and the herbicide alachlor did not stimulate the proliferation of breast cancer cells (3). Recently, alachlor was reported to have weak estrogenic activity with the human estrogen receptor (hER) in breast cancer cells and yeast using reporter-gene assays (4).

Chemicals are usually found as mixtures in the environment and the hormonal activity of mixtures has been investigated only to a limited extent. Soto et al. demonstrated that a combination of 10 chemicals enhanced the proliferation of MCF-7 cells in the E-SCREEN in a greater than additive manner (2). Sumpter and Jobling have shown that vitellogenin induction in fish hepatocytes was stimulated in a synergistic fashion by a combination of environmental chemicals (5). An in vivo example of mixtures of environmental chemicals having synergistic activity is a study using turtle eggs performed in collaboration with Bergeron et al. (6). The sex of turtle eggs is temperature dependent; eggs incubated at 26 or 31°C develop into males or females, respectively. Male-determined turtle eggs can be sex reversed by estradiol or estrogen hydroxylated polychlorinated biphenyls (PCBs). In fact, PCBs in combination were effective in sex reversal at concentrations that had little or no activity individually.

These observations of the apparently synergistic estrogenic activity of combinations of environmental chemicals stimulated the experiments described in this paper. We tested the possibility that synergistic estrogenic activity was in part mediated by the estrogen receptor (ER) interacting with multiple environmental chemicals. We show that ER from the American alligator (aER) and humans can interact with environmental chemicals in a manner that may indicate that the ER contains more than one ligand-binding site. Our observations provide a mechanistic basis for understanding the combined actions of multiple environmental chemicals on estrogen target tissues.

Methods

Chemicals

17β-[3,4,6,7,3H]estradiol (84 Ci/mmol) was obtained from DuPont NEN Products (Boston, MA). Radioinert 17β-estradiol was purchased from Sigma Chemical (St. Louis, MO). Alachlor [2-chloro-N-(2,6-diethylphenyl)-N-(methoxy-methyl)acetamide (dissolved in ethanol)], chlordane [1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methano-1H-indene (dissolved in isocetane)], dieldrin

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The was EDTA, assays at bated with collected, manufacturer toxaphene-complex mix of at least 177 C10 polychloro derivatives (dissolved in isoc tane) were purchased from AccuStandard (New Haven, CT). All of the chemicals were at least 99% pure as stated by the manufacturer with the exception of toxaphene (not available).

**Preparation of Alligator Cytosol**

A protein cytosol for estrogen binding assays was prepared from the frozen oviductal tissue of adult female alligators (*Alligator mississippiensis*) captured from several lakes in central Florida. The oviduc tal tissue was thawed on ice, placed into buffer A (20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 NaVO4, 10 mM NaPO4, 50 mM NaF, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM leupeptin, 2 mg/ml pepstatin, and 50 µl/ml aprotinin), minced by a Polytron homogenizer, and centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was collected, 400 mM KCl added, and incubated at 4°C for 15 min. The supernatant was centrifuged at 15,000 rpm for 10 min at 4°C and the protein cytosol was flash frozen on dry ice.

**[3H]17β-Estradiol Binding Assays with Alligator Cytosol**

The [3H]17β-estradiol binding assays used 35 µl of protein cytosol dissolved in 165 µl of buffer A. For the competition binding assays, alligator protein cytosol was incubated with 2.5 nM [3H]17β-estradiol for 1 hr at 25°C. Free [3H]17β-estradiol was removed by incubation with chardex (5% activated charcoal and 0.5% dextran sus pended in homogenization buffer) for 10 min at 4°C and centrifuged for 3 min at 15,000×g. Samples were then incubated at 25°C for 1 hr with either vehicle, radioinert environmental chemical(s), or a 300-fold excess of radioinert estradiol, to measure nonspecific binding. Combinations of chemicals were mixed prior to their addition in the reaction. The vehicle was composed of the appropriate concentrations of the various solvents used for dissolving the chemicals. The concentration of solvent was less than 1% in all reactions. Free [3H]17β-estradiol was again removed by incubation with chardex for 10 min at 4°C and centrifuged for 3 min at 15,000×g. The bound [3H]17β-estradiol was measured by scintillation counting. The data are representative of at least three independent experiments with three replicates.

**Preparation and [3H]17β-Estradiol Binding of the Human Estrogen Receptor**

The recombinant hER was extracted from SF9 insect cells infected with a baculovirus containing the cDNA of the hER and prepared as ammonium sulfate precipitates. The recombinant hER at a concentration of approximately 0.4 nM was dissolved in buffer A for 1 hr at 25°C with 2.5 nM [3H]17β-estradiol in the presence or absence of radioinert environmental chemical(s), or a 300-fold excess of radioinert estradiol, to determine nonspecific binding. The preparation of the combinations of the chemicals and the removal of the free [3H]17β-estradiol are the same as described for the alligator cytosol.

**Results**

**Competition Binding Assays with Combinations of Environmental Chemicals**

To measure the interaction of selected environmental chemicals alone and in combination, an *in vitro* competition binding assay was used with cytosol containing the ER from the American alligator (7). We have shown that the affinity of the aER for estradiol is similar to the hER (7). The aER was incubated with [3H]17β-estradiol in the presence or absence of radioinert estradiol or selected environmental chemicals, individually or in combination. The environmental chemicals used were dieldrin, toxaphene, and chlordane; concentrations used in the binding assays were derived from a study examining environmental chemicals in alligator eggs. This study measured the levels of dieldrin (630 nM), chlordane (220 nM), and toxaphene (200 nM) in alligator eggs collected from Lake Apopka, Florida (8). As expected, estradiol strongly inhibited the binding of [3H]17β-estradiol to the aER (Figure 1). Chlordane, dieldrin, or toxaphene individually did not decrease the binding of [3H]17β-estradiol. This is consistent with the lack of activity of these chemicals in the E-SCREEN at the concentrations used in the binding assay (2). Interestingly, a combination of two environmental chemicals inhibited [3H]17β-estradiol binding. For example, a combination of toxaphene and dieldrin decreased [3H]17β-estradiol binding by 20%, whereas a mixture of toxaphene and chlordane reduced binding by 40%. A combination of all three chemicals did not appear to decrease binding to a greater extent than a combination of two chemicals.

To determine if the synergistic binding of environmental chemicals was limited to the aER, a competition binding assay with the hER was also used (Figure 2A). As observed with the aER, chlordane, dieldrin, or toxaphene alone did not interact with the hER. A combination of two chemicals inhibited [3H]17β-estradiol binding in a synergistic manner. A combination of toxaphene or dieldrin and chlordane reduced binding by 25%. A combination of three chemicals was as effective as two chemicals.

Additionally, we examined the interaction of alachlor, a recently identified chemical with weak estrogenic activity (9), with the hER, to determine if interaction could be enhanced in the presence of a second chemical, dieldrin (Figure 2B). Individually, dieldrin (630 nM) and alachlor (1 µM) had no appreciable interaction with the hER. A mixture of alachlor and dieldrin decreased the binding of [3H]17β-estradiol to the hER by approximately 25%.

**Discussion**

We have shown that some combinations of environmental chemicals displace [3H]17β-estradiol from the aER and hER in a synergistic manner compared to the...
action of individual chemicals. The binding of multiple chemicals with the aER or hER performed in this study does not indicate that this interaction will have estrogenic or antiestrogenic activity.

This study demonstrates that the aER and hER can interact with the same chemicals in a synergistic manner. We have shown that affinity of the aER for estradiol is similar to the hER (7). The classic environmental estrogens kepone and o,p'-DDT are recognized by the aER. However, the aER did appear to interact with DDOH and o,p'-DDD, metabolites of DDT that have been classified in rodent studies as nonestrogenic (9). It is not surprising that some combinations of chemicals would interact in a synergistic fashion with either the aER or the hER but not both, based on the differences between the aER and hER in recognizing some environmental estrogens (7). These unique chemical combinations may account for the apparent sensitivity of some species to the potential health effects associated with environmental estrogens (10).

The results presented in this paper are consistent with a previous report from our laboratory describing the synergistic estrogenic effects of some environmental chemicals in yeast and mammalian cells containing the hER and an estrogen-sensitive reporter (11). We observed that treatment of yeast with combinations of chlordane, dieldrin, or toxaphene produced a greater than additive increase in the activity of the reporter as compared with activity produced by any of the chemicals alone.

We also showed that two hydroxylated PCBs produced a synergistic estrogenic response in yeast and mammalian cells (11). This finding provides some molecular detail for the fact that combinations of PCBs were effective in sex-reversing turtle eggs at concentrations that had no activity alone. We hypothesize that the synergistic estrogenic effects produced by combinations of some environmental chemicals are in part mediated by their interaction at multiple sites on the hER. The presence of multiple binding sites for environmental chemicals on the hER would explain the synergistic binding, transcriptional activity, and sex reversal observed with multiple chemicals in yeast and mammalian cells and turtle eggs.

The presence of multiple binding sites on the hER that could lead to the production of estrogenic responses by weakly estrogenic or inactive chemicals has profound implications for the identification of environmental chemicals with hormonal activity. For this purpose, it would be necessary to identify the regions of the ER responsible for binding multiple chemicals and to identify the endogenous ligands for the sites.

The synergistic activity of the ER with environmental chemicals suggests that other steroid receptors that interact with environmental chemicals may also have synergistic activity. For example, the antiandrogens p,p'-DDT and vinclozolin may function more effectively in combination than individually (12,13). Nonetheless, the results presented in this paper and studies from other laboratories suggest that we are just beginning to discover the sophisticated interactions between environmental signals and the endocrine system.

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