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Novel Actions of an Estrogen Mimicking Xenobiotic

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Abstract: Several environmental contaminants have been shown to induce estrogen-like responses in the breast and uterus. Included in this list of xenoestrogen contaminants are ß-hexachlorocyclohexane (ß-HCH) and o,p'-dichlorodiphenyltrichloroethane (o,p'-DDT) two organochlorine pesticide isomers. These chemicals are ubiquitous in the environment and due to their estrogenic activity they may be involved in the etiology of cancers of uterus and breast. Both ß-HCH and o,p'-DDT stimulate gene expression and cell proliferation in MCF-7 and T47D human breast cancer cells. ß-HCH also supports the growth of MCF-7 cells grown as solid tumors in athymic mice. In the uterus, these compounds moderately increase uterine weight and increase cellular height in the epithelium. Similar to estradiol (E2), o,p'-DDT binds to and activates the estrogen receptor (ER). In contrast ß-HCH does not bind to or activate the ER in the classical manner. ß-HCH enhances gene transcription through an estrogen-responsive, complex promoter such as the prolactin gene or the pS2 gene but, unlike E2, it is ineffective on a simple estrogen response element.

Obesity is a risk factor for both uterine and breast cancer; xenoestrogens may play a role in this risk factor. Because both ß-HCH and o,p'-DDT are sequestered and stored in body fat, it was hypothesized that fasting would induce a release of these xenoestrogens from fat stores, thereby stimulating estrogen target tissues. To test this hypothesis ovariectomized mice were loaded with three daily doses of either o,p'-DDT or ß-HCH and two weeks later they were either fed *ad libitum* as usual or they were fasted for two days. Animals that had been loaded with ß-HCH and then fasted had increased uterine weights and epithelial cell height as compared to control animals; there was no indication of estrogenic stimulation in fasted animals that had been loaded with either o,p'-DDT or E2. These results suggest a novel mechanism by which xenoestrogens stimulate estrogen target tissues.

INTRODUCTION

The continuously climbing incidence of breast cancer may also be linked to environmental factors, particularly man-made estrogenic compounds that contaminate our water and food supplies. Estrogens stimulate the growth of estrogen receptor (ER)-positive uterine and breast tumors and therefore have been implicated in the etiology of neoplastic and benign tumors of these tissues[1-6]. Increased risk for endometrial and breast cancer is associated with obesity, anovulatory infertility, late menopause, polycystic ovary syndrome, steroid-secreting ovarian tumors, and use of estrogen as hormonal replacement therapy[1-8]; the risk is believed to derive from an increase in circulating estrogen associated with these conditions. In the case of obesity, it is postulated that increased estrogens stem from the capacity of fat cells to produce estrogens from circulating androgens[9, 10]. It may also be that the link is related to exposure to environmentally derived estrogenic compounds.

Several pesticide residues have been identified as estrogen mimetics; these xenoestrogens, such as the organochlorine compounds o,p'-DDT and ß-hexachlorocyclohexane (ß-HCH), persist in the environment, bioaccumulate in the food chain[11-22], and have produced dramatic endocrine disruptive effects in wildlife[18]. The lack of consensus over whether exposure to xenoestrogens is related to breast cancer incidence[23-26] may be due to how exposure was measured. Environmental contaminants tend to accumulate in fatty tissues of the body[22, 27-29] and, although blood levels are believed to reflect tissue levels[30], concentrations found in fat per se may be a more appropriate measure of exposure[23, 24]. Indeed, mammary adipose tissue from breast cancer patients exhibits significantly higher levels of isomers of DDT, ß-HCH and PCBs than does the breast fat from control subjects[27, 28]; in fact, the odds ratio was greater than 10.5 that fat from cancerous breasts would have

high levels of ß-HCH compared to that from non-cancerous breasts[27]. These latter observations suggest that estrogenic environmental contaminants may be responsible in part for the increase in breast cancer.

Most xenoestrogens have been shown to act as ligands for ER[11, 12]. However, one report suggested that although ß-HCH stimulates rat uterus and human breast cancer cells in an estrogen-like manner, it may not interact directly with the ER[31, 32]. Our investigations were designed to further examine the mechanisms by which ß-HCH acts on estrogen target cells; results show that this compound is unique in its mode of action. In addition, we have examined the question of whether o,p'-DDT and ß-HCH can be released from fat stores in biologically active amounts; results suggest that this mode of delivery may be important to target tissue growth.

EXPERIMENTAL RESULTS

Estrogen-like Effects of ß-HCH and o,p'-DDT in Mammary Cancer Cells. Both o,p'-DDT and ß-HCH stimulate cell proliferation and gene expression in MCF-7 and T47D cells, two human ER-positive breast cancer cell lines and these effects are blocked by concomitant treatment with an antiestrogen[33]. In contrast, these compounds had no estrogenic effects in the ER-negative MDA-MB-231 cells[33]. ß-HCH also stimulated growth of the MCF-7 cells grown as tumors in athymic hosts (Fig. 1). The final tumor sizes and weights were similar in both ß-HCH- and estradiol-treated hosts. Furthermore, host animals treated with ß-HCH had uterine weights approximately twice those of vehicle-treated hosts (not shown).

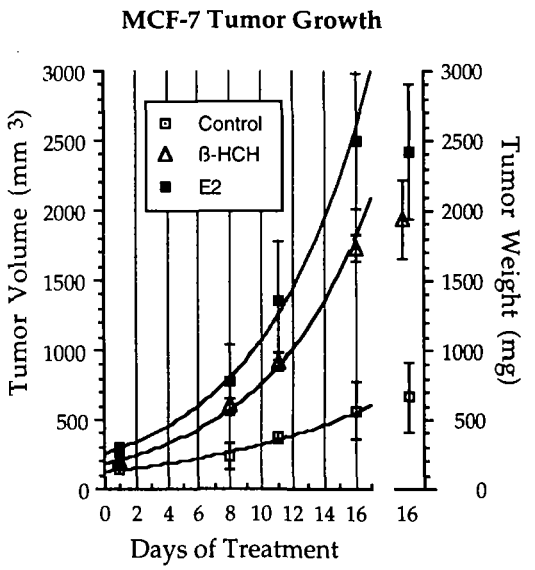

Fig. 1. MCF-7 Tumors Grown in Athymic Mice. Female athymic mice were inoculated with MCF-7 tumor tissue, sc. The inoculate was derived from a tumor that had been growing in an athymic host; it was chopped and sieved through a 40 mesh wire grid. The resultant slurry was washed and resuspended; 100 µl of this suspension was injected into each host. Ten days later the host animals were ovariectomized and a silastic capsule containing 20 mg of test compound was placed sc. Tumor was measured periodically using calipers, commencing on the first day of treatment. After 16 days the hosts were killed and the tumor was excised and weighed. (Reproduced, with permission, from Cancer Research 56:5403, 1996.)

Interaction with Estrogen Receptor. Natural estrogen, and all xenoestrogens studied to date, either bind directly to ER or are metabolically converted to a ligand capable of activating ER[34-40]. This can be demonstrated by determining the localization of ER during cellular fractionation into nuclear and cytosolic compartments. When cells containing ER are homogenized, non-activated ER is spuriously extracted from the nucleus and is found in the cytosolic preparation[41]. On the other hand, if the ER is ligand-activated it is bound more tightly to DNA and therefore remains in the nuclear fraction during homogenization where it can be extracted using a "high salt" solution. Thus, a decrease in the amount of cytosolic ER coupled with an increase in the amount of nuclear ER indicates ligand-induced receptor activation. While this type of shift is seen following treatment with estradiol, ß-HCH had no effect on 'compartmentalization' of ER (Fig. 2). Thus ß-HCH appears to be distinct; it does not displace E2

from ER[32, 33], at least not at the site occupied by E2, nor does it serve as a proestrogen, i.e. it is not metabolically converted to an ER-activating ligand. o,p'-DDT on the other hand does exhibit weak competitive binding to ER and increases ER concentration in the nuclear fraction[33, 34].

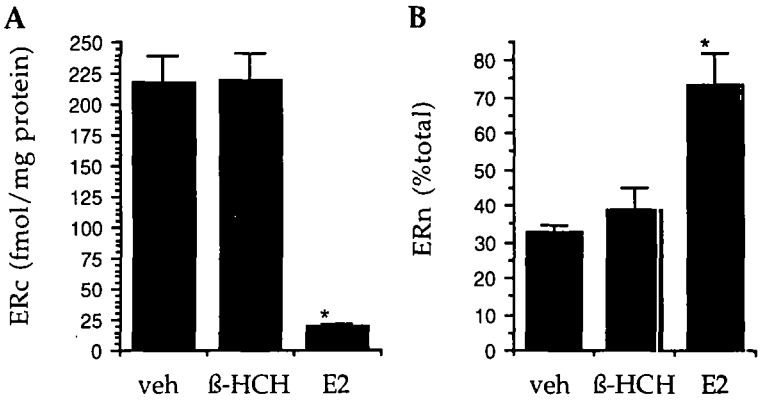

Fig. 2. Effect of ß-HCH on the relative cytosolic and nuclear fractionation of ER. MCF-7 cells were grown in phenol red-free MEM plus 3% charcoal-stripped FBS for 1 week and treated with vehicle (veh), 1 M ß-HCH, or 1 nM estradiol (E2) for 24 h. Cells were washed 4 times with cmf-PBS, removed from the culture dish in 10 mM EDTA, and homogenized in tris-EDTA buffer. The homogenate was assayed for ER binding using the dextran-coated charcoal method (--) and the maximum binding was determined by Scatchard analysis. Estradiol reduced cytoplasmic ER (ERc) (A) and increased relative nuclear ER (ERn) (B) while ß-HCH was without effect. (Adapted, with permission, from Cancer Research 56:5403, 1996.)

Transcriptional Activation by ß-HCH. Ligand activated ER acts as a transcription factor, it binds to a specific cis-acting DNA sequence, the estrogen response element (ERE), and induces transcription of ER-responsive genes . The regulatory regions of most genes contain multiple cis-acting elements such as AP-1, CREB, and Sp1 binding sequences. Expression of the pS2 gene is enhanced by both o,p'-DDT and ß-HCH in MCF-7 cells[33]. To further examine the capacity of ß-HCH to regulate transcription of estrogen-responsive genes, we tested its capacity to activate 2 different chimeric, estrogen-responsive gene constructs transfected into cultured cells.

The rat prolactin (PRL) gene is regulated by a complex promoter/enhancer region containing a single functional ERE and multiple other cis-elements. A reporter gene construct (PRL-luc), kindly provided by Dr. R. Maurer[42], composed of the luciferase gene under the control of the endogenous PRL gene regulatory region, was used to transfect the rat pituitary lactotroph cell line, GH3. In transient transfection assays, E2, ß-HCH, and o,p'-DDT stimulated expression of this reporter gene (Fig. 3A). In contrast, E2 is effective while ß-HCH fails to activate a reporter gene construct containing only a simple promoter driven by a single ERE, but devoid of additional cis-elements (Fig.3B).

Estrogen-like Effects of ß-HCH and DDT in Mouse Uterus and Vagina. Estrogens increase uterine weights in ovariectomized rodents by initially increasing water imbibition, and then by increasing protein synthesis and cellular proliferation (growth response) . Both ß-HCH and o,p'-DDT act like estrogens in this regard. Dry weight of the uterus is a measure of the growth response. Uterine dry weight was increased when either o,p'-DDT or -HCH were administered at 100 mg/kg to adult ovariectomized mice for 3 days (Fig. 5A). Additionally, o,p'-DDTcaused the vaginal epithelium to increase from a 2 to a 6-7 cell thick layer with a keratinized surface, the typical estrogenic response. ß-HCH produced an epithelium that was 4-5 cells thick but with a mucified surface layer (not shown). The response to ß-HCH in the vaginal epithelium may indicate that the compound has both estrogenic and progestogenic character. Thus, there are qualitative and quantitative differences in the response to stimulation by o,p'-DDT or ß-HCH.

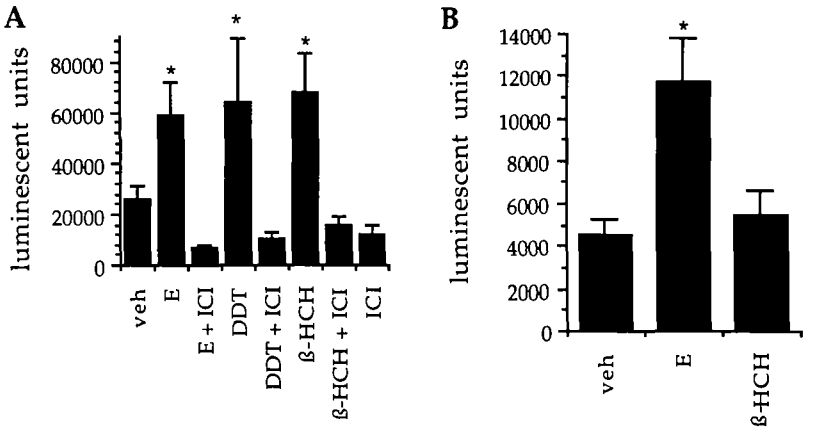

Fig. 3. Effects of -HCH on estrogen-responsive reporter gene constructs. GH3, rat pituitary cells were transfected with one of two different luciferase reporter constructs: A, a 2500-bp region of the prolactin (PRL) gene 5' regulatory region plus the coding region of firefly luciferase (luc) (PRL/luciferase); and B, a 15 bp wild type consensus estrogen response element (ERE) 5' of a minimal promoter region of the thymidine kinase gene (-105 to +36) plus the coding region of luc (EREwtc/Luciferase)> Cells were transfected by electroporation, plated in a 96-well plates, and the following treatments were added: vehicle (veh), 10 nM estradiol (E), 1 µM ß-HCH, or 1 µM o,p'-DDT (DDT), alone or in combination with the antiestrogen, ICI164384 (ICI) at 1 µM. Results shown represent the means from a single representative experiment, with five replicate cultures per treatment group. Bars, SEM, Each transfection experiment was repeated 3-5 times with similar results. (Reproduced, with permission, from Cancer Research 56:5403, 1996.)

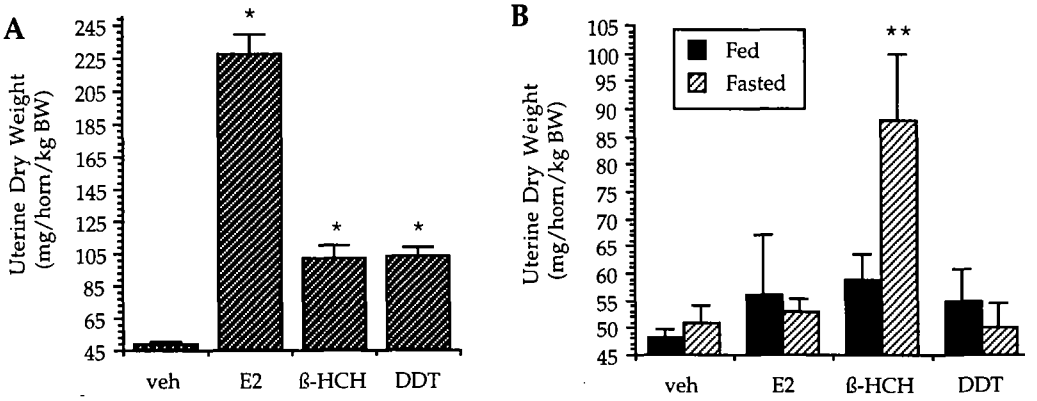

Fig. 4. Uterine Effect of Fast-induced Mobilization of -HCH and o,p'-DDT. (A) Adult mice were ovariectomized and 3 weeks later they received 3 daily injections (ip) of vehicle (veh, dimethylsulfoxide), 10 g/kg estradiol-17 (E2), 100 mg/kg ß-HCH (HCH), or 100 mg/kg o,p'-DDT (DDT). Groups of animals were sacrificed at 24 h after the last treatment injection and one uterine horn was weighed, dried and then re-weighed (mg uterine weight/g body weight). (B) Another group of animals was ovariectomized, and one week later they were dosed with compound as above but then left untreated for two weeks. These animals were then divided into two groups: animals were either fasted for 2 days or maintained on feed *ad libitum*. Uterine weights of ß-HCH-loaded, fasted animals were increased above control levels and above the corresponding uterine weights of fed animals. (*, $p < 0.05$ treatment vs. corresponding vehicle group; **, $p < 0.05$ vs. corresponding vehicle group or corresponding fed group).

Estrogenic Response following Fat Mobilization. We have tested whether fat stores of either ß-HCH or o,p'-DDT are mobilized during fasting and whether release of these stores induce a growth response in the uterus. Ovariectomized animals that had been "loaded" with E2, ß-HCH, or o,p'-DDT were left untreated for 2 weeks; one group was then fasted for 2 days and the other fed *ad libitum*. Fasted animals weighed 4.1 g less than the fed animals (25.9 ± 1.89 g vs. 30.0 ± 2.82 g) and had visibly less intraperitoneal fat than fed animals. In ß-HCH loaded animals, fasting resulted in significant growth of the uterus (Fig. 4B). Note that the degree of growth induction above control (vehicle) was similar following the initial ß-HCH treatment (A) and following the 2-day fast in ß-HCH-loaded animals (B). Fasting also increased the degree of water imbibition in the uterus of ß-HCH-loaded animals (% water/uterine tissue: 79.7 ± 1.49 for ß-HCH-loaded, fasted vs. 73.1 ± 1.33 for vehicle-loaded, fasted, $p<0.05$). There were no similar effects of fasting in o,p'-DDT- or estradiol-loaded animals.

DISCUSSION

The term xenoestrogen is operationally defined as a non-steroidal compound that produces an estrogen-like effect in a bioassay system, such as uterine growth in the ovariectomized mouse. While, ß-HCH is a xenoestrogen, its mechanism of action does not conform to that of other estrogenic compounds. The above studies show that ß-HCH stimulates growth of cultured human breast cancer cells and increases uterine weights and uterine epithelial cell height in ovariectomized mice. Other xenoestrogens, such as o,p'-DDT, that exhibit similar activity do so by directly interacting with the ER or they are metabolized to active ER ligands, competing with E2 in ER binding assays[34-40]. In contrast, we have shown that ß-HCH does not compete with E2 for the ER binding and the lack of an significant increase in nuclear ER during stimulation by ß-HCH indicates that there is no metabolic conversion of ß-HCH to an ER-binding ligand. These results suggest that: 1) ß-HCH exhibits estrogen-like actions in the breast and uterus and may act as a promoter in the formation of estrogen-responsive tumors and 2) the estrogen-like activity of ß-HCH is not mediated by the classic ligand-activated ER pathway. Thus, ß-HCH may represent a new category of xenobiotic capable of promoting uterine cancer.

Organochlorine compounds such as o,p'-DDT and ß-HCH accumulate in fat tissue of animals and man, and fat levels of these compounds are correlated with an increased risk of breast cancer[23-27]. In addition, there is ample evidence to suggest that obesity is a risk factor for both breast and uterine cancers[6-10]. It has been suggested that the link between obesity and estrogen-responsive cancers is due in part to increased non-ovarian estrogen production[9, 10]. Unexplored is the possibility that fat stores of xenobiotics are released in obese patients who are periodically subjected to dietary restrictions in an attempt to reduce their weight and that such a release of bioactive compounds may serve as a source of tumor promoter activity.

Animal studies have shown that fat stores of organochlorine compounds such as DDT, DDE, HCH, or dieldrin are mobilized during periods of dietary restriction[43-45] but that the actual amount of residue that is released into the systemic circulation is a determined, in part, by the chemical structure of the compound[45]. Our data are consistent with this notion of differential mobilization in that fasting produced an estrogenic effect in the ß-HCH-loaded animals but not in the o,p'-DDT-loaded animals. However, this differential effect may have merely resulted from differences in loading of the fat; it will be necessary to measure fat levels of these compounds in future experiments to define the true pharmacokinetics of these compounds.

The breast may pose a very different situation with regard to fat mobilization. Adipose tissue acts as the stroma for the mammary epithelium[46]. Although o,p'-DDT mobilized during lipolysis was not effective in stimulating the uterus, the proximity of the fat to the mammary epithelium may allow locally high concentrations of o,p'-DDT to be achieved. Again, these questions require further investigation.

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