Xenobiotics Released from Fat during Fasting Produce Estrogenic Effects in Ovariectomized Mice

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ABSTRACT

The pesticide residues 1-(o-chlorophenyl)-1-(p-chlorophenyl)-2,2,2-trichloroethane (o,p'-DDT) and β-hexachlorocyclohexane (β-HCH) act as weak estrogens, producing uterotrophic responses in ovariecctomized rodents and stimulating human breast cancer cells in culture. Such activity suggests that these compounds may act as tumor promoters in estrogen-responsive tissues. Organochlorine compounds such as o,p'-DDT and β-HCH are concentrated in body fat. The present report tests whether sufficient compound can be released from fat depots to produce estrogenic effects in uteri of ovariectomized mice. Adult animals were “loaded” with test compound by three daily injections of vehicle (DMSO), 17β-estradiol (E2), β-HCH, or o,p'-DDT. Uterotrophic effects were assessed at 24 h after the last loading dose of test compound and at 2 weeks after the loading regimen, with or without a prior 2-day period of fasting. The initial 3-day treatment with either β-HCH or o,p'-DDT doubled the relative dry weight of the uterus; 102 ± 8.6 mg/kg body weight (BW) and 104 ± 4.4 mg/kg BW for β-HCH and o,p'-DDT, respectively, compared to 49 ± 1.9 mg/kg BW for vehicle-treated animals. E2-treated animals had uterine dry weights of 228 ± 11 mg/kg BW. After 2 weeks without further treatment, a 2-day fast produced a decrease in body mass of 4.1 g/animal. Animals that had been loaded with β-HCH and fasted had uterine weights (88 ± 12 mg/kg BW) significantly greater (P < 0.05) than those of vehicle-loaded, fasted animals (51 ± 2.9 mg/kg BW) or of β-HCH-loaded, fed animals (59 ± 4.6 mg/kg BW). The uterine weights of the fasted and fed o,p'-DDT-loaded or E2-loaded animals were not different from those of control weights. The difference between wet and dry weights showed that fasting of β-HCH-loaded animals also increased water imbibition in the uterus; there was no effect from fasting in the other groups. Generally, epithelial cell height reflected the same responses as uterine weight with the exception that cell heights of β-HCH-loaded animals were slightly higher (P < 0.05) than corresponding controls, indicating that there may have been some active compound available to the tissues even without fasting. The effects of fasting show that during periods of lipolysis β-HCH can be released in quantities sufficient to stimulate estrogen target tissues, suggesting a novel mechanism linking obesity and the progression of estrogen-responsive tumors. The lack of effect from fasting in o,p'-DDT-loaded animals indicates that these compounds are differentially mobilized from fat depots.

INTRODUCTION

Estrogens have been implicated in the etiology of benign and neoplastic tumors of the uterus and breast (1–3). Increased risk for these hormonally sensitive cancers is associated with obesity, anovulatory infertility, late menopause, polycystic ovary syndrome, and steroid-secreting ovarian tumors; increased risk of endometrial carcinoma is also associated with the use of estrogen as postmenopausal hormonal replacement therapy (1, 2). The basis of each of these risk factors is believed to be an increase in circulating estrogen levels. In the case of obesity, the higher levels of estrogen that contribute to increased risk are believed to derive from conversion of adrenal androgens to estrogens in fat cells (4, 5).

Epidemiological data suggest that there was a climbing incidence of uterine cancer from the mid-1940s to the 1980s (6); incidence of breast cancer has also continued to increase during the past several decades (7, 8). Although the acceleration in incidence of uterine cancers between 1968 and 1980 has been attributed to the increased use of estrogen replacement therapy in postmenopause (6), this cannot explain the progressive increase in incidence seen during the earlier years. The widespread use of estrogens and progestins confounds epidemiological data of recent years, particularly since progestins protect against endometrial cancer (1, 9). Increased total lifetime exposure to endogenous estrogens due to early menarche, age at first full-term pregnancy, age at menopause, and postmenopausal obesity has been proposed as the basis for the increased incidence of breast cancer over the past 50 years (6). However, international differences in incidence patterns cannot be fully accounted for by such a hypothesis, suggesting that established risk factors do not explain the increase in breast cancer incidence (7).

It has been suggested that increased incidence of endometrial and breast cancers are linked to environmental factors (10–13). Man-made estrogenic compounds are likely contributors to such an environmental effect. Several organochlorine compounds, including pesticide residues and hydroxylated PCBs, have been characterized as estrogenic (14, 15). These xenoestrogens persist in the environment, have endocrine-disruptive effects in wildlife (14). Furthermore, most organochlorine pesticides are fat soluble, facilitating their tendency to persist in the environment, bioaccumulate in the food chain, and concentrate in human tissues, although their use has been banned in developed countries for more than two decades (11, 17–22).

The present report tests the hypothesis that xenoestrogens stored in body fat will be released during periods of fasting and that this released material will induce estrogenic effects in the uterus. Ovariectomized mice had their fat loaded with the prototypical xenoestrogen o,p'-DDT (23) or with β-HCH, a less well-studied compound with weak estrogenic activity in the mouse uterus (24). It was found that uteri were stimulated following fast-induced lipolysis in β-HCH-loaded animals but not in o,p'-DDT-loaded animals. Such a finding suggests a novel mechanism linking obesity with cancers of hormonally sensitive tissues.

MATERIALS AND METHODS

All procedures involving animals were approved by the Institutional Animal Use and Care Committee. Adult ICR mice (25–30 g BW) were ovariectomized under general anesthesia (ketamine). Groups of animals were left untreated for 3 weeks and then given three daily injections of 5 ng/g BW E2, 100 μg/g BW o,p'-DDT, or 100 μg/g BW β-HCH. Compounds were dissolved in DMSO and delivered i.p. in 100 μl. Control animals received an injection of DMSO. Groups of animals were sacrificed by cervical dislocation on the day following

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2 The abbreviations used are: PCB, polychlorinated biphenyls; BW, body weight; β-HCH, β-hexachlorocyclohexane; o,p'-DDT, 1-(o-chlorophenyl)-1-(p-chlorophenyl)-2,2,2-trichloroethane; DDE, 1,1-dichloro-2,2-bis( p-chlorophenyl)ethylene; E2, 17β-estradiol; ER, estrogen receptor.
the last injection of test compound. Other animals were ovariectomized, treated with compound, and then left untreated for 2 weeks. These animals were then split into groups which were either fasted for 48 h or fed ad libitum as usual, and then they were sacrificed as described above. All animals were weighed at the time of sacrifice.

One uterine horn from each animal was processed for histological examination. It was fixed in buffered formalin, embedded in paraffin, and cut into 6-μm sections. The sections were stained with H&E, and the height of the luminal epithelium was determined with the aid of an image analysis system (IPLab Spectrum; Signal Analytics Corp., Vienna, VA); the height of 75–100 cells was assessed for each specimen. The other horn was used to determine wet and dry weights. This horn was dissected out of each animal, trimmed of mesentery, slit longitudinally, blotted to remove luminal fluid, and then weighed. After weighing, the tissues were desiccated in a 65°C oven for 3 days and weighed again; the dry weight was expressed relative to the animal’s body mass (mg uterine weight/kg BW). The difference between wet and dry weights allowed for determination of the percentage of weight represented by tissue water. Determinations of the epithelial cell height and tissue wet weights were performed in a blinded fashion to avoid technician bias.

Results were subjected to ANOVA and differences between individual treatment means were tested with Fisher’s PLSD. These analyses were carried out with the aid of the Statview statistics program (version 4.02; Abacus Concepts, Berkeley, CA).

RESULTS
Estrogens increase uterine weights in ovariectomized rodents by increasing water imbibition (edema), increasing cell size and protein synthesis (hypertrophy), and inducing cellular proliferation (hyperplasia). Both β-HCH and o,p'-DDT act like estrogens in this regard except that the effect is only a fraction of that produced by the natural estrogen E2. The uterine dry weight, a crude measure of the hypertrophic and hyperplastic responses, was increased when either o,p'-DDT or β-HCH was administered at 100 μg/g BW to adult ovariectomized mice for 3 days (Fig. 1A); in a preliminary study, we found that 10 μg/g BW of either compound was not sufficient (data not shown).

Animals that had been loaded with E2, β-HCH, or o,p'-DDT were left untreated for 2 weeks and then one group was fasted for 2 days and another group left on feed ad libitum. Fasted animals weighed 4.1 g less than the fed animals (25.9 ± 1.89 g versus 30.0 ± 2.82 g) and, although not quantified, there was visibly less i.p. fat in the fasted animals. There were no differences in body weights due to loading treatments. Fasting resulted in growth of the uterus of animals that had been loaded with β-HCH (Fig. 1B). The degree of growth induction above control (vehicle) was nearly equivalent following the initial β-HCH treatment (Fig. 1A), and following the 2-day fast in β-HCH-loaded animals (Fig. 1B), an increase of approximately 60 mg/horn/kg BW and 40 mg/horn/kg BW, respectively, was apparent. Fasting also increased the degree of water imbibition in the uterus of β-HCH-loaded animals (percentage of water/uterine tissue: 79.7 ± 1.49 for β-HCH-loaded, fasted versus 73.1 ± 1.33 for control, fasted, P < 0.05). There were no such effects from fasting on uterine dry weight or the proportion of water in tissues of o,p'-DDT- or E2-loaded animals.

The hypertrophic response to estrogen is reflected in the height of the uterine luminal epithelial cells, and this measurement proves to be a very sensitive indicator of estrogenic stimulation (25–27). As expected, E2 dramatically increased the size of the epithelial cells in the uterine lumen; β-HCH and o,p'-DDT also produced significant increases in the cell height, albeit a fraction of the effect of the natural estrogen (Fig. 2A). In fed, β-HCH-loaded animals, the epithelium was still above control levels, indicating that there was a continued low level of stimulation in these animals 2 weeks after the last injection (Fig. 2B). Fasting of the β-HCH-loaded animals induced a further increase in cell height (Figs. 2B and 3); the degree of this fast-induced response was the same as that seen immediately after 3 days of treatment with β-HCH (Fig. 2).

DISCUSSION
The results of these studies confirm the uterotrophic effects of o,p'-DDT and β-HCH (23, 24). They also indicate that fat stores of β-HCH but not of o,p'-DDT can be mobilized in a biologically significant manner during fast-induced lipolysis. Earlier studies had shown that fat stores of organochlorine compounds such as DDT, DDE, HCH, or dieldrin are mobilized during periods of dietary restriction (19, 28—31). In one study, insecticide residues released from adipose tissue caused nerve damage (28). In another study, dietary restriction that resulted in a 50% decrease in total body fat reduced the total fat content of HCH and dieldrin but not DDT or DDE, the concentration of the latter two compounds doubled in the fat that remained (31). Such results indicate that xenobiotics can be differentially mobilized during lipolysis, some being released in a way...


that allows for redistribution in the body and others are merely resorbed in the remaining fat. Although blood and tissue levels were not measured in the present study, our results are consistent with this notion of differential mobilization of organochlorine compounds from fat depots. Further testing is required to determine the extent to which such differential mobilization occurs with varying body burdens of each compound.

There is ample evidence to suggest that obesity is a risk factor for both breast and uterine cancers (32—37). Results of the present study suggest a novel mechanism as the basis of this risk. It has been suggested that the link between obesity and estrogen-responsive cancers is due to an endocrine-related mechanism such as increased nonovarian estrogen production (4, 5) or insulinemia (36). It has also been suggested that the link with cancer is due to other commonalities between the two pathologies such as increased dietary fat intake (38). The present study suggests a new possibility: It may be 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. Our results in ovariectomized mice suggest that such a hypothesis warrants further testing, including examination of the blood levels of these compounds in women before and during a period of diet-induced weight loss. The hypothesis also suggests that diet-induced fat loss, not obesity per se, may be correlated to tumor etiology; thus, a woman’s dieting habits may become an important factor for determining risk of estrogen-responsive cancers.

\[ \text{Fig. 2. Luminal epithelial cell heights following loading treatments and after fasting of loaded animals. The contralateral uterine horn from treatments as described for A and B in Fig. 1 was used in histological examination. Tissues were fixed, paraffin embedded, and 6-\mu m sections were stained with H&E. The height of 75—100 luminal cells was determined for each animal. }^* P < 0.05 \text{ compared to the corresponding vehicle control; }^{**} P < 0.05 \text{ compared to both the appropriate vehicle control and to the corresponding fed group.} \]

\[ \text{Fig. 3. Uterine histology of fed and fasted } \beta\text{-HCH-loaded mice. Shown are representative views of histological sections used to generate cell height measurements depicted in Fig. 2. Tissues were derived from } \beta\text{-HCH-loaded animals that were either fed (A) or fasted (B). Bars, 10 } \mu \text{m.} \]

\[ \text{\beta-HCH is one of the persistent environmental contaminants that may increase the risk for estrogen-responsive cancers, and release from fat may be an important mechanism involved in this effect. In a recent report, } \beta\text{-HCH levels in breast fat were strongly linked with cancer incidence; fat from cancerous breast was more likely to have a high level of } \beta\text{-HCH than fat of noncancerous breast, with an odds ratio of more than 10:1 (13). } \beta\text{-HCH is a component of the pesticide lindane that was widely used in agriculture for several years in this country. Lindane is a mixture of isomers of hexachlorocyclohexane: the } \gamma\text{-isomer being the active pesticide comprises approximately 10—18% of the total mix; } \beta\text{-HCH makes up about 5—12% of the total mix (39). Manufacture of the technical grade lindane mixture was approximately 12 million pounds/year during the 1960s when it was used extensively as a farm pesticide (39). Although lindane is still used as medication for head and body lice, the medication is composed of purified } \gamma\text{-HCH; of course, the manufacture of } \gamma\text{-HCH still requires production of the other isomers as by-products. Like other organochlorine compounds, the isomers of HCH have accumulated in the environment. As recently as 1992, lindane residues were found in the waters of Resolute Bay, Northwest Territories, Canada; the concentrations of HCH had changed little since last tested in 1986 (40). } \beta\text{-HCH has been measured in fish (17, 18), the meat of game animals (16), and blood, fat, and milk of humans (21, 41—43). In fact, } \beta\text{-HCH bioaccumulates at a rate higher than the other HCH isomers (43, 44). Thus, } \beta\text{-HCH is present in the food chain, is measurable in human fat, and is linked to the incidence of estrogen-responsive cancer. We have} \]

caused uterotrophic responses in ovariectomized mice. Such a finding suggests a new mechanism underlying the well-established link between breast cancer and estrogen-like activity of $\beta$-HCH is not mediated by the classic ligand-activated ER pathway. Thus, $\beta$-HCH may represent a new category of xenobiotic capable of promoting uterine cancer.

Epidemiological studies examining a potential link between exposure to organochlorine compounds and development of estrogen-responsive tumors have yielded controversial results (53—55). Levels of DDT have been reported to be higher in leiomyomas, a benign uterine tumor, than in nontumorous tissue (56); there have been no reported epidemiological studies of endometrial cancer and estrogenic xenobiotics. Analysis of serum levels of DDT showed linkage (10) or a lack of linkage (57) to breast cancer. Tissue levels of $\beta$-HCH but not DDT were higher in fat surrounding cancerous breasts than in fat of noncancerous breasts (13). Such epidemiological analyses based on measurement of tissue and blood levels may never adequately describe the risk from these organochlorine molecules. Numerous studies confirm that people from all parts of the world have these compounds in their fat and blood (58). With such a ubiquitous background level, it is not possible to compare exposed and unexposed cohorts of patients. In addition, the results of the present study suggest that bioavailability of these compounds may be regulated by the integrity of the fat stores, and individual compounds may be affected differently during diet-induced lipolysis, further confounding attempts at correlation analysis using blood and tissue levels.

Mobilization of the weak xenoenestrogen, $\beta$-HCH, from fat depots caused uterotropic responses in ovariectomized mice. Such a finding suggests a new mechanism underlying the well-established link between obesity and estrogen-sensitive cancers of the breast and uterus. Further testing will be required to determine whether such a mechanism is at work in women.

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