Effects of Pesticides on the Ratio of 16α/2-Hydroxyestrone: A Biologic Marker of Breast Cancer Risk

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Xenobiotic estrogens are external compounds with estrogenic activity that may thereby affect the risk of breast cancer. This paper describes a mechanism by which xenoestrogens may affect the development of breast cancer. Estradiol metabolism proceeds by hydroxylation at one of two mutually exclusive sites at C-2 and C-16α. The catechol pathway yields the weakly estrogenic 2-hydroxyestrone (2-OHE_1), which inhibits breast cell proliferation. In contrast, the alternative pathway yields the genotoxic 16α-hydroxyestrone (16α-OHE_1), which enhances breast cell growth, increases unscheduled DNA synthesis, and oncogene and virus expression, and increases anchorage-independent growth. Using a radiometric assay that measures the relative formation of 16α-OHE_1 versus 2-OHE_1 from specifically tritiated estradiol in (ER+) MCF-7 cells, we compared the ratio of 16α-OHE_1/2-OHE_1 observed after treatment with the known rodent carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) with the ratios after treatment with DDT, atrazine, γ-benzene hexachloride, kepone, coplanar PCBs, endosulfans I and II, linoleic and eicosapentenoic acids, and indole-3-carbinol (I3C). These pesticides significantly increase the ratio of 16α-OHE_1/2-OHE_1 metabolites to values comparable to or greater than those observed after DMBA. In contrast, the antitumor agent I3C increased 2-OHE_1 formation and yielded ratios that are 1/3 of those found in unexposed control cells and 1/10th of those found in DMBA-treated cells. Thus the ratio of 16α-OHE_1/2-OHE_1 may provide a marker for the risk of breast cancer. Assays of this ratio, which can be measured in spot urines, may prove useful for a variety of *in vitro* and *in vivo* studies bearing on breast cancer risk. — Environ Health Perspect 103(Suppl 7):147–150 (1995)

Key words: estrogens, P450 hydroxylation, pesticides, cancer risk, hydroxyestrogens

Introduction

Changes in screening practices or in known or suspected risk factors cannot completely account for recently observed increases in the incidence of breast, prostate, and testicular cancer or suspected increases in male and female reproductive disorders, such as reduced sperm count, increased reproductive failures, endometriosis, and ovarian fibroid tumors. With respect to breast cancer, most of the known risk factors for the disease other than genetic makeup can be related to cumulative lifetime exposure to estrogen (*1,2*).

We have recently hypothesized that foreign compounds with estrogenic activity can affect the risk of breast cancer (*3*). Estradiol metabolism predominantly proceeds via two mutually exclusive pathways: one pathway yields the catechol estrogen 2-hydroxyestrone (2-OHE_1), which is weakly antiestrogenic and nongenotoxic (*4*); the alternative pathway yields 16α-hydroxyestrone (16α-OHE_1), a fully potent estrogen, which is tumorigenic and genotoxic and causes increased cell proliferation (Figure 1) (*5*). The reasons for suspecting that alterations in endocrine function underlie estrogen-mediated cancers, such as those of the breast and endometrium, have evolved from diverse observations in endocrinology, biochemistry, and epidemiology. Some 30 years ago, it was calculated that on a molar basis 2-OHE_1 possessed about 0.25% of the uterotrophic activity of estradiol (*6*). Indeed, some studies suggest that 2-OHE_1 is a weak antiestrogen (*7,8*). Others have noted that 4-OHE_2 is carcinogenic in the Syrian hamster kidney model where it is a major metabolite (*9*), but it is a minor metabolite in people. In contrast, 16α-OHE_1 covalently binds with estrogen receptors and amino functions on DNA (*10,11*) and exerts persistent biological responses (*12*). A number of lines of evidence suggest that 16α-hydroxylation is a biological marker of risk for breast cancer and may directly contribute to the initiation and progression of the disease. 16α-OHE_1, the product of the 16α-hydroxylation pathway of E_2 metabolism, causes prolonged growth responses by virtue of its ability to bind covalently to the estrogen receptor (*10*). This paper presents results of an established assay of estradiol metabolism applied to a number of organochlorine pesticides. Materials that increase the ratio of 16α-OHE_1/2-OHE_1 should be regarded as potential breast carcinogens.

Methods

Estradiol metabolism via the C-2 and C-16α hydroxylation pathways was assayed radiometrically (*13*). We measured the amount of estradiol metabolized by these two pathways in estrogen receptor positive (ER+) human breast cell cultures (MCF-7) in the presence and absence of 7,12-dimethylbenz[*a*]anthracene (DMBA) and linoleic acid as positive tumorigenic

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Abbreviations used: 2-OHE_1, 2-hydroxyestrone; 16α-OHE_1, 16α-hydroxyestrone; ER, estrogen receptor; DMBA, 7,12-dimethylbenz[*a*]anthracene; E_2, estradiol; ER+, estrogen receptor positive; MEM, Eagle's minimum essential medium; FBS, fetal bovine serum; ^3H_2O, tritiated water; DDT, dichlorodiphenyltrichloroethylene.

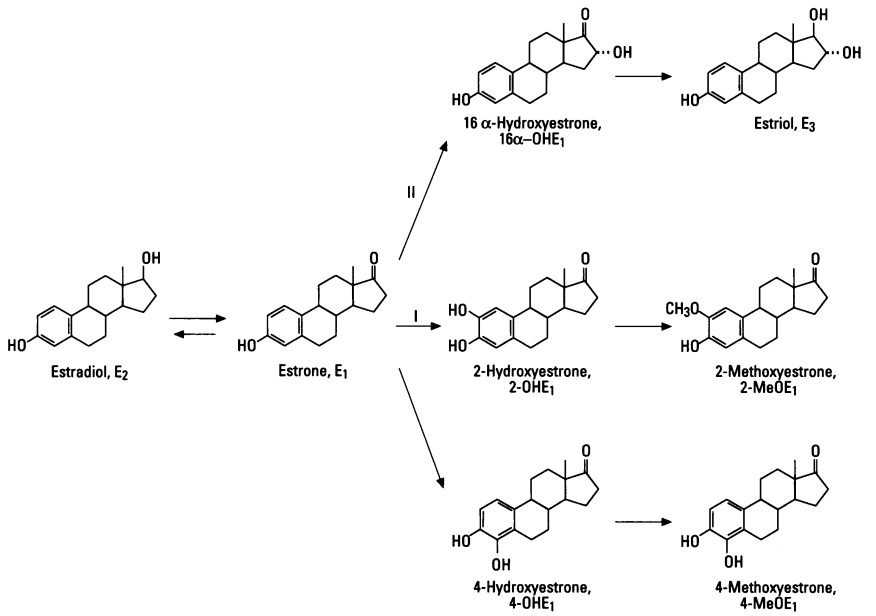

Figure 1. Metabolic pattern for estrogen metabolism pathway I yields the catechol estrogen antagonist 2-hydroxyestrone (2-OHE_1), which is rapidly converted to 2-methoxyestrone; pathway II yields the estrogen agonist 16α-hydroxyestrone (16α-OHE_1), which is ultimately converted to estriol.

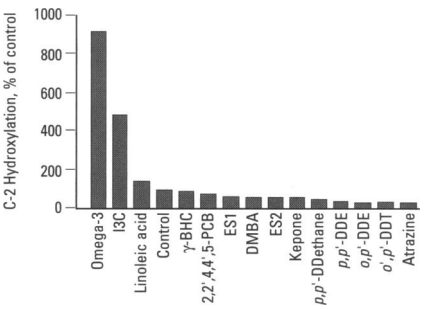

Figure 2. Effect of pesticides on C-2 hydroxylation of estradiol in MCF-7 cells. Abbreviations: ES1, Endosulfan 1; ES2, Endosulfan 2; γ-BHC, γ-benzene hexachloride. Percent of C-2 estradiol hydroxylation induced in MCF-7 cells per million cells. Assay incubation was carried out for 48 hr. Mean values are averages of triplicates from three experiments.

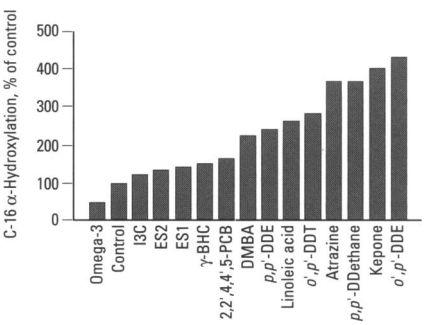

Figure 3. Effect of pesticides on C-16α-hydroxylation of estradiol in MCF-7 cells. Percent of C-16α-hydroxylation per million cells. Assay incubation was carried out for 48 hr. Mean values are averages of triplicates from three experiments.

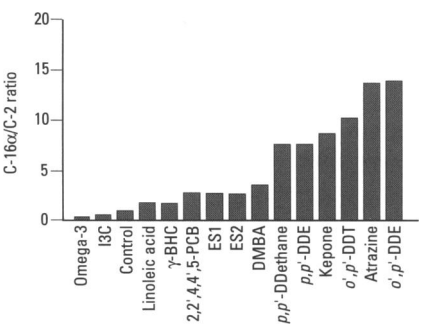

Figure 4. Effect of pesticides on the 16α/2 estrogen metabolite ratio. (Calculated from mean values, Figures 2 and 3.)

controls, indole-3-carbinol and eicosapentenoic acid as negative (antitumor) controls, and a variety of chlorinated pesticides. The MCF-7 cells were plated at a density of 10^5 cells per milliliter in MEM + 10% FBS into 24-well plates and underwent an attachment period of 24 hr. After 24 hr the medium was aspirated from the wells and replaced with fresh medium containing 10^{-5} M of the various pesticides and either C-2 or C-16α tritiated estradiol at a concentration of 10^{-7} M. The cells were then incubated for a further 48 hr. At the end of the incubation period, 0.1 ml of the medium in each well was aliquoted into counting vials containing 5 ml of scintillant, which were counted in a Packard 300 counter. An additional 0.5 ml of the medium was diluted with 3 ml of distilled water and lyophilized to separate the tritiated water (^3H_2O) from the residual bound label. One-milliliter aliquots of the sublimed water were counted in duplicate. From the relative amount of tritiated water formed, the extent of conversion of E_2 to 2-OHE_1 and 16α-OHE_1, respectively, can be calculated. In addition, the cells were washed with buffer to remove residual protein and were then analyzed for DNA. The data in the figures are expressed as the percent conversion of the tracer to the two metabolites (Figures 2 and 3) and the ratio of C-16α/C-2 hydroxylation, with the ratio for the unexposed control cells set equal to 1 (Figure 4).

Results

All of the organochlorine pesticides tested in the present study decreased the amount of 2-hydroxyestrone formed (Figure 2) and significantly increased the amount of 16α-hydroxyestrone formed (Figure 3) relative to untreated control cells by 3- to 4-fold. The greatest effects were observed with DDT, *o,p*-DDE, kepone, and atrazine, which caused substantially greater conversion to 16α-OHE_1 and lower conversion to 2-OHE_1 than was observed with DMBA, a known carcinogen. The exact extent of the change in the metabolic pattern varied with the structure of the compound being tested. The DDT-related compounds had the greatest potency. The combined effect of the changes in both reactions is shown in Figure 4, which shows the change in the ratio of C-16α/C-2 metabolites in response to the various compounds that were tested.

The chlorinated pesticides that we have tested greatly increased the ratio of C-16α/C-2 metabolites relative to the ratio in the untreated control breast cancer cell cultures. Other compounds, such as indole-3-carbinol and eicosapentenoic acid, reduced the ratio to levels lower than those that occurred in unexposed control cells. In general, these organochlorine pesticides produced increases in 16α-OHE_1 comparable to or severalfold greater than that produced by the positive control compound, DMBA. Atrazine and DDE appear

to be 3 to 4 times more potent inducers of 16α-OHE_1 than DMBA. I3C and eicosapentenoic acid indirectly inhibited 16α-OHE_1 formation by substantially increasing C-2-hydroxylation, yielding ratios that were 1/10 and 1/2 that of DMBA, respectively.

Discussion

A number of common organochlorine pesticides alter estrogen metabolism in ER+ cell cultures by increasing the production of the estrogen agonist 16α-OHE_1 and reducing that of the weak antagonist 2-OHE_1. When compared with the ability of DMBA or linoleic acid (*14,15*) to increase 16α-OHE_1, the pesticides tested appear to have similar or greater activity. Previous studies (*5*) have demonstrated that 16α-OHE_1 is genotoxic to normal mammary epithelium and that an elevated ratio of 16α/2 is associated with breast and other cancers in animals. 16α-OHE_1 increases unscheduled DNA synthesis, hyperproliferation, and increased anchorage-independent growth. In contrast, the alternative metabolite 2-OHE_1, which exhibits none of the above properties (*4*), appears to be weakly antiestrogenic (*7,8*). In addition, *in vivo* and *in vitro* studies with carcinogens, oncogenes, and tumor viruses have also produced between 10- and 200-fold higher levels of 16α-OHE_1 compared to unexposed controls (*16,17*). Fifty percent elevated levels of 16α-hydroxylation have been found in human breast cancer as compared to control cases (*18*). Imoto (*19*) has recently reported a major increase in the C-16α/C-2 metabolite ratio in the metabolites isolated by HPLC directly from tumor tissue compared to normal control tissue. The C-16α/C-2 urinary metabolite ratio in women with carcinoma *in situ* in the cervix and in control subjects also differed significantly ($p = 0.008$) (*20*). There is general agreement that most known risk factors for breast cancer can be tied to total lifetime exposures to estrogens (*1,2*). Our findings, and those of other researchers, indicate that a number of environmental chemicals affect endogenous hormone production and metabolism. Other studies have indicated that an elevated ratio of 16α-OHE_1/2-OHE_1 is associated with a number of toxic processes. Our results indicate that materials such as the organochlorine pesticides tested here may increase the risk of breast cancer by altering that ratio. The higher the ratio, the greater the effect on breast cancer cell proliferation, development, and promotion.

Despite this evidence, some have argued that the catechol estrogens play an important role in producing cancer (*21*). They maintain that the catechol estrogens form quinones and semiquinones, which can form adducts with DNA and which may serve as the initiating events that lead to cancer. These researchers have tended to lump all catechol estrogens together as a class; these include: 4-hydroxyestradiol, 2-hydroxyestradiol, and 2-hydroxyestrone. The former two have hydroxy groups at the 17 position, while the latter has a ketone at this same position.

In fact, the three common catechol estrogens have distinctly different properties. Recently, some researchers (*22*) have shown that only 4-hydroxyestradiol is carcinogenic and only in the rather unique male Syrian hamster kidney model. There is no evidence that this compound is even formed in humans *in vivo*. Moreover, no human or animal evidence indicates that carcinogenic effects arise from the catechol estrogen, 2-OHE_1, and it does not show any of the genotoxic or proliferative effects that have been associated with 16α-hydroxyestrone. Recent dietary studies further strengthen the case for concluding that 2-hydroxyestrone is protective against both breast and colon cancer. Both human and animal *in vivo* studies have found that diets high in materials that stimulate 2-OHE_1 are protective against cancer. Animal studies from three laboratories have shown that induction of 2-hydroxylation with indole-3-carbinol results in a decrease in tumor formation (*23–26*). Rates of these two cancers were inversely related to estimated cabbage consumption in 27 European countries (L Kohlmeier, private communication). It is well known that diets high in cabbage and other cruciferae induce P450 1A1 and 1A2, which carry out 2-hydroxylation of estradiol. Under these conditions, there is no induction of 4-hydroxylation (*27*). Further support for this protective effect in humans comes from recent observations from this laboratory on patients with laryngeal papillomas. Consumption of foods that induce estradiol 2-hydroxylation have markedly reduced the rate of growth and recurrence of laryngeal tumors in more than 20 patients observed to date.

Thus, it is important to realize that not all catechol estrogens have similar biological properties. The one form of catechol estrogens that appears to be carcinogenic in the male Syrian hamster kidney model has not been shown to circulate in humans. For all of these reasons, we believe that 2-OHE_1 plays a protective, rather than a harmful, role for human cancer development. We recognize that the relative importance of specific estradiol metabolites and pathways will ultimately be determined by continued laboratory investigations. However, one point on which all investigators agree at this time is that the greater the lifetime exposure to estradiol, the greater the risk of developing breast cancer.

Some recent epidemiologic studies have found that higher serum levels of organochlorines are tied to a 4-fold increase in breast cancer (*28–30*). One study on this point was negative (*31*), but in this study there were equal groups of Asian, African–American, and Caucasian women. Estradiol metabolism appears to be different in Asian women than in white and black women (*32*). When the 150 Asian, white, and African–American breast cancer cases in this negative study were analyzed as three distinct racial/ethnic groups, black women and white women had higher organochlorine levels and 2 to 3 times more breast cancer. Asians showed no effect, consistent with other observations that they may have dietary or genetic protective factors (*33*). Our assay suggests that these same materials that have been associated with increased breast cancer in women also alter estradiol metabolism by increasing the ratio of 16α-OHE_1/2-OHE_1. Thus an elevated ratio constitutes a biological marker for the risk of breast cancer.

Assays of this ratio, which can be efficiently measured in urine, may prove useful for screening new chemicals for their potential risk for breast cancer and for prognostic indices, developing and testing new cancer chemotherapies for breast cancer patients and those at elevated risk, and generating preventive nutritional strategies and interventions.

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