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Estrogenic Activity of Natural and Synthetic Estrogens in Human Breast Cancer Cells in Culture

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Abstract

We investigated the estrogenic activity of various environmental pollutants (xenobiotics), in particular the xenoestrogen *o,p*-DDT, and compared their effects with those of endogenous estrogens, phytoestrogens, and mycoestrogens on estrogen receptor binding capacity, induction of estrogen end products, and activation of cell proliferation in estrogen-sensitive human breast cancer cells in monolayer culture. We also quantified the levels of phytoestrogens in extracts of some common foods, herbs, and spices and in human saliva following consumption of a high phytoestrogen food source (soy milk) to compare phytoestrogen abundance and bioavailability relative to the reported xenoestrogen burden in humans. Results show that natural endogenous estrogens, phytoestrogens, mycoestrogens, and xenoestrogens bind estrogen receptor (ER) in intact cells, but demonstrate marked differences in their ability to induce end products of estrogen action and to regulate cell proliferation. All of the different classes of estrogens stimulated cell proliferation at concentrations that half-saturated ER, but only some classes were able to induce estrogen-regulated end products. Genistein, a common phytoestrogen found in soy foods, differed from the xenoestrogen DDT in its effects on cell proliferation and ability to induce estrogen-regulated end products. Moreover, we found that many of the foods, herbs, and spices

commonly consumed by humans contain significant amounts of phytoestrogens, and consumption of soy milk, a phytoestrogen-rich food, markedly increases the levels of phytoestrogens in saliva. In conclusion, our *in vitro* results predict that a diet high in phytoestrogens would significantly reduce the binding of weak xenoestrogens to ER in target tissues *in vivo*. -- *Environ Health Perspect* 105(Suppl 3):637-645 (1997)

Key words: xenoestrogens, phytoestrogens, endogenous estrogens, synthetic estrogens, breast cancer, reproduction

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Abbreviations used: ß-BHC, ß-hexachlorocyclohexane; BPBS, 0.1% bovine serum albumin in PBS; BSA, bovine serum albumin; CT-FRI, charcoal-treated fetal bovine serum (FBS) in RPMI media with insulin; dH_2O, deionized water; ER, estrogen receptor; ERBA, estrogen receptor binding assay; HCB, hexachlorobenzene; HMEC, normal human mammary epithelial cells; OC, oxychlordane; PBS, phosphate-buffered saline; PCB-126, pentachlorobiphenyl; PCB-153, hexachlorobiphenyl; PR, progesterone receptor; TBPS, 0.1% Tween-20 in PBS; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Introduction

Each year billions of pounds of pesticides, herbicides, solvents, detergents, and other chemical wastes spill into our ecosystem and accumulate in the air, water, and, food chain (1,2). Environmental scientists have recently raised concern that many of these industrial wastes are weakly estrogenic and, by acting as estrogen mimics (xenoestrogens), disrupt normal endocrine function, leading to reproductive failure and cancer of estrogen-sensitive tissues (1-3). Recent scientific reviews (4,5) have contested the notion that trace levels of petrochemicals to which humans are inadvertently exposed act as endocrine disruptors or increase the incidence of cancer. This counterargument suggests that xenoestrogen levels to which humans are exposed daily are trivial relative to the daily exposure to natural phytoestrogens found

abundantly in common foods consumed by humans.

Many estrogen mimics are produced by plants and by the petrochemical and pharmaceutical industries. These can be categorized broadly as natural or synthetic (Table 1). Natural estrogens are those that humans, and all other animal species, have been exposed to throughout their evolution. They are manufactured by the body (endogenous estrogens), or by plants (phytoestrogens), or fungi (mycoestrogens). In contrast, synthetic estrogens are those that only recently have been introduced into the ecosystem either purposely as pharmaceuticals (drugs) or inadvertently by the petrochemical industry (xenoestrogens). Phytoestrogens are found abundantly in foods, herbs, and spices commonly consumed by humans (6,7), whereas mycoestrogens (8) and xenoestrogens (1,2,4) gain entry into the food chain as contaminants of air, water, and food.

Table 1.

The molecular structure of exogenous natural and synthetic estrogens may be very similar to, or strikingly different from, the parent hormone estradiol (4,5,7,9). Despite their structural diversity, all of the exogenous estrogens, when consumed either as natural components (phytoestrogens) or contaminants (xenoestrogens, mycoestrogens), have the capacity at some concentration to bind to estrogen receptors (ERs) in target cells of the body and initiate (agonist) or inhibit (antagonist) estrogenlike actions (10,11). In doing so, estrogen mimics have the potential to alter, either in a beneficial or harmful manner, the growth, development, and function of estrogen target tissues.

In this study we compared the relative estrogenic potency of various well-known environmental petrochemical pollutants with natural endogenous estrogens, phytoestrogens, and mycoestrogens in order to shed light on possible differences in molecular actions that might account for the putative harmful effects of xenoestrogens on reproductive function and cancer. We investigated differences in the ability of these structurally diverse compounds to bind ER, induce estrogen-regulated end products, and activate cell proliferation in well characterized estrogen-sensitive human breast cancer cell lines *in vitro*. We also used an estrogen radioreceptor assay to quantitate the levels of phytoestrogens present in common foods, herbs, and spices in order to compare their abundance relative to the reported burden of xenoestrogens in humans (1,2,4,5). In addition, we developed a saliva radioreceptor assay to monitor the bioavailable fraction of phytoestrogens in the bloodstream following consumption of phytoestrogen-rich foods, herbs, or spices. As a paradigm for this system we measured the total estrogen content of saliva following consumption of soy milk, a phytoestrogen-rich food source.

Methods

Chemicals, Hormones, and Reagents

Propidium iodide, sulforhodamine B, calf thymus DNA, Triton X-100, thimerosol, bovine serum albumin (BSA), estradiol, progesterone, ß-naphthoflavone, hesperitin, genistein, equol, and kaempferol were purchased from Sigma Chemical Co. (St. Louis, MO). *o,p*-DDT, *o,p*-DDE, hexachlorobenzene (HCB), ß-hexachlorocyclohexane (ß-BHC), oxychlordane (OC), pentachlorobiphenyl (PCB-126), hexachlorobiphenyl (PCB-153), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were a gift from D. Hayward (Hazardous Materials Laboratory, California Department of Health Services). 4-Hydroxytamoxifen (ICI 79280) was a gift from Stuart Pharmaceuticals (Wilmington, DE).

Cells and Culture Conditions

ER positive MCF-7 and T47D breast carcinoma cell lines were purchased from American Type Culture Collection (Rockville, MD). The breast cancer cell lines were grown and passaged routinely as monolayer cultures in T-25 flasks in RPMI-1640 medium (Sigma R-7880) with 5% fetal bovine serum (FBS; Hyclone A-1115) containing 0.2 ng/ml insulin (Sigma I-2767) at 37°C in 5% CO_2.

Cell Proliferation Studies

For growth studies, cells were removed from their growth chamber with trypsin/EDTA (Sigma T-5775), counted, diluted to 8000 to 20,000 cells/ml in growth media and 250 µl seeded into 96-well tissue culture plates. Twenty-four to 48 hr after cell plating, media was completely removed and cells were switched to 250-µl well of phenol-red-free RPMI-1640 medium (Sigma R-8632) containing 1 to 2% charcoal-treated FBS (CT-FRI) (Hyclone A-1120) (1% CT-FRI) and insulin, as above, with or without test compounds in a final ethanol concentration of 0.1%. Controls contained 0.1% ethanol. For growth studies, media was changed after the first 4 days, then every 2 days thereafter.

Hormone-treated cells (in triplicate) were allowed to reach near confluence, formalin-fixed *in situ* with 10% formaldehyde/phosphate-buffered saline (PBS) for 15 min at room temperature, and then analyzed *in situ* for DNA and total cellular protein as previously described (12).

Estrogen Receptor Binding Assay

Preparation of Test Compounds. Test compounds were prepared in 100% ethanol as 1000 X stocks ranging from 1 nM to 10 mM. For the assay, stock solutions were diluted 250-fold in 1% CT-FRI. Control tubes contained 1% CT-FRI with 0.4% ethanol; final concentration in the incubation was 0.1%. Duplicate 50-µl aliquots of test compounds were delivered into 12 X 75 mm glass tubes followed by 50 µl of ^{125}I-17ß-estradiol (E_2) (NEX-144, 2200 Ci/mmol, NEN-Dupont, Wilmington, DE) diluted in 1% CT-FRI to a concentration of 0.4 nM. The tubes were equilibrated in the cell culture incubator at 37°C in 5% CO_2 while the cells were prepared.

Preparation of Cells. MCF-7 cells were fed with 5% CT-FRI at least 3 days prior to the assay. Cells were removed with trypsin/EDTA and, after dispersion to single cells, diluted in 1% CT-FRI to 1.75 (±0.5) X 10^6 cells/ml.

Assay Proper. Diluted cells (100 µl) were added to tubes containing test compounds and ^{125}I-E_2. The contents were mixed by gently shaking the tube rack and incubated at 37°C with 5% CO_2 for 45 min, with gentle mixing every 15 min. Following incubation, the cells were sedimented at 600 X g for 5 min at 4°C. The supernatant was removed and the tubes plunged into an ice-water bath. Within 5 min, 1 ml of ice-cold TPSG (0.2% Triton X-100 in PBS containing 0.1 M sucrose and 10% glycerol) was added as described (12). Tubes were vigorously vortexed then allowed to incubate 5 min before intact nuclei were centrifuged for 5 min at 4°C. The supernatant was discarded, and ^{125}I-E_2 remaining in the washed nuclei was measured in a gamma counter. Results are expressed as percent ^{125}I-E_2 binding to ER in the nucleus in the absence (control=100%) or presence of increasing concentrations of test compounds.

pS2 Measurement in Cell Growth Media by Radioimmunoassay

MCF-7 cells were grown in 96-well plates as previously described in "Culture Conditions." Approximately 48 to 72 hr (established as maximal time point for pS2 induction) following addition of 250 µl of the test compounds, 125 µl of growth media was removed and transferred to a parallel plate. Sodium azide (10%) was added to 0.05% final concentration, and the plate was sealed and stored at 4°C until pS2 could be assayed (usually within 1 week). The remaining 125 µl of growth media covering the cells was discarded. The cells were then formalin fixed and assayed for DNA and protein content as described above. pS2 was measured by a two-site enzyme-linked immunosorbent assay format as previously described (12).

Immunocytochemical Analysis of pS2, Estrogen Receptor, and Progesterone Receptor

in Formalin-fixed/Paraffin-embedded Cells

Preparation of Cells. Cells were grown in T-25 flasks in 5% CT-FRI in the presence and absence of chemicals as indicated in the text. Following incubation for 2 to 3 days, cells were gently scraped from the flask with a plastic cell rake into the growth media and transferred to a glass tube. Cells were washed twice with PBS and centrifuged at 500 X g. Cells were fixed overnight at 4°C in 10% formalin/PBS, then dehydrated in graded ethanol and xylene. The dehydrated cell pellet was embedded in paraffin, cut into 5-μm sections, and mounted on glass slides (Probe-On Plus, Fisher Biotech, Fisher Scientific, Pittsburgh, PA, 15-188-52) for immunocytochemical analysis.

Immunocytochemical Analysis. Glass slide-mounted thin sections were air-dried overnight and dewaxed with Hemo-DE (Fisher 15-182-507A) followed by rehydration in 100 and 95% ethanol and PBSTX (PBS containing 0.1% Triton X-100 and 0.01% thimerosol). Each treatment was repeated 5 times for 5 min. The specimen slides were then treated with 1% hydrogen peroxide in deionized water (dH_2O) for 15 min and washed 5 times with PBSTX. All treatments were followed by five washes in PBSTX unless indicated otherwise.

For pS2 analysis, the slides were incubated with rabbit polyclonal antibody to pS2 (Novacastra, Burlingame, CA, NCL-pS2) at 1/400 dilution in PBS buffer containing 1% BSA followed by biotinylated antirabbit IgG (H+L, Vector Laboratories, Burlingame, CA), horseradish peroxidase streptavidin (Vector Laboratories, SA-5004). pS2 was then revealed in the cells by incubation with the aminoethylcarbazole peroxidase substrate (Zymed, San Francisco, CA, 00-2007). Cells were counterstained with 10% Harris hematoxylin (Sigma GHS-3-16), washed with water, covered with Crystal/Mount (Biomeda, Fisher Scientific) and dried for 20 min at 80°C.

For detection of ER and progesterone receptor (PR) in formalin-fixed/paraffin-embedded cells by immunocytochemistry, slides containing thin sections were treated as above for pS2 with the exception that primary antibodies were mouse monoclonals (ER, Immunotech, Inc., Westbrook, ME, 1344; PR, Signet Laboratories, Inc., Dedham, MA, 521-26) and the secondary antibody a biotinylated goat antimouse IgG (Zymed 62-6540).

Immunocytochemical staining for pS2 (cytoplasmic granular staining) and for ER and PR (nuclear staining) was evaluated by light microscopy at 100 X and 250 X as follows: none or less than 10% (-), 10-40% (+), or >40% (++) of cells staining at any intensity (weak to strong).

Preparation of Herbal Extracts (Tinctures)

Dried herbs were extracted in 50% ethanol/dH_2O (2 g herbs/10 ml) for 2 days and the insoluble contents sedimented by centrifugation at 1500 X g for 15 min at room temperature. The soluble extract was removed and transferred to a 15-ml polypropylene tube and stored at 4°C. Unless otherwise indicated, herbal tinctures were combined with 1% CT-FRI (4 ml extract/ml) to determine their estrogen content by estrogen receptor binding assay (ERBA).

Collection and Processing of Saliva

Before collecting saliva, volunteers rinsed thoroughly with cool water and chewed sugar-free gum for about 5 min to stimulate saliva flow. Saliva (5 ml) was then collected in polypropylene tubes. Sodium azide was added to a final concentration of 0.1% and the saliva frozen, thawed, and clarified by centrifugation at 1500 X g for 10 min. Saliva (1 ml) was extracted twice with 1 ml of diethylether and the ether evaporated to dryness under nitrogen. The dried ether extract was then reconstituted in 1.0 ml of 0.1% BSA/dH_2O and assayed for estradiol equivalents by the ERBA.

Statistics

Data are presented as the mean ± SD. Statistical significance was determined using the Student's *t*-test with $p<0.05$ evaluated as statistically significant.

Results

Binding of Natural and Synthetic Compounds to Estrogen Receptor in Intact MCF-7 Breast Cancer Cells

Table 1 compares the ER binding capacity of several endogenous and exogenous estrogens. The concentrations of estradiol, genistein, zearalenone, and *o,p*-DDT required to inhibit the binding of a single subsaturating concentration of radiolabeled estradiol to ER in intact MCF-7 cells are shown in Figure 1. The concentration of these and other endogenous and exogenous natural and synthetic estrogens and antiestrogens required to reduce the binding of ^{125}I-17ß-estradiol to ER by half is summarized in Table 2, and expressed relative to estradiol (100%). The endogenous estrogen metabolites 16α-hydroxyestrone and 2-hydroxyestradiol were 20 and 0.1% as potent as estradiol. The most potent natural exogenous estrogens tested were the mycoestrogens zearalenone and its metabolite zearalenol, which were 2 and 100% as potent as estradiol. The isoflavonoids genistein and equol were 0.1% as potent as estradiol, whereas the bioflavonoids kaempferol and quercetin were very weak ER binders, with relative potencies of 0.01 and 0.001%. Neither hesperitin, which is a 3'-methylated form of quercetin, nor naphthoflavone, which has no phenolic groups, were able to compete with estradiol binding to ER at concentrations up to 10 mM.

Figure 1

Figure 1. Competition of natural and synthetic estrogens for [^{125}I]-E_2 binding to nuclei of intact MCF-7 cells. Cells were incubated with [^{125}I]-17ß-estradiol and increasing concentrations of the test compounds as described in "Methods." Nuclear radioactivity was determined and results expressed as percent [^{125}I]-E_2 binding to ER in the presence of test compounds. Results are representative of at least two other experiments.

Table 2.

Of the seven industrial xenobiotics tested, only *o,p*-DDT and *o,p*-DDE significantly competed with estradiol binding to ER with relative binding affinities similar to those of bioflavonoids such as kaempferol and quercetin, or approximately 0.01% lower than that of estradiol. None of the other xenobiotics tested at single concentrations of 1 μM were able to compete with estradiol binding to ER and therefore were not considered for further testing. Higher concentrations (>1 μM) of these highly nonpolar xenobiotics were not tested because most were insoluble in the low serum (1%) cell growth media used for the ERBA.

Estrogen End Product Induction by Natural and Synthetic Estrogens

In MCF-7 cells, pS2 and PR are estrogen-regulated proteins (11,13). PR is an intracellular protein localized to the nuclear compartment whereas pS2 is a secretory protein, about half of which is released into the growth medium within 24 to 48 hr. ER is also sensitive to estrogens and is downregulated by estrogen exposure (11). Therefore, the upregulation of pS2 and PR and the downregulation of ER were used as indices of the estrogenic activity of test compounds from each category listed in Table 2. pS2 was measured both as a secreted protein in the growth media by enzyme immunoassay (12) and as an intracellular protein by immunocytochemistry. ER and PR also were measured semiquantitatively by immunocytochemistry. Each compound was tested at a concentration determined to approximately half-saturate the nuclear ER binding sites (Figure 1, Table 1).

Results in Table 3 demonstrate that estradiol, zearalenol, zearalenone, genistein, equol, and kaempferol significantly increased both the extracellular and the intracellular concentration of pS2. These estrogens also increased the intracellular immunostaining for PR, and decreased the immunostaining for ER, consistent with their actions as estrogen agonists (11). As might be expected for estrogen antagonists (12) such as tamoxifen and hydroxytamoxifen and non-ER-binding ligands such as progesterone and cortisol, there was no increase in pS2 or PR. Neither *o,p*-DDT nor quercetin, at concentrations that were shown to half-saturate ER, increased pS2 or PR; however, both of these weak estrogens downregulated ER.

Table 3.

Effect of Natural and Synthetic Estrogens on Cell Proliferation

The effects of DDT, estradiol, and several phytoestrogens on cell proliferation of the ER(+) human breast cancer cell line T47D, are shown in Figure 2. The initial concentrations for each test compound were one log lower than the concentrations determined in Table 2 to half-saturate ER. Estradiol was not tested above 10 nM because higher concentrations are not relevant to expected *in vivo* exposure in humans (8).

Figure 2.

Figure 2. Effects of natural and synthetic estrogens on proliferation of ER-positive T47D human breast cancer cells. Cells were exposed to different concentrations of estrogens for 11 days (Figure 3). The effects of test compounds on cell growth were evaluated by measuring the DNA content of cells *in situ* as described in "Methods." Results are expressed as micrograms of DNA per well (mean ± SD) and are representative of at least two other experiments.

Cell growth, measured as DNA or total cellular protein concentration (protein data not shown), increased with all ER-binding compounds tested. With some estrogens (genistein and kaempferol) the response was biphasic and resulted in stimulation at ER-saturating doses (1 and 10 μM, respectively), but abrupt growth inhibition at a slightly higher concentration (20 μM). In contrast, other ER-binding

compounds (DDT, equol), although they showed similar growth stimulation at concentrations that approximately half-saturated ER (i.e., 1 and 10 nM, respectively), continued to stimulate growth at higher concentrations (10-20 nM). When hydroxytamoxifen at 100 nM was combined with ER-saturating doses of 1 nM estradiol and 1 nM equol, genistein, and DDT, the growth stimulation seen with these compounds was inhibited to near control levels (respectively, 0.24 +/0.04, 0.31 +/0.03, 0.46 +/0.07, and 0.17 +/0.05 ng DNA/well), indicating that the actions of all of these estrogens were ER mediated.

Estrogenic Content of Common Foods, Herbs, and Spices

Nature produces a broad array of phytoestrogens in foods, herbs, and spices commonly consumed by humans (6,7,14,15). These phytoestrogens might be expected to compete with endogenous estrogens, as well as exogenous xenoestrogens, for binding to ER sites in target cells. To provide some appreciation for the abundance of natural phytoestrogens, relative to the reported burden of xenoestrogens in blood and adipose tissue of humans (1,2,4,5), we used the ERBA to quantify the phytoestrogen content in extracts of several foods, herbs, and spices commonly consumed by humans.

Our laboratory has tested over 200 foods, herbs, and spices for estrogen content (DT Zava, unpublished data); 12 with the highest ER-binding activity are listed in Table 4. Not unexpectedly, soy milk, which is known to contain high levels of the phytoestrogens genistein and daidzein (14), had the highest level of phytoestrogens. Licorice, *Glycyrrhiza glabra,* and red clover, *Trifolium pratense,* both of which, like soybeans, belong to the *Leguminosae* family, also contained high levels of phytoestrogens, as reported previously (6). Thyme, *Thymus vulgaris*, and tumeric, *Curcuma longa*, common spices in foods, and hops, *Humulus lupulus,* used in beer production, were also relatively high in phytoestrogens. Other herbs used as phytomedicines (15,16), such as mandrake, *Podophylum peltatum*, bloodroot, *Sanguinaria canadensis*, verbena, *Verbena hastata,* yellow dock, *Rumex crispus*, and sheep sorrel, Rumex acetosella, also contained significant levels of phytoestrogens.

Table 4.

Use of Saliva to Measure *in vivo* Bioavailability and Estrogenic Content of Exogenous Estrogens

in vivo, endogenous and exogenous natural and synthetic estrogens are constantly competing for the same ER sites in target cells. Access to ER will depend on the estrogen's abundance, affinity, and bioavailability to target cells (4,5). Saliva is a natural ultrafiltrate of blood and has been shown to contain the bioavailable or free fraction of nonpolar, low molecular weight molecules such as steroid hormones (17,18). Xenobiotics, as well as phytoestrogens, fall into this class of low molecular weight, nonpolar molecules (7,9,16), and are detectable in saliva. To confirm that the saliva assay can detect an increase in the levels of ER-binding components following consumption of a phytoestrogen food source, we measured estradiol and total estrogen levels in saliva after ingestion of soy milk, which contains high levels of phytoestrogens (Figure 3A-D).

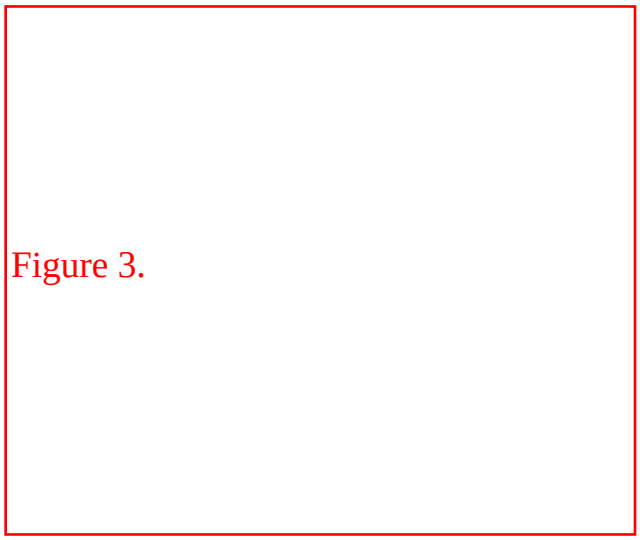

Figure 3. Saliva estrogens following soy milk consumption. Volunteers drank 200 cc of soy milk and collected saliva samples at hourly intervals over 24 hr. Saliva was processed and assayed for ER-binding components by the ERBA and for estradiol by conventional radioimmunoassay as described in "Methods."

Four laboratory volunteers drank 200 cc of soy milk, then collected saliva at hourly intervals over 24 hr. The volunteers refrained from consumption of other soy foods over this time course, but otherwise ate as usual. Saliva was extracted, reconstituted, then analyzed for estradiol by conventional radioimmunoassay and for total estrogens by ERBA. In three of four individuals soy milk ingestion resulted in a rapid increase in total salivary estrogens within 1 hr, followed by a rapid decline over 5 hr. In several individuals a second peak was seen at 5 to 12 hr, followed by a return to baseline within 24 hr. Note that very low levels of salivary estrogens were seen in one individual (Figure 3B) following soy milk consumption. Salivary estradiol was also quantified in the same saliva samples and did not change significantly over the 24-hr time course. No changes in total salivary estrogens were seen over the same time course when volunteers drank water or cow's milk (data not shown).

Discussion

In regions where wildlife has been heavily exposed to industrial pollutants, reproductive tract failures are commonplace, threatening survival of many animal species (1,2,9,19,20). These forewarnings have

led environmental scientists to conjecture that the bioaccumulation of petrochemical pollutants with estrogenlike activity in our ecosystem and food chain could also account for the reported increase in reproductive dysfunction (3), lower intelligence and behavioral disorders in children (21,22), and cancers of the breast and reproductive organs (23-25). This argument has been refuted (4,5) on the grounds that natural phytoestrogens, which bind with much higher affinity to ER, and are consumed in much higher quantities in common foods than are xenoestrogens, would diminish xenoestrogen binding to ER in target cells to insignificant levels. Hence, by competitively inhibiting xenoestrogen binding to ER, phytoestrogens in food would suppress any toxic effects of xenoestrogens as endocrine disruptors or cancer promoters.

The daily exposure from xenoestrogenic pesticides such as DDT, dieldrin, endosulfan, and methoxychlor is approximately 2.5 mg/day (4,5). The serum concentration of DDT in women ranges from 2 to 15 nM, which is approximately 5 to 40 ppb (23-26). Our *in vitro* cell culture results (Table 2) demonstrate that the concentration of DDT required to half-saturate ER is about 1000-fold higher (1 mM) than the reported levels of DDT in serum (23-26). Based on this information we would not predict that DDT, or other xenoestrogens with similar binding affinities to ER, would have significant estrogenic impact on target tissues *in vivo*. However, xenoestrogens and other xenobiotics are very nonpolar molecules and resist metabolic activation/elimination, which allows them to bioaccumulate and persist in fatty tissues of the body for years (23-27). In fact, the tissue burden of DDT (2 ppm) and other xenobiotics such as PCBs (5-6 ppm) in adipose breast tissue of humans is nearly 1000-fold higher than their serum levels (23-27). This biomagnification of xenoestrogens in fatty tissues or organs of the body could effectively raise the local concentrations of xenoestrogens such as DDT to the low micromolar range, which could have estrogenic effects on target tissues, according to our data (Table 2, Figure 2).

Because xenobiotics bioaccumulate in fatty tissues of the body over a lifetime, most currently available assays that assess the short-term estrogenicity of a xenobiotic in animals *in vivo*, or in human cells in culture *in vitro*, probably fall short of portraying the true estrogenic potential of an environmental pollutant in humans *in vivo*. Moreover, a recent study found that the cumulative effects of several pesticides commonly found as pollutants in the environment at low levels are over 1000 times more potent as xenoestrogens than the individual molecules.

It has been suggested (4,5) that xenoestrogens would not be expected to have a significant impact on estrogen target tissues because their effects would be competitively blocked from ER sites by the much higher concentrations of phytoestrogens consumed in foods. Indeed, our results confirm that the amounts of phytoestrogens consumed in common foods, herbs, and spices (Table 4, Figure 3) would be much greater than the estimated daily exposure to xenoestrogens (e.g., DDT) and other xenobiotics found in the air, water, and food chain (4,5). Moderate consumption of soy foods in amounts common to Asian diets (12,14) results in intake of about 10 to 20 mg of phytoestrogens such as genistein and daidzein (14,29). Based on the results presented in Figure 3, the bioavailable levels of estrogens increase from less than 50 to about 200 to 1000 pg/ml following soy milk consumption. From this we estimate that the bioavailable level of phytoestrogens would range from 200 to 1000 ng/ml, or 1 to 3 mM. Hence, the phytoestrogen concentration after consumption of a soy meal might be expected to be at least 100 times

the reported serum concentrations of xenoestrogens (i.e., 1-3 μM vs 2-15 nM). Since the phytoestrogens bind to ER with about 10-fold greater affinity than DDT and other xenoestrogens [(7,9,30); Table 2], this would reduce the apparent binding of xenoestrogens such as DDT to ER nearly 1000-fold. Other common spices (e.g., turmeric and thyme) and hops, which are used copiously in many foods and beverages (e.g., beer) could also significantly contribute to the phytoestrogen content *in vivo* (Table 4).

Although our *in vitro* results concur with Safe's hypothesis (4,5) that the estrogenic actions of xenoestrogens *in vivo* would be expected to be insignificant relative to endogenous estrogens and dietary phytoestrogens, it is important to underscore that significant differences in the bioaccumulation and metabolism of xenoestrogens *in vivo* may significantly increase their biological impact on estrogen target tissues. As mentioned, the tissue burden of DDT and other xenobiotics such as PCBs (24,26) can be much higher than serum levels (25,27), and many of these chemicals persist for years in fatty tissues of the body such as the liver, breast, testes, ovaries, and brain. In sharp contrast to the very long biological half-lives of most xenobiotics, our studies (Figure 3) and others (29,31) demonstrate that phytoestrogens such as those found abundantly in soy foods have very short biological half-lives and are inactivated, metabolized, or eliminated within hours after consumption.

Our studies have also revealed that xenoestrogens such as DDT can have very different effects on cell proliferation and estrogen-regulated end products than estradiol or phytoestrogens such as genistein. For example, estradiol and genistein at concentrations that half-saturated ER were equipotent estrogen agonists; they both induced several well-characterized estrogen-regulated proteins (pS2 and PR), downregulated ER, and stimulated cell proliferation (Table 3, Figure 2). In contrast, DDT did not stimulate the synthesis of the estrogen-regulated proteins despite its ability to stimulate cell proliferation (Table 3). Moreover, DDT differed from genistein in its effects on cell proliferation over a broad concentration range. Genistein demonstrated a biphasic effect on cell proliferation, stimulating proliferation up to 1 μM concentration and abruptly inhibiting proliferation at 10 to 20 μM. In contrast, DDT persistently stimulated cell proliferation over the same dose range. Equol, which is structurally very similar to genistein (7,12), does not possess the same growth inhibitory properties at the high (10-20 μM) concentration. We previously speculated that structural features endow genistein with both relatively potent estrogenic and growth inhibitory properties that are unique among the flavonoids (12).

Immunocytochemical staining of formalin-fixed cells with a pS2-specific antibody confirmed that only those compounds that induced pS2 in the growth media increased specific intracellular cytoplasmic pS2 antibody staining in MCF-7 cells (Table 3). Nearly all of the estrogens (excepting equol) that increased both the intracellular cytoplasmic and extracellular (growth media) levels of pS2 also increased specific nuclear staining for PR, consistent with the notion that both of these proteins are estrogen-regulated. Most of the chemicals tested that downregulated ER also induced pS2 and PR, as would be expected for a pure estrogen agonist such as estradiol. However, two of the estrogens that downregulated ER (DDT and quercetin) did not induce pS2 or PR, indicating a divergence in the molecular pathways regulating cell proliferation and estrogen end-production induction.

Although our *in vitro* tests would predict that the impact of xenoestrogens on estrogen target tissues

would be trivial relative to phytoestrogens, it is important to recognize that some petrochemicals perturb endocrine pathways by disrupting other hormone receptor systems or by modifying the metabolic disposition of steroid hormones. For example, xenobiotics such as PCBs and DDE have antiandrogen activity (32,33). Other xenobiotics such as TCDD indirectly affect estrogen action by downregulating ER, thus acting as an antiestrogen (4,5). We (Table 2) and other investigators (4,5) have found that TCDD does not bind directly to ER. However, it does bind to arylhydrocarbon receptor and induces cytochrome P450 enzymes that enhance estradiol metabolism (4,5). Many of the xenobiotics have similar actions on estradiol metabolism (1,34-36), and therefore could affect the formation of highly reactive forms of endogenous estrogens that could potentially damage cells, leading to mutations and eventually to cancer. For example, hydrocarbon-induced 16α-hydroxyestrone (34) covalently binds to ER, and estrogen-3,4-quinone, a by-product of estrogen metabolism, binds DNA and has been shown to be carcinogenic in animal models (36).

Another weakness of using *in vitro* models to predict the impact of xenoestrogens *in vivo* is that these model systems do not take into account how bioaccumulation of xenobiotics in hormone-synthesizing organs (adrenals, ovaries, testes, thyroid) might alter the ability of those organs to manufacture endogenous hormones (e.g., estradiol, progesterone, testosterone, cortisol, dehydroepiandosterone, thyroxine). Epidemiologic and clinical studies have closely linked imbalanced hormone production to reproductive dysfunctions, cancers (breast, uterus, and prostate), premenstrual syndrome, menopausal symptoms, and numerous other diseases in humans (heart disease, osteoporosis, Alzheimer's, immune dysfunction) (17,37-42).

Experiments on nonhuman primates have demonstrated that petrochemical pollutants can have a profound impact on hormone-synthesizing organs (1,2,43,44). For example, hexachlorobenzene (HCB) has been shown to suppress luteal progesterone synthesis in cynomolgus monkeys (43). We and other investigators (9) have not found that HCB binds to ER (Table 1), which would categorize it as a potential endocrine disruptor but not xenoestrogen. HCB has also been found in human serum, fat, and ovarian follicular fluid of *in vitro* fertilization patients, and therefore could potentially suppress ovarian progesterone synthesis in humans. Interestingly, the stable isomer of lindane, BHCH, was recently shown to induce estrogenlike effects through estrogen responsive genes without apparent binding to ERs (45). We also found that BHCH does not bind estrogen receptors but did not test it further for effects on cell proliferation.

Although it is well recognized that failed production of progesterone by the corpus luteum during the first trimester of pregnancy leads to miscarriage (17) and that progesterone is essential for proper neuronal development (46), virtually nothing is known about what effect a more subtle and persistent suppression of progesterone synthesis by xenobiotics might have on fetal development or on a woman's overall health, well-being, and susceptibility to breast and endometrial cancers. Lower ovarian progesterone production in adult women has been associated with increased bone loss (41,42) and estrogen dominance (37)--risk factors for osteoporosis and cancers of the endometrium and breast.

Because studies show that xenobiotics suppress progesterone synthesis in nonhuman primates (43), it is tempting to speculate that reported lower birth weight, smaller skull circumference, lower intelligence,

and serious attention deficit disorders (21,22) in children whose mothers periodically ate fish highly contaminated with PCBs and other xenobiotics might have resulted from xenobiotic-suppressed synthesis of progesterone, or some other vital hormone (e.g., thyroid) essential for proper neuronal development during gestation. Lowered ovarian progesterone synthesis during gestation could have significant impact on a child's intellectual capacity and overall demeanor. This is supported by a study showing that children born to women given progesterone supplementation for preeclampsia during pregnancy were remarkably more intellectually advanced and socially well adapted than normal children or children whose mothers had preeclampsia and did not take progesterone (47). It remains to be determined if the bioaccumulation of xenobiotics in organs such as the ovaries and testes is partly responsible for the reported increase in reproductive failures in animals and humans (1,2), as well as the alarming increase in attention deficit disorders in children.

In summary, we find that xenoestrogens, phytoestrogens, and mycoestrogens, relative to the parent estrogen estradiol, may have very different effects on estrogen-induced end products and regulation of cell proliferation depending on the chemical's structure and concentration. Our *in vitro* results concur with those of Safe's hypothesis that phytoestrogens, commonly consumed in foods, would block binding of xenoestrogens to ER in target tissues and thereby negate any harmful effects of xenoestrogens. However, this concept is flawed because it is based solely on *in vitro* studies and does not take into consideration the long half-life, bioaccumulation, and non-ER-mediated actions of xenobiotics on hormone-synthesizing organs in the human body. Future studies should focus not only on the direct estrogenic actions of chemicals as xenoestrogens, but also on the actions of the broader array of xenobiotics that contaminate our ecosystem as endocrine disruptors via altered synthesis and metabolism of natural endogenous hormones. Broad field studies of this sort should be feasible with simple noninvasive saliva assays for steroid hormones (17).

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