Dietary Estrogens Stimulate Human Breast Cells to Enter the Cell Cycle

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It has been suggested that dietary estrogens neutralize the effect of synthetic chemicals that mimic the effects of estrogen (i.e., xenoestrogens, environmental estrogens). Genistein, a dietary estrogen, inhibits the growth of breast cancer cells at high doses but additional studies have suggested that at low doses, genistein stimulates proliferation of breast cancer cells. Therefore, if dietary estrogens are estrogenic at low doses, one would predict that they stimulate estrogen-receptor positive breast cancer cells to enter the cell cycle. Genistein and the fungal toxin zearalenone were found to increase the activity of cyclin dependent kinase 2 (Cdk2) and cyclin D_1 synthesis and stimulate the hyperphosphorylation of the retinoblastoma susceptibility gene product pRb105 in MCF-7 cells. The steroidal antiestrogen ICI 182,780 suppressed dietary estrogen-mediated activation of Cdk2. Dietary estrogens not only failed to suppress DDT-induced Cdk2 activity, but were found to slightly increase enzyme activity. Both zearalenone and genistein were found to stimulate the expression of a luciferase reporter gene under the control of an estrogen response element in MVLN cells. Our findings are consistent with a conclusion that dietary estrogens at low concentrations do not act as antiestrogens, but act like DDT and estradiol to stimulate human breast cancer cells to enter the cell cycle. — *Environ Health Perspect* 105(Suppl 3):633–636 (1997)

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Estrogenic chemicals present in the human diet or environment have been called xenoestrogens or environmental estrogens. Recently it has been suggested that xenoestrogens may have a role in the etiology of breast cancer (*1,2,3*). Compounds that have been suggested to be xenoestrogens include pesticides, dyes, pollutants, plasticizers and food preservatives (*2,4–7*). For example, the pesticide DDT and the food colorant Red Dye No. 3 have been shown to bind to the estrogen receptor (ER) and to stimulate proliferation of ER-positive breast cancer cells (*2*). At the molecular level, DDT and Red Dye No. 3 mimic the effects of estradiol by stimulating breast cancer cells to enter the cell cycle (*2,8*). Entry into the cell cycle requires cyclin D_1 synthesis, activation of cyclin-dependent kinase 2 (Cdk2), and retinoblastoma protein (pRb105) hyperphosphorylation (*2,8*). Phosphorylation of pRb105 by activated cyclin-dependent kinase can be detected as a migration shift using Western blot analyses after breast cancer cells are treated with estradiol, Red Dye No. 3, or DDT (*8–11*).

Whether xenoestrogens have a role in the etiology of human breast cancer remains controversial (*1,12–16*). It has been suggested that exposure to xenoestrogens cannot produce adverse effects on reproductive tissue because they are neutralized by estrogenic compounds derived from dietary sources (*17*). Therefore, if dietary estrogens have chemopreventive activity by antagonizing the effects of estrogen, one would predict that cellular processes associated with entry into the cell cycle (e.g., cyclin-dependent kinase activation, cyclin D_1 synthesis) would be blocked by dietary estrogens. Genistein has recently been shown to be a potent inhibitor of ER-positive breast cancer cell growth when added at high concentration (>10 μM) (*18,19*). However, genistein at lower concentrations has also been shown to increase the growth of ER-positive cells (*19,20*).

In this study, we examined the effects of dietary estrogens on cyclin D_1 synthesis, Cdk2 activity, and pRb105 phosphorylation. By performing Cdk2 assays on MCF-7 breast cancer cells that were treated with low concentrations of genistein or zearalenone in addition to DDT, we also determined if genistein or zearalenone exhibited antiestrogenic activity. The ability of zearalenone and genistein to stimulate the expression of a reporter gene under the control of an estrogen response element in human breast cells stably transfected with this construct was also determined.

Materials and Methods

Chemicals

ICI 182,780 was a gift from Alan Wakeling, Zeneca Pharmaceuticals (Macclesfield, England). Dietary estrogens and estradiol were purchased from Sigma Chemicals (St. Louis, MO). Dietary estrogens and DDT were concentrated solutions in absolute ethanol. An equal amount of ethanol was added to all control cells.

Human Breast Cancer Cells

MCF-7 cells were maintained in Dulbecco's Modified Minimal essential medium/ Hams F12 1:1 without phenol red (Sigma Chemicals). Both cell lines were incubated at 37°C in a 5% CO_2 atmosphere with 10% fetal bovine serum (FBS). Prior to studies on Cdk2 activation, MCF-7 cells were growth arrested by removal of serum and transfer into methionine-free medium for 72 hr before exposure. During dietary

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Abbreviations used: A/G, albumin–globulin; ATP, adenosine triphosphate; Cdk2,cyclin-dependent kinase 2; ER, estrogen receptor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; pRb105, retinoblastoma susceptibility gene product; t_0, time zero.

estrogen exposure, breast cells were maintained in methionine-containing DMEM/F12 (phenol red- and FBS-free).

MVLN cells were provided by M.J. Duchesne, Montpelier, France. These cells have been stably transfected with a firefly-luciferase reporter gene that is under the control of an estrogen response element of the *Xenopus vitellogenin A2* gene and a thymidine kinase promoter of the Herpes simplex virus (*21*).

Estrogenicity Assay

The ability of zearalenone and genistein to stimulate the expression of luciferase production under the control of an estrogen response element was performed by depriving MVLN cells of estrogen. Estrogen deprivation in MVLN cells was accomplished by growing them in culture with phenol red-free DMEM/F12 medium containing 5% charcoal-stripped, delipidated calf serum. After gradual withdrawal of the serum, the cells were left in the medium alone for 24 hr more and then treated with estradiol (1 nM), estradiol (1 nM) and ICI 182,780 (100 nM), zearalenone (10 nM), zearalenone (10 nM) and ICI 182,780 (100 nM), genistein (0.1 μM), or genistein (0.1 μM) and ICI 182,780 (50 nM). The medium alone was used as the control. After 24 hr of treatment the cells were lysed and the luciferase assay performed using Promega's Luciferase Assay kit (Madison, WI) per the kit protocol. Light emission from treated MVLN cell extracts was measured using a scintillation counter in out-of-coincidence mode.

Cyclin-dependent Kinase 2 Assays

MCF-7 cells for Cdk2 analysis were exposed to estrogens for 20 hr. After incubation, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed by the addition of cold lysis buffer (Tris 20 mM, pH 7.5, NaCl 250 mM, 0.1% NP-40, NaF 10 mM, $NaVO_3$ 1 mM, PMSF 1 mM). After 15 min on ice, the lysates were centrifuged at 20,000× g for 15 min (4°C). Cdk2 was precipitated from equal amounts of cell extracts using purified rabbit anti-Cdk2 (Santa Cruz Biotechnology, Santa Cruz, CA) and protein albumin-globulin (A/G) agarose. Immunoprecipitates were washed three times with the lysis buffer and twice with kinase buffer (Tris 40 mM, pH 7.5, $MgCl_2$ 10 mM). The immunoprecipitates were suspended in 30 μl of kinase buffer supplemented with 400 μg/ml histones (Sigma Chemicals type II-SS), 5 μM adenosine triphosphate (ATP), 0.5 mM dithiothreitol, 0.5 mM ethylene glycol tetraacetic acid, and 5 μCi γ-[^{32}P]-ATP for 20 min at room temperature. The reaction was stopped using gel electrophoresis sample buffer, and the reaction products were separated on a 14% polyacrylamide gel (Novex, San Diego, CA).

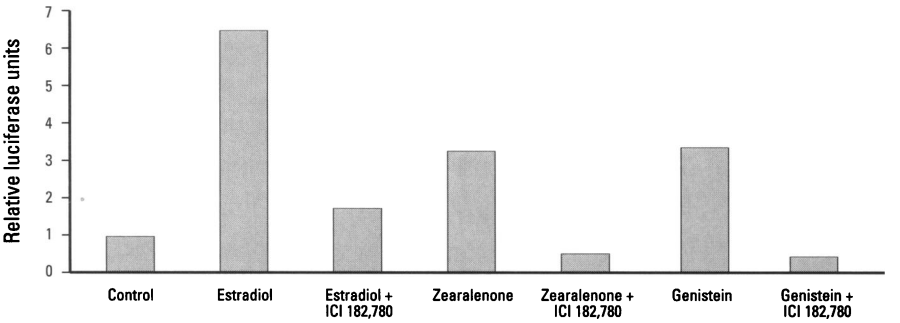

Figure 1. Genistein (0.1 μM) and zearalenone (10 nM) at low concentrations mimic effects of estradiol by stimulating luciferase production in MVLN cells where the reporter gene is under the control of an estrogen-response element. The steroidal antiestrogen ICI 182,780 (100 nM) blocks genistein- and zearalenone-stimulated luciferase production.

Cyclin D_1 Synthesis

MCF-7 cells were plated in six well culture plates, grown to 60 to 80% confluency, and growth arrested as described for kinase assays. Deficient medium was replaced and the dietary estrogens added as indicated. At appropriate time points, medium in wells to be radiolabeled was changed to methionine-free DMEM/F12 (no serum or phenol red). After 15 min at 37°C, this medium was removed and replaced with methionine-free DMEM/F12 containing 50 μCi of Trans ^{35}S-label (1000 Ci/mmol). Following incubation for 2 hr at 37°C, labeling medium was removed and the monolayers were washed twice with ice-cold PBS. Lysates were prepared as given for kinase assays and stored at –80°C for later use. For immunoprecipitation analysis, lysates were equalized based on protein concentration and precleared with 30 μl of protein A/G agarose beads followed by incubation with antibodies to cyclin D_1 (mab HD-11, Santa Cruz Biologicals, Santa Cruz, CA), 1 μg per sample, and protein A/G agarose beads. After 18 hr at 4°C, immune precipitates were washed 5 times with wash solution, suspended in 1X sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, boiled, and separated on 12% polyacrylamide gels. Following electrophoresis, the gels were fixed, washed in water, and soaked in 1 M sodium salicylate (pH 6.0) before drying. Fluorography was performed using Kodak XAR film at –80°C.

Results

Stimulation of Estrogen Receptor-controlled Luciferase Production

We hypothesized that if dietary estrogen exposures were mitogenic, they acted through the ER. Therefore, the expression of a reporter gene (luciferase) under the control of an estrogen response element must be increased in MVLN cells exposed to dietary estrogens. (Figure 1) shows that exposure of MVLN cells to genistein (0.1 μM) or zearalenone (10 nM) stimulates the production of a luciferase reporter gene that is under the control of a *Xenopus* estrogen response element. The steroidal antiestrogen ICI, 182,780 (100 nM) inhibited luciferase production in MVLN cells stimulated by all of the estrogens, demonstrating the requirement for ER in xenoestrogen action (Figure 1).

Stimulation of Cyclin D_1 Synthesis

Synthesis of cyclin D_1 occurs early in G_1 phase prior to activation of Cdk2 (*9–11*). Estradiol and xenoestrogens (e.g., DDT, Red Dye No. 3) (*2*) induce increased synthesis of cyclin D_1 in G_1-arrested MCF-7 cells (*8*). Increased synthesis of cyclin D_1 protein induced by genistein and zearalenone confirm that both dietary estrogens regulate expression of this protein critical for progression through the cell cycle at low concentration (0.5 μM) (Figure 2).

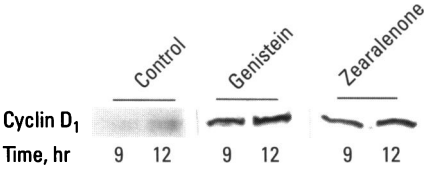

Figure 2. Increased cyclin D_1 synthesis is found in MCF-7 cells treated with genistein or zearalenone at low concentrations (0.5 μM).

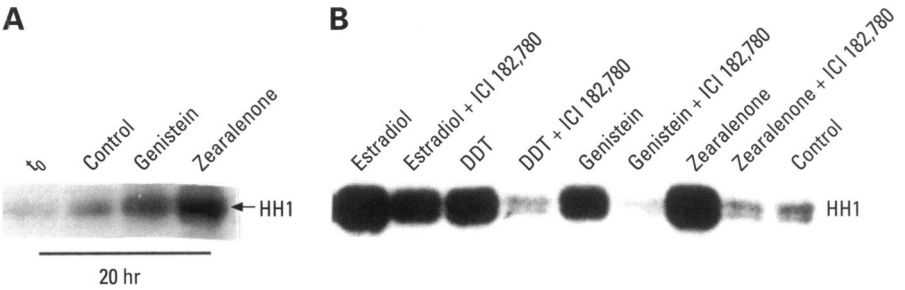

Figure 3. (*A*) Genistein and zearalenone increase Cdk2 activity in MCF-7 breast carcinoma cells; (*B*) increased activity in Cdk2 activity in genistein-treated (1 μM) and zearalenone-treated (1 nM) MCF-7 cells was completely inhibited by the steroidal antiestrogen ICI 182,780 (50 nM). Cdk2 activity stimulated by DDT (0.3 μM) was also completely inhibited by ICI 182,780 (50 nM). However, the same concentration of ICI 182,780 only partially suppresses Cdk2 activation stimulated by estradiol (10 nM).

Stimulation of Cdk2 Activation

Subsequent to cyclin D_1 synthesis and activation of Cdk4, transit of cells through G_1 and entry into *S* phase requires Cdk2 activation (*9–11*). Both genistein and zearalenone stimulated Cdk2 activity when added to growth-arrested MCF-7 cells (Figure 3A). Increased Cdk2 activity induced by genistein or zearalenone could be detected as early as 12 to 16 hr after they were added to MCF-7 cells (data not shown). However, maximum levels of dietary estrogen-stimulated Cdk2 activity occurred 18 to 22 hr after they were added to human breast cancer cells (Figure 3A).

The steroidal antiestrogen ICI 182,780 (50 nM) partially inhibits Cdk2 activation stimulated by estradiol (10 nM) and completely inhibits Cdk2 activation by DDT (0.3 μM), genistein (1 μM), and zearalenone (1 nM) (Figure 3B). Both genistein and zearalenone slightly increased Cdk2 activity induced in MCF-7 cells by DDT (Figure 4).

Phosphorylation of pRb105

Cdk activation results in the hyperphosphorylation and inactivation of pRb105, allowing for release of transcription factors of the E2F family, which are required for *S* phase entrance (*10*). Therefore, if dietary estrogens mimic the effects of estradiol on the cell cycle, then increased phosphorylation of pRb105 should occur in breast cancer cells treated with dietary estrogens. Figure 5 shows that low levels of dietary estrogens (0.5 μM) induce hyperphosphorylation of pRb105 that can be detected as a mobility shifted form of pRb105 by Western blot analyses.

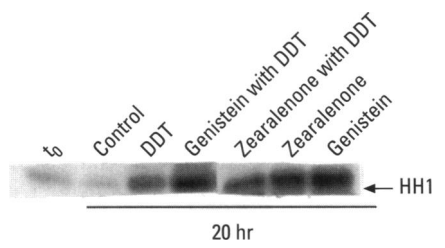

Figure 4. Cdk2 activity in MCF-7 cells is increased by DDT, genistein, and zearalenone. When added with low concentrations of DDT, genistein and zearalenone increase the Cdk2 activity above that produced by DDT, genistein, or zearalenone alone.

Discussion

The role, if any, that xenoestrogens have in the etiology of human breast cancer is controversial (*1,2,15,17,22*). Some investigators have proposed that exposure to xenoestrogens may enhance the risk of developing breast cancer (*1,2,22*). Some epidemiologic studies have supported this hypothesis (*3*), but others do not find any correlation with xenoestrogen exposure and breast cancer (*14*). Additionally, it has been proposed that exposure to estrogenic chemicals in the diet may neutralize or somehow prevent xenoestrogens from having any significant biologic effect (*17*).

Human diets contain plant-derived nonsteroidal estrogenic compounds (e.g., genistein). Other estrogenic molecules in the diet may include compounds produced by fungi (e.g., zearalenone) (*20*). Additional estrogenic compounds in the diet may include zeranol, a synthetic derivative of zearalenone, which has been used as a hormonal growth-promoter in cattle (*20*). Dietary derived estrogenic compounds like genistein have been proposed to act in preventing proliferation of ER-positive breast cells (*17*). However, one previous report failed to find any chemopreventive action of genistein or zearalenone because these compounds stimulated the growth of MCF-7 human breast cancer cells (*20*). More recent reports suggest that genistein is a potent inhibitor of MCF-7 cells (*18,19*). The apparent disparity in the reported effects of genistein on MCF-7 cells may depend on the concentration of genistein used. Genistein is a potent inhibitor of protein tyrosine kinases and can inhibit cell cycle progression in tumor cells independent of action at the ER (*23,24*). Other inhibitors of MCF-7 cells have been shown to have concentration-dependent estrogenic effects and can also display antiestrogenic activity (*25*). We surmised that dietary estrogens may have estrogenic activity at low concentrations and antiestrogenic activity or toxicity at higher concentrations.

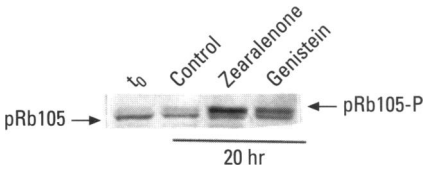

Figure 5. Genistein and zearalenone induce hyperphosphorylation of pRb105. Hyperphosphorylated pRb105 is evident as a slower migrating band.

It has been previously demonstrated that estrogen induces Cdk2 activity, cyclin D_1 synthesis, and hyperphosphorylation of pRb105 in growth-arrested MCF-7 cells (*8*). Two xenoestrogens also have been shown to mimic estrogen's ability to stimulate MCF-7 cells to enter the cell cycle (*2*). Therefore, using Cdk2 activation, cyclin D_1 synthesis, and hyperphosphorylation of pRb105, we examined the effects of low concentrations of genistein and zearalenone on growth-arrested MCF-7 cells.

In all assays the dietary estrogens simulated molecular changes, indicating that they stimulated cell cycle entry. Additionally, rather than antagonizing the effects of DDT, both dietary estrogens stimulated Cdk2 activation. Hyperphosphorylation of pRb105 was induced by both genistein and zearalenone. Flow cytometric data has confirmed that both genistein and zearalenone induce *S* phase entry in MCF-7 cells as has been demonstrated with estradiol (*8*) (data not shown). Therefore, at low concentrations, dietary estrogens appear to stimulate MCF-7 cells to enter the cell cycle. In addition, the steroidal antiestrogen ICI 182,780 was found to inhibit stimulation of Cdk2 activity by genistein and zearalenone (Figure 3B). Genistein and zearalenone also stimulated the production of a reporter molecule under the control of an estrogen response element that was also inhibited by ICI

182,780 (Figure 1). Therefore, genistein and zearalenone stimulation of human breast cancer cells to enter the cell cycle is mediated through the dietary estrogens' effects on the ER and transcriptional control via estrogen-responsive elements. Further studies are required to determine if genistein and zearalenone inhibit Cdk2 activity, cyclin D_1 synthesis, and hyperphosphorylation of pRb105 at higher concentrations.

Our studies using molecular assays to evaluate the effects of dietary estrogens agree with previous reports (*19,20*) that at low concentrations, genistein and zearalenone produce proliferative effects on human breast cancer cells. The effects appear to be concentration dependent, which would agree with a recent study that shows stimulation of MCF-7 cell growth at genistein concentrations of less than 10 μM. Our studies do not support the suggestion that dietary estrogens neutralize the effects of DDT. In contrast, the effects of dietary estrogens at low concentrations on DDT-induced Cdk2 activation appear to be additive or perhaps synergistic (Figure 4).

Under the proper conditions and concentrations, genistein has been reported to be a potent inhibitor of MCF-7 cell growth (*18,19*). However, our studies suggest that women should not consume particular foods (e.g., soy-derived products) to prevent breast cancer. Further, our results suggest that low concentrations of genistein may stimulate MCF-7 cells to enter the cell cycle. If the amount of estrogens that can be derived from the dietary sources does not contribute a level high enough to suppress ER-positive cell growth, dietary estrogens may increase the risk of breast cancer, especially in combination with dietary derived xenoestrogens. The risk of exposure to xenoestrogens and low levels of dietary estrogens may be further increased if developing fetal tissues are exposed (*23*).

The molecular effects of dietary derived and xenoestrogens on ER-positive breast cancer cells appear to be complex. The effects of dietary estrogens may be concentration dependent and may interact with synthetic and natural estrogens. It may be premature at this time to suggest dietary changes that significantly alter the amount of dietary derived estrogens until additional research can fully elucidate the effects they have on reproductive tissues in terms of dose, tissue-specific effects (like tamoxifen), and potential interactions with other estrogenic compounds. It remains to be determined if dietary estrogens are beneficial or, as suggested by our *in vitro* studies, an additional carcinogenic risk factor for tissues where proliferation is controlled by estrogens.

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