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Effects of environmental estrogens on tumor necrosis factor α-mediated apoptosis in MCF-7 cells

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Environmental estrogens represent a class of compounds which have been shown to mimic the effects or activity of the naturally occurring ovarian hormone 17β-estradiol. Given the role of 17β-estradiol in cell survival in a number of systems, we wished to determine if environmental estrogens protect MCF-7 cells from apoptosis. Here we demonstrate that the organochlorine pesticides *o, p′* DDT and alachlor, like 17β-estradiol, have the ability to suppress tumor necrosis factor α (TNF)-induced apoptosis in estrogen receptor (ER)-positive MCF-7 breast carcinoma cells. These compounds, however, did not affect TNF-induced apoptosis of the ER-negative MDA-MB-231 cell line. The ability of these compounds to suppress apoptosis in MCF-7 cells was correlated with an ER-dependent increase in Bcl-2 expression. Taken together these results demonstrate that estrogenic organochlorine pesticides like *o, p′* DDT and alachlor may partially mimic the primary endogenous estrogen, 17β-estradiol, and function to suppress apoptosis in ER-responsive cells.

Introduction

Apoptosis is a process of cellular suicide by which specific cells undergo a programmed series of biochemical events culminating in the elimination of those cells (1,2). Apoptosis is a normal physiological process that functions to control cell populations during embryogenesis, immune responses, hormone withdrawal from dependent tissues and normal tissue homeostasis (1–6). Recent studies have suggested that apoptosis may play a critical role in the generation and progression of cancer and may have potential applications in cancer therapy (1,4–7).

Accumulating evidence suggests that steroid hormones regulate apoptosis in hormone-responsive tissues. Both prostate and mammary epithelial cells undergo apoptosis upon removal of testosterone and estrogen, respectively (3,8–11). This dependence upon hormone for survival and proliferation extends to neoplasms arising from these tissues (3,9,12,13). The MCF-7 breast cancer cell line has been shown to form tumors in nude ovariectomized mice only in the presence of estrogen. Upon removal of estrogen the tumor cells begin to undergo apoptosis, leading to tumor regression (12). Additionally, recent studies have shown that pretreatment of MCF-7 cells grown *in vitro* with estrogen reduces the induction of apoptosis by cytotoxic drug treatment as well as tamoxifen (14–16). These reports also show that one mechanism by which estrogens may affect apoptosis is through the increased expression of Bcl-2, a member of a family of apoptosis regulating proteins whose expression has been shown to suppress MCF-7 cell apoptosis (17). These studies provide evidence that estrogens may play a role in both tumorigenesis and drug resistance through suppression of apoptosis.

Environmental estrogens represent a class of compounds, both natural and synthetic, which can mimic the function or activity of the endogenous estrogen 17β-estradiol. These environmental estrogens may function as endocrine disrupters both in wildlife and humans, leading to developmental defects, disease and, potentially, cancer (18–23). Recently, a number of organochlorine pesticides, including DDT, (3α,5aβ,6β,9β, 9aβ)-6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide (endosulfan II) and 2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide (alachlor), have been shown to mimic estrogen and are capable of binding to the estrogen receptor (ER), causing transcription from estrogen response elements (ERE) in DNA and causing proliferation of MCF-7 cells *in vitro* (24–26). The potential exists that these compounds, acting through the ER, can affect the apoptotic pathways of estrogen-responsive cells. With mounting evidence for the role of estrogen in the regulation of apoptosis, we suggest that these environmental estrogens can act like endogenous estrogen to inhibit tumor necrosis factor α (TNF)-induced apoptosis in ER-positive breast cancer cells.

Abbreviations: alachlor, 2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)-acetamide; *o,p′* DDT, 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl) ethane; DMEM, Dulbecco's modified Eagle's medium; endosulfan II, (3α,5aβ,6β,9β,9aβ)-6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide: ER, estrogen receptor; ERE, estrogen response element; FBS, fetal bovine serum; TNF, tumor necrosis factor α.

Materials and methods

Cell culture

MCF-7 cells (N variant, passage 50) were generously provided by Louise Nutter (University of Minnesota) (27). MDA-MB-231 cells were obtained from The American Type Culture Collection (Rockville, MD). MCF-7 and MDA-MB-231 cells were routinely maintained and grown in high glucose-containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), BME amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (Gibco BRL, Gaithersburg MD) and 1×10^{-10} M porcine insulin (Sigma Chemical Co., St Louis, MO) (10% DMEM). Cells were maintained in 75 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Prior to experiments both cells lines were placed in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (5% DCC-FBS) supplemented with BME amino acids, MEM amino acids, L-glutamine, penicillin/streptomycin, sodium pyruvate (Gibco BRL) (5% CS-DMEM) for 48 h prior to plating. The cells were plated in 6-well plates at 5×10^5 cells/well in the same medium and allowed to attach overnight. Following this, cells were treated accordingly.

17β-Estradiol was purchased from Amersham Corp. (Arlington Heights, IL), 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane (*o,p′* DDT) was purchased from Sigma, alachlor and endosulfan II were purchased from AccuStandard (New Haven, CT).

Luciferase assays

Estrogen-responsive reporter gene analysis was performed as described by Klotz *et al*. (26). Briefly, MCF-7 cells were placed in 5% CS-DMEM for 48 h prior to plating. The cells were plated in 6-well plates at 5×10^5 cells/well in the same medium and allowed to attach overnight. The next day the cells were transfected for 5 h in serum/supplement-free DMEM with 2 μg of plasmid pERE2-luciferase, which contains two copies of the vitellogenin ERE linked to the luciferase gene, and 1 μg of plasmid pCMV-β-galactosidase using 12 μl of lipofectamine (Gibco BRL). After 5 h, the transfection medium was removed and replaced with phenol red-free DMEM supplemented with 5% DCC-FBS containing vehicle, 17β-estradiol or environmental estrogen and incubated for 18 h at 37°C.

After 18 h the treatment-containing medium was removed and 250 μl of 1× lysis buffer (Analytical Luminescence Laboratory, Ann Arbor, MI) was added per well and incubated for 15 min at room temperature. The cell debris was then pelleted by centrifugation at 15 000 *g* for 5 min. The cell extracts were normalized for protein concentration using the Bio-Rad Reagent following the supplied protocol (Bio-Rad, Hercules, CA). For β-galactosidase assays, the cell extract was placed in 500 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM β-mercaptoethanol), 100 μl of *o*-nitrophenyl-β-D-galactopyranoside at 4 mg/ml in Z-buffer was added to each reaction and the tubes placed at 37°C. The addition of 400 μl of 1 M Na_2CO_3 terminated the reactions. The β-galactosidase activity of each reaction was measured at an absorbance of 420 nM. Luciferase activity for the cell extracts were determined using Luciferase Substrate (Promega, Madison, WI) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

Viability assay

Cell viability was determined using the Trypan blue assay as described previously (27). Briefly, MCF-7 or MDA-MB-231 cells were plated at 5.0×10^5 cells/ml in 10 cm^2 wells in 5% CS-DMEM. The cells were allowed to adhere for 24 h before treatment with vehicle (1% DMSO), 17β-estradiol (1 nM), *o,p′* DDT (100 nM) or alachlor (1 μM) for 24 h. Following this, cells were treated with TNF (10 ng/ml) (R&D systems, Minneapolis MN) and harvested 24 h later for viability analysis using the Trypan blue exclusion method. Percent viability was expressed as the percentage of viable cells in the treated samples as compared with control viability, with 500 cells counted per sample.

DNA fragmentation analysis

Following treatment, cells were harvested for DNA as described previously (27). Briefly, $1–2\times10^6$ cells were pelleted and resuspended in lysis buffer (10 mM Tris–HCl, 10 mM EDTA, 0.5% w/v SDS, pH 7.4) to which RNase A (100 μg/ml) was added. After incubation for 2 h at 37°C, proteinase K (0.5 mg/ml) was added and the lysates were heated to 56°C for 1 h. NaCl was then added (final concentration 1 M) and lysates were incubated overnight at 4°C. Lysates were centrifuged at 15 000 *g* for 30 min and nucleic acids in the supernatant were precipitated in 2 vol of ethanol with 3 M Na acetate. Isolated DNA was then separated by electrophoresis on 1.5% agarose gels for 2 h and visualized by ethidium bromide staining.

Immunoblot analysis

MCF-7 cells (5×10^6) were grown for 3 days and then harvested in sonicating buffer (62.5 mM Tris–HCl, pH 6.8, 4% w/v SDS, 10% glycerol. 1 mM phenylmethylsulfonyl fluoride, 25 mg/ml leupeptin, 25 mg/ml aprotinin) and sonicated for 30 s. Following centrifugation at 1000 *g* for 20 min, 50 μg of protein was resuspended in sample loading buffer (62.5 M Tris–HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 3 min and electrophoresed on a 15% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with phosphate-buffered saline/0.05% Tween, 5% low fat dry milk solution at 4°C overnight. The membrane was subsequently incubated with a solution of rabbit antisera (anti-Bcl-2 1:4000) and incubated for 2 h at room temperature. Blots were washed in phosphate-buffered saline/Tween solution and incubated with goat anti-rabbit antibodies conjugated to horseradish peroxidase (1:30 000 dilution; Oxford Scientific, Oxford, MI) for 30 min at room temperature. Following four washes with PBS/Tween solution, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham) and recorded by fluorography on Hyperfilm, according to the manufacturer's instructions. After analysis, membranes were stained with Ponceau S to verify equal loading and transfer.

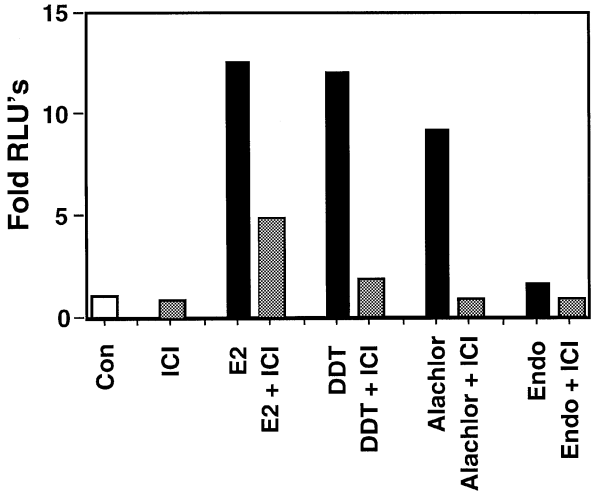

Fig. 1. Reporter gene activity of environmental estrogens. MCF-7 cells were transfected with 2 μg of ERE–luciferase containing reporter gene constructs. Cells were then treated with vehicle (control), 1 nM 17-β-estradiol (E2), 100 nM *o,p′* DDT (DDT), 1 μM alachlor (Ala) or 1 μM endosulfan II (Endo) in the presence or absence of 100 nM ICI 182,780 (+ICI). Cells were harvested 18 h later for luciferase assays. Data is expressed as fold relative light units (RLU) over control from a representative experiment of three independent determinations.

Results

Several reports in the literature have addressed the estrogenicity of endocrine-disrupting chemicals in breast cancer cells. Klotz *et al*. showed that some organochlorine pesticides such as DDT metabolites and alachlor are capable of binding to the ER as measured by tritiated 17β-estradiol displacement (26). This group also showed that these compounds are capable of driving reporter gene transcription from ERE elements. These studies indicate the role of environmental estrogens in signaling through estrogenic pathways and that they, like 17β-estradiol, possess the ability to induce proliferation of MCF-7 cells (24–26). Based on these data we examined the estrogenic activity of estrogen and the environmental estrogens *o,p′* DDT and alachlor on MCF-7 cells. Using an estrogen-responsive reporter gene assay, 17β-estradiol (1 nM) treatment was shown to result in a 12.5-fold increase in luciferase activity (Figure 1). *o,p′* DDT (100 nM) resulted in an equivalent 12-fold increase in luciferase activity, while alachlor (1 μM) induced a 9.1-fold increase in luciferase activity. Addition of the pure anti-estrogen ICI 182,780 with either 17β-estradiol, *o,p′* DDT or alachlor reduced luciferase activity to 4.8-, 1.8-, 0.79- and 0.81-fold, respectively, indicating that the effect of these compounds on ERE-mediated transcriptional activity was dependent on the estrogen receptor. These results demonstrate that the two environmental estrogens *o,p′* DDT and alachlor, at doses 100 nM and 1 μM, respectively, activate ERE-mediated gene expression roughly equivalent to a 1 nM dose of the ovarian estrogen 17β-estradiol.

Based on the observation that estrogen is a survival factor in MCF-7 cells, we examined the ability of 17β-estradiol and the environmental estrogens *o,p′* DDT and alachlor to inhibit TNF-mediated cell death. We previously demonstrated that TNF strongly induced apoptosis in MCF-7 cells (27). Cell viability assay was used to assess the anti-apoptotic effects of 17β-estradiol and the environmental estrogens *o,p′* DDT and alachlor. TNF (10 ng/ml) caused a decrease in viability from 100% in the control to 33 ± 4.46% at 24 h. Pretreatment of

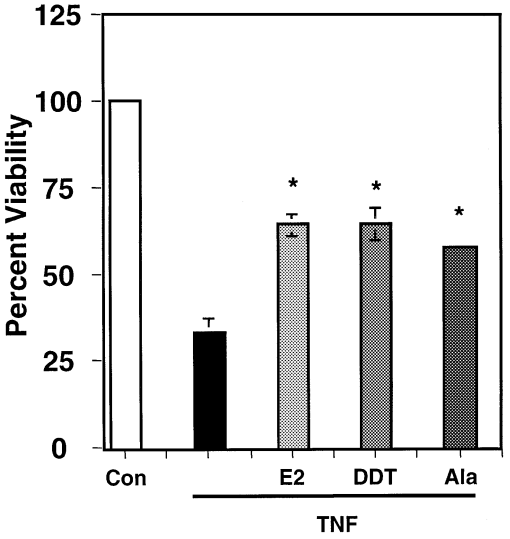

Fig. 2. Effects of estrogens on TNF-induced cell death in MCF-7 N cells. MCF-7 cells were treated with vehicle (Con), 1 nM 17-β-estradiol (E2), 100 nM *o,p′* DDT (DDT) or 1 μM alachlor (Ala) for 24 h prior to the addition of TNF (10 ng/ml). Cell were harvested 24 h later and viability was assessed using the Trypan blue method. Error bars represent standard deviation of the mean for five independent experiments performed in duplicate. Absence of error bars indicates <2.5% SD. Statistical significance (*) as compared with TNF treatment was determined using Student's *t*-test with $P < 0.02$ for Ala + TNF and $P < 0.005$ for 17β-estradiol + TNF and DDT + TNF.

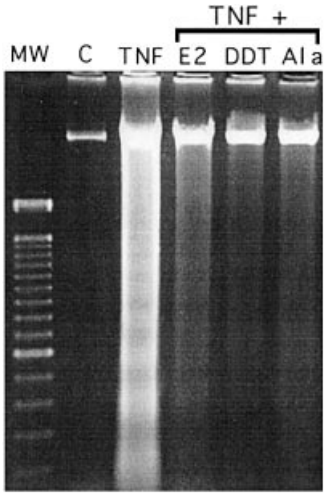

Fig. 3. Effects of estrogens on TNF-induced DNA fragmentation in MCF-7 N cells. MCF-7 cells were treated with vehicle (C), 1 nM 17-β-estradiol (E2), 100 nM *o,p′* DDT (DDT) or 1 μM alachlor (Ala) for 24 h prior to the addition of TNF (10 ng/ml). Cells were harvested 48 h later for DNA fragmentation analysis. Molecular weight marker is shown as MW.

MCF-7 cells for 24 h with 1 nM 17β-estradiol resulted in a 31% inhibition of TNF-induced cell death, restoring viability to 64 ± 3.1% (Figure 2). Similarly 100 nM *o,p′* DDT reduced TNF cell death by 30.5%, restoring viability to 63.5 ± 4.46%. Treatment with 1 mM alachlor was able to inhibit cell death by 24%, restoring viability to 57 ± 1.33%. DNA fragmentation analysis was used to demonstrate that the suppression of TNF-induced loss of viability was due to induction of apoptosis (Figure 3). Consistent with previous results, TNF induced strong DNA fragmentation at 48 h (27). A 24 h pre-treatment with 17β-estradiol (1 nM), *o,p′* DDT (100 nm) or alachlor

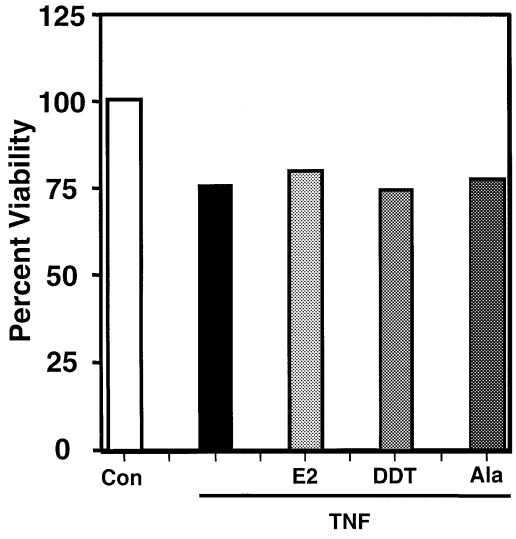

Fig. 4. Effects of estrogens on TNF-induced cell death in MDA-MB-231 cells. Cells were treated with vehicle (Con), 1 nM 17-β-estradiol (E2), 100 nM *o,p′* DDT (DDT) or 1 μM alachlor (Ala) for 24 h prior to the addition of TNF (10 ng/ml). Cells were harvested 24 h later and viability was assessed using the Trypan blue method. Data are expressed as percent viability from a representative experiment of two independent determinations.

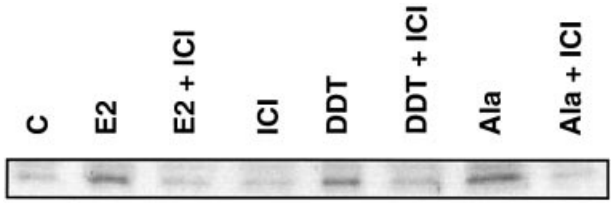

Fig. 5. Estrogen-induced Bcl-2 expression. MCF-7 cells were grown in 5% CS-DMEM medium for 5 days and subsequently treated with vehicle (C), 1 nM 17-β-estradiol (E2), 100 nM *o,p′* DDT (DDT) or 1 μM alachlor (Ala) in the presence or absence of 100 nM ICI 182,780 (+ICI) as shown above. Cells were harvested 24 h later for western blot analysis of Bcl-2 expression.

(1 μM) all suppressed TNF-induced DNA fragmentation to near control levels.

To determine if the anti-apoptotic effects of these chemicals are exclusive to ER-positive breast cancer cells, we examined their effects on TNF-induced apoptosis in the ER-negative MDA-MB-231 breast cancer cell line. TNF (10 ng/ml) treatment induced a 25% decrease in MDA-MB-231 cell viability as compared with control cells at 24 h (Figure 4). Pretreatment of these cells with 17β-estradiol (1 nm), *o,p′* DDT (100 nM) or alachlor (1 μM) for 24 h prior to the addition of TNF (10 ng/ml) resulted in 21.2, 26.7 and 23.3% losses of viability, respectively, demonstrating that these compounds do not possess survival effects in ER-negative breast cancer cells.

Given that Bcl-2 is an estrogen-responsive gene, we examined the abilities of 17β-estradiol and the environmental estrogens *o,p′* DDT and alachlor to increase expression of this gene. MCF-7 cells were treated for 48 h with 17β-estradiol (1 nM), *o,p′* DDT (100 nM) or alachlor (1 μM) with or without pre-exposure to ICI 182,780 (100 nM). Western blot analysis revealed an increase in expression of Bcl-2 with all three compounds which was inhibited by ICI 182,780 treatment, indicative of a specific ER-mediated pathway (Figure 5).

Discussion

Environmental estrogens represent a class of compounds which possess the ability to mimic the activity and effects of endogenous 17β-estradiol. In addition to their role as endocrine disrupters in wildlife, these compounds may also affect humans, resulting in developmental defects, disease and, potentially, cancer. 17β-Estradiol induces proliferation of the ER-positive MCF-7 breast cancer cell line and also acts as a survival factor in these cells in response to treatment with the anti-estrogen tamoxifen, as well as with chemotherapeutic drugs (14–16). The ability of environmental estrogens to mimic 17β-estradiol and cause proliferative and estrogenic effects has been previously analyzed in MCF-7 breast cancer cells (24–26). Given the previously described estrogenicity of these compounds and the role of 17β-estradiol in cell survival, we examined the effects of two organochlorine pesticides on suppression of apoptosis in human breast cancer cells.

We have previously demonstrated that TNF acts as a potent inducer of apoptosis in sensitive MCF-7 cells (27). Consistent with the previous demonstration of the survival effect of estrogen, we show that 1 nM 17β-estradiol is capable of partially suppressing TNF-induced apoptosis in MCF-7 cells. The abilities of both *o,p′* DDT and alachlor to protect against TNF-induced cell death in MCF-7 cells closely correlate with the relative estrogenic potential of these compounds. The most estrogenic compounds in the reporter gene assay, 17β-estradiol (at 1 nM) and *o,p′* DDT (at 100 nM), exerted the greatest survival effects against TNF-induced cell death. While alachlor, a less potent ER agonist even at 1 μM, exerted the least effect on suppression of TNF cytotoxicity. However, upon analysis of DNA fragmentation induced by TNF we observed a significant reduction in apoptosis by pretreatment with all three agents, suggesting that subtle differences in viability may not directly correlate with qualitative analysis of apoptosis.

Recently, Shen *et al*. demonstrated that an isomer of DDT, *p,p′* DDT, was capable of activating cellular signaling events in ER-negative MCF-10A cells, suggesting that some organochlorine pesticides or potentially environmental estrogens may function through other signaling pathways (28). To investigate the possibility that either alachlor or *o,p′* DDT affects apoptosis in an ER-independent manner, we used the ER-negative MDA-MB-231 cell line. ER-negative MDA-MB-231 breast cancer cells were not as sensitive to the cytotoxic effects of TNF as MCF-7 cells. As expected, in ER-negative MDA-MB 231 cells 17β-estradiol exerted no protective effect against TNF-induced cell death. Similar to the effects of 17β-estradiol, pretreatment of these cells with either *o,p′* DDT or alachlor did not affect the ability of TNF to induce cell death, suggesting that at the concentrations used and under the conditions tested here the effects observed on apoptosis are occurring through an ER-dependent pathway. However, we cannot rule out the possibility that these compounds may possess anti-apoptotic effects not observed here which occur through an ER-independent mechanism.

Recent reports show that one mechanism by which estrogens may affect apoptosis is through increased expression of Bcl-2, a member of a family of apoptosis regulating proteins (14–16). Additionally, Jaattela *et al*. have demonstrated that overexpression of Bcl-2 in MCF-7 cells resulted in resistance to TNF-induced apoptosis (17). The data presented here show that, like estrogen, the two organochlorine compounds *o,p′* DDT and alachlor both increase Bcl-2 expression in an ER-dependent manner, suggesting that the mechanism of organochlorine suppression of apoptosis is in part mediated through increased expression of Bcl-2.

We have provided evidence that the organochlorine compound *o,p′* DDT, which can function as an environmental estrogen, is capable of suppressing TNF-induced apoptosis in human breast cancer cell lines and this effect is correlated with increased expression of Bcl-2. We have also demonstrated that the anti-apoptotic effect of this compound is observed in ER-positive MCF-7 cells but not in ER-negative MDA-MB-231 cells, suggesting specificity of this effect for the ER-mediated pathway. Environmental estrogenic compounds which mimic estrogen have been shown to induce proliferative responses and expression of estrogen-responsive genes and promoters. Here we demonstrate that certain environmental estrogens are also capable of mimicking estrogen in their ability to suppress apoptosis.

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