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DDT Stimulates c-erbB2, c-met, and STATS Tyrosine Phosphorylation, Grb2-Sos Association, MAPK Phosphorylation, and Proliferation of Human Breast Epithelial Cells

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Several environmental organochlorines, some of which exhibit estrogenic activity, have been detected in human breast tissue and have been suggested as having a role in tumorigenesis. In this communication, we report the effects of DDT on c-erbB2 and c-met growth factor receptor tyrosine kinase and STATS signal transduction processes in human breast epithelial MCF-10A cells. p,p′-DDT at physiologically relevant concentrations (i.e. 10 nM) elevated c-erbB2, c-met and STAT1α (p84/91) tyrosine phosphorylation, stimulated Grb2-Sos1 association and elevated MAPK phosphorylation. In contrast, o,p′-DDT under identical conditions failed to stimulate either c-erbB2 or c-met tyrosine phosphorylation, demonstrating a structural specificity for this effect. p,p′-DDT also stimulated breast epithelial cell proliferation, as evidenced by ^{3}H thymidine incorporation and analysis of cell doubling times. These results provide evidence of additional pathways by which environmental chemicals may stimulate cell proliferation and/or tumorigenesis and thereby function as xenomitogens. © 1997 Academic Press

Although several epidemiological studies have correlated the presence of DDT and PCBs in breast adipose tissue with breast cancer, substantial controversy exists on this issue (1,2) and the role of environmental organochlorines in breast cancer is the subject of continuing debate and research. Epidemiological studies have reported numerous lipophilic environmental chemical contaminants to be present in normal and cancerous breast tissue, including the pesticides DDT and hexachlorobenzene, as well as the PCBs (3,4). DDT, which consists of a mixture of p,p-DDT (85%) and o,p′-DDT (15%), and its metabolites are lipophilic and sequestered in adipose tissue with long half-lives (2-4). o,p′-DDT and its metabolites DDE and DDD have been identified as "xenoestrogens", or endocrine disrupters (5-7), based on their ability to bind the estrogen receptor and activate estrogen-responsive genes, whereas p,p′-DDT and its metabolites have been reported to bind the androgen receptor and inhibit androgen binding (6). More recently, multiple weakly estrogenic chemicals have been reported to interact with, and synergistically activate, the estrogen receptor (8).

Breast cancer is a proliferative disease of breast epithelial cells and estrogen has been shown to stimulate proliferation of breast epithelial cells in culture and in nude beige mice (9,10). Hence, environmental estrogenic chemicals, such as the o,p′-isomer of DDT and DDE, have been postulated to stimulate cell proliferation through activation of the estrogen receptor. However, other signaling pathways which control cell proliferation also exist and cross-talk between receptor systems occurs.

Whereas substantial research has been directed towards characterization of the estrogenicity of environmental organochlorine contaminants, little attention has been directed towards the potential mitogenic effects of these chemicals, whereby activation of growth factor receptors and cytokine receptor-mediated signal transduction processes results in altered cell proliferation and differentiation. In addition, a majority of the previous studies on estrogenicity focused on yeast model systems and tumor cell lines (8-10), whereas little attention has been directed towards human breast epithelial cells. In this communication, we provide evidence that p,p′-DDT stimulates c-erbB2 and c-met growth factor receptor and STATS signal transduction processes and human breast epithelial cell prolifera-

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Abbreviations: p,p′-DDT, 1,1,1-dichloro-2,2-bis(p-chlorophenyl)-ethane; PCB, polychlorinated biphenyls (aroclor); RTK, receptor tyrosine kinase; JAK, Janus protein tyrosine kinases; STAT, signal transducer and activator of transcription; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; Grb2, growth factor receptor binding factor; Sos, son of sevenless.

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tion. The results of our research suggest that multiple signaling mechanisms exist by which chemicals may stimulate cell proliferation and, potentially, tumorigenesis.

MATERIALS AND METHODS

Cell lines. MCF-10A cells were used in the current studies. The MCF-10A line is a spontaneously immortalized, untransformed human breast epithelial cell line obtained from a patient with mild fibrocystic disease (11). These cells do not express the estrogen receptor.

Cell culture and chemical treatment. MCF-10A cells were cultured in DMEM/F12 media containing 10 mg/ml of human insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, 0.5 mg/ml hydrocortisone, 5% horse serum and 100 units/100 μg penicillin/streptomycin (GIBCO), to 100% confluence. Cells were serum- and EGF-starved for 18 to 24 h, then treated for 24 h, with 10 nM of p,p′-DDT, or o,p′-DDT in 0.1% 1,4-dioxane, which was added to the serum- and EGF-deprived media, unless otherwise indicated. Untreated cells and solvent (0.1% dioxane)-treated cells served as controls. In selective experiments, cells were treated for 10 min either with 20 ng/ml EGF, or 20 ng/ml HGF.

Immunoprecipitation. Equal amounts of whole cell lysate (200 mg to 1 mg) were incubated with 1 mg/ml of PY20 anti-phosphotyrosine antibody (Transduction Laboratories) or anti-Grb2 antibody (Santa Cruz Biotechnology), in lysis buffer (50 mM HEPES, pH 7.2, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton-X-100, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM $MnCl_2$, 100 mM ATP, 10 mg/ml leupeptin, 2 mM phenylmethyl sulfonyl fluoride, 200 units of aprotinin) at 4°C for 2 h. Protein A-sepharose was added and the samples were allowed to incubate for 1 h. The samples were centrifuged at 10,000 xg and washed with lysis buffer 3 times and centrifuged. The pellet was resuspended in Western blot loading buffer (125 mM Tris-HCl, 2% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.0025% bromophenol blue) and heated at 95°C for 5 min and centrifuged. The supernatant was loaded onto a 4 to 20% or a 5 to 10% gradient SDS-PAGE gel for Western blot analysis.

Western blot analysis. Whole cell lysate (20 to 100 mg) or immunoprecipitate was separated using 5 to 10% or 4 to 20% gradient SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane and gel were stained to check for even loading and transfer of protein. The membrane was blocked in PBST (1× PBS, 0.05% Tween 20), 5% nonfat dry milk at room temperature for 1 h, washed twice with PBST, and hybridized with 0.5 to 1 mg/ml of primary antibody in PBST containing 0.5% nonfat dry milk at room temperature for 1 to 16 h. The blots were washed twice with PBST and incubated with a 1:3000 to 1: 5000 dilution of secondary antibody in PBST, 5% non-fat dry milk for 1 h, washed three times with PBST, and exposed and developed using the enhanced chemiluminescence (ECL) technique (Amersham).

Methyl-^{3}H-thymidine incorporation assay. 6×10^5 MCF-10A cells were plated on a 60 mm plate and allowed to attach to the plate in complete media for 24 h. The cells were then grown in serum- and EGF-deprived media for 24 h, followed by treatment with chemicals (22 hrs) or with 20 ng/ml of EGF or HGF for 10 minutes in serum- and EGF-deprived media. Following 22 h treatment with p,p′-DDT, 5 mCi of methyl-^{3}H-thymidine was added to each plate, and the cells were allowed to incubate for an additional 2 h. Cells were washed twice with PBS, and harvested by scraping into PBS. The cell pellet was incubated with 5% cold TCA for 2 h at 4°C, and centrifuged at 3000 rpm, followed by sequential washes with 5% TCA until the counts remaining in the wash fluid were comparable to those of the reagent control. The cell pellet was then washed once with 100% cold ethanol, centrifuged and allowed to air dry. The cell pellet was subsequently dissolved in 50 ml of the lysis buffer used for Western blot analysis and a sample was subjected to liquid scintillation counting, while an equal aliquot was analyzed for protein content by the BCA protein assay. Cell proliferation was calculated as ^{3}H-thymidine cpm/mg protein.

TABLE 1

Cell Doubling Time Analysis

Additions	Average doubling time (hr)†
None	32.4 ± 1.36
EGF	27.1 ± 0.85*
Solvent + EGF	27.3 ± 0.85*
p,p′-DDT (10 nM) + EGF	23.7 ± 0.04**

Equal numbers of MCF-10A cells were plated in T_{25} flasks in media containing the respective agents and allowed to grow for 120 h as described in Materials and Methods. Cells were trypsinized and counted. Cell doubling time was calculated as described in Materials and Methods. p,p′-DDT decreased the average cell doubling time, relative to the other treatments.

† Represents mean ± S.E.M. of triplicate experiment.

* Significantly different from untreated, $p < 0.05$.

** Significantly different from solvent treated + EGF, $p < 0.01$.

Cell doubling time analysis. Equal number of MCF-10A cells were plated in T_{25} tissue flask in complete media deprived of EGF, or in complete media, or in complete media supplemented with 0.1% dioxane, or 10 nM p,p′-DDT in 0.1% dioxane. After 120 h of culture, cells were trypsinized and cells were counted using a hemocytometer. The average cell doubling time was calculated as: $t_D = 120 \text{ h} / [(\log x_e / x_s) / \log 2]$; where x_s = starting number of cells and x_e = ending number of cells (Table 1).

RESULTS AND DISCUSSION

The tyrosine kinase receptors of the growth factors, such as epidermal growth factor receptors (EGFR), c-erbB2, hepatocyte growth factor receptor (HGFR/c-met), and insulin-like growth factor (IGFR), have been found to be amplified or mutated in a significant proportion of primary and malignant breast lesions (12-14) and overexpression of the growth factor receptor c-erbB2 and c-met predicts for poor clinical prognosis (12,13). Thus, the effects of p,p′-DDT on c-erbB2 and c-met protein levels and tyrosine phosphorylation were examined. p,p′-DDT failed to affect either c-erbB2 or c-met growth factor receptor protein levels (Fig. 1A and 1B). However, c-erbB2 tyrosine phosphorylation was increased ~5-fold in response to 10 nM p,p′-DDT treatment, compared to the solvent control (Fig. 2A). p,p′-DDT at 10 nM also produced an ~2-fold increase in c-met tyrosine phosphorylation (Fig. 2B). In contrast, o,p′-DDT failed to effect tyrosine phosphorylation of either c-erbB2 (Fig. 2B) or c-met (Fig. 2D) growth factor receptors, suggesting a structural specificity for this process. These results showed that p,p′-DDT selectively stimulated c-erbB2 and c-met RTK activity.

A critical downstream effector of RTK signal trans-

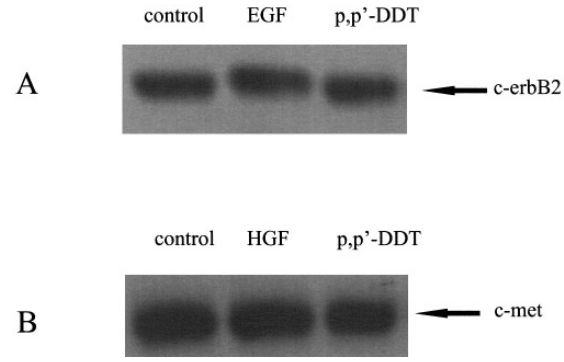

FIG. 1. p,p′-DDT effects on c-erbB2 and c-met protein levels. Cells were treated with the chemicals and cell lysate obtained and separated by SDS–PAGE, as described in Materials and Methods. c-ErbB2 and c-met protein levels were examined by Western blot analysis using the respective antibodies. Panel A: c-erbB2 protein levels. Lane 1: solvent-treated cells; Lane 2: EGF-treated cells; Lane 3: 10 nM p,p′-DDT-treated cells. Panel B: c-met protein levels. Lane 1: solvent-treated cells; Lane 2: EGF-treated cells; Lane 3: 10 nM p,p′-DDT-treated cells. p,p′-DDT failed to effect either c-erbB2 or c-met protein levels.

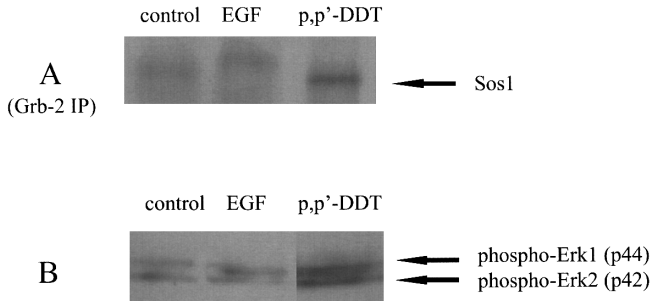

FIG. 3. p,p′-DDT effects on Grb2-Sos1 association and MAPKs phosphorylation. Panel A: Grb2-Sos1 association. Cell lysate was immunoprecipitated with anti-Grb2 antibody and the immunoblot probed with anti-Sos1 antibody as described in Materials and Methods. Lane 1: solvent-treated cells; Lane 2: EGF-treated cells; Lane 3: 10 nM p,p′-DDT-treated cells. Panel B. MAPK (ERK1, ERK2) phosphorylation. Cell lysate was separated by SDS–PAGE, and the immunoblot probed with anti-phosphoMAPK antibody as described in Materials and Methods. Lane 1: solvent-treated cells; Lane 2: EGF-treated cells; Lane 3: 10 nM p,p′-DDT-treated cells. p,p′-DDT increased Grb2-Sos1 association and ERK1, ERK2 phosphorylation.

duction is Ras, and Ras signaling requires the activity of the son of sevenless (Sos), which is a guanine nucleotide exchanger that, upon association with Grb2 and Shc, is recruited to the activated site of the RTK, becomes activated, and exchanges GDP on the Ras protein for GTP, thereby activating Ras (15,16). Consequently, measurement of p,p′-DDT effects on Sos association with Grb2 provides an index of DDT effects on ras protein activation and on immediate downstream events resulting from RTK activation. Sos1 was co-immunoprecipitated with anti-Grb2 antibody, and the immunoprecipitate was examined for Grb2-Sos1 association by probing the immunoblot with anti-Sos1 antibody. EGF failed to affect the level of Grb2 association with Sos1 in MCF-10A cells. However, EGF did increase the association of Grb2 with phosphorylated Sos1, likely reflecting the interaction of Grb2-Sos1 complex with the activated RTKs (Fig. 3A, lane 2). p,p′-DDT at 10 nM, however, produced an ~2.5-fold increase in Grb2-Sos1 association (Fig. 3A, lane 3). The results suggest that cells treated with p,p′-DDT may have an increased Ras signaling as a result of increased activation of Sos1 through association with Grb2.

In growth factor receptor-mediated signal transduction, Ras activation results in Raf-1 protein and MEK/MAPKK phosphorylation and activation, which in turn results in the phosphorylation of ERKs/MAPKs that causes their activation. Subsequently, activated MAPKs/ERKs translocate to the nucleus where they phosphorylate and activate transcription factors which regulate gene expression and cell proliferation (16,17).

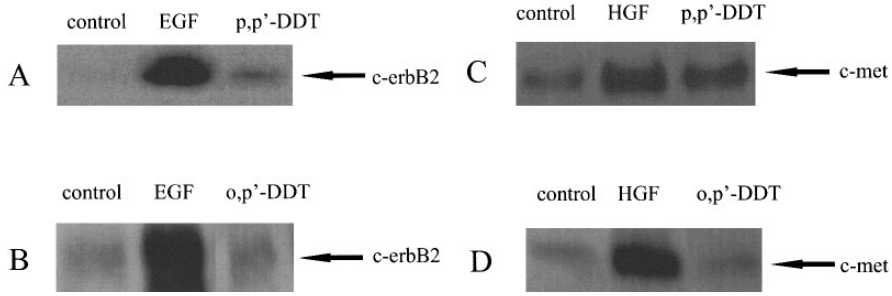

FIG. 2. p,p′-DDT and o,p′-DDT effects on c-erbB2 and c-met tyrosine phosphorylation. Cells were treated as described, cell lysate was immunoprecipitated with PY20, the immunoprecipitate collected, separated by 4–20% SDS–PAGE and transferred to nitrocellulose membrane for immunoblot analysis as described in Materials and Methods. Western blot analysis was performed using anti-c-erbB2 or anti-c-met antibodies. Panel A: c-erbB2 tyrosine phosphorylation. Lane 1: solvent-treated cells; Lane 2: EGF-treated cells; Lane 3: 10 nM p,p′-DDT-treated cells. Panel B: Lane 1: solvent-treated cells; Lane 2: EGF-treated cells; Lane 3: 10 nM o,p′-DDT-treated cells. Panel C: c-met tyrosine phosphorylation. Lane 1: solvent-treated cells; Lane 2: HGF-treated cells; Lane 3: 10 nM p,p′-DDT-treated cells. Panel D: Lane 1: solvent-treated cells; Lane 2: HGF-treated cells; Lane 3: 10 nM o,p′-DDT-treated cells. p,p′-DDT increased c-erbB2 and c-met tyrosine phosphorylation, while o,p′-DDT failed to cause a significant effect.

MAPK/ERK phosphorylation was examined by immunoblot analysis using anti-phosphoMAPK antibody that specifically recognizes the phosphorylated form of MAPK/ERK. p,p′-DDT at 10 nM increased the phosphorylation of MAPKs ~ 6-fold, relative to solvent-treated cells (Figure 3B). These results suggest that increased MAPK phosphorylation, and consequently, increased MAPK activation occurs in response to p,p′-DDT treatment.

Activation of the receptor protein tyrosine kinases and downstream Ras-Raf-MAPK serine threonine kinase cascade is a common and essential signaling pathway by which activation of the nuclear transcriptional factors that regulate cell proliferation and differentiation occurs. Another pathway, frequently employed by cytokines to regulate cell proliferation, is the JAK-STATS signaling pathway (18). Tyrosine phosphorylation of cytoplasmic transcription factors STATS (signal transducers and activators of transcription) by Janus protein-tyrosine kinases (JAKs) results in activation of the STATS which translocate to the nucleus and activate gene expression (18).

To examine whether DDT activated this pathway to effect cell proliferation, the effects of p,p′-DDT on STAT1α (p84/91) was examined by immunoprecipitating cell lysate with anti-phosphotyrosine antibody and probing the blot with anti-STAT1α antibody. p,p′-DDT at 10 nM increased STAT1α (p84/91)tyrosine phosphorylation ~ 3-fold (Fig. 4). This result suggests that p,p′-DDT may activate transcription factors not only through the RTK-Ras-MAPK pathway, but also through the JAKs-STATS pathway.

One of the ultimate results of RTK- and JAKs-STATS-associated signal transduction is cell proliferation. p,p′-DDT effects on MCF-10A cell proliferation were measured by methyl-^{3}H-thymidine incorporation and by analysis of cell doubling time. Short term stimulation with EGF for 2 h did not cause a significant increase in the rate in ^{3}H-thymidine incorporation. However, 10 nM p,p′-DDT at 24 h increased cell proliferation by ~1.5-fold (Fig. 5). To study further the effects of p,p′-DDT exposure on cell proliferation, cell doubling time analysis was performed. Cells were cultured in EGF-deprived MCF-10A media, or in complete

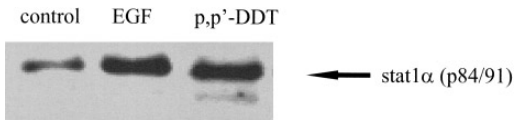

FIG. 4. p,p′-DDT effects on STAT1α p(84/91) tyrosine phosphorylation. Cell lysate was immunoprecipitated with PY20, the immunoprecipitate collected, separated by SDS–PAGE and immunoblot analysis performed using an anti-STAT1α p(84/91) antibody, as described in Materials and Methods. Lane 1: solvent-treated cells; Lane 2: EGF-treated cells; Lane 3: 10 nM p,p′-DDT-treated cells. p,p′-DDT increased STAT1α p(84/91) tyrosine phosphorylation.

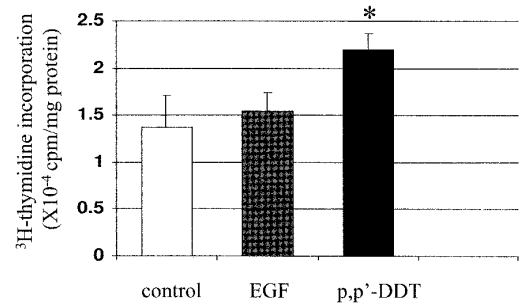

FIG. 5. Cell proliferation. Cell proliferation measured by methyl-^{3}H-thymidine incorporation. Cells were treated with p,p′-DDT and ^{3}H-thymidine incorporation was assayed as described in Materials and Methods. Cell proliferation was calculated as ^{3}H-thymidine cpm/mg protein. Error bars indicate the standard error of the mean (SEM) from triplicate experiments. Bar 1: solvent-treated cells; 2: EGF-treated cells; 3: 10 nM p,p′-DDT-treated cells. *Significantly different from the solvent-treated cells $p<0.05$.

MCF-10A media containing solvent (0.1% dioxane) or 10 nM p,p′-DDT for 120 h, and the average cell doubling time calculated. Cells grown in the absence of EGF showed an average doubling time of ~32.4 h, while cells grown in complete media containing solvent showed an average doubling time of ~27.3 h. Cells grown in complete media containing 10 nM p,p′-DDT, however, showed a further decrease in cell doubling time, with an average doubling time of ~23.7 h. Therefore, treatment of cells with p,p′-DDT appeared to increase further the rate of cell replication. These results confirm the mitogenic effects of p,p′-DDT.

MCF-10A cells are negative for estrogen receptor expression. p,p′-DDT is only weakly estrogenic, o,p′-DDT however, is a strongly estrogenic chemical (6). The average level of DDT in human breast adipose tissue is ~5 μM, and 0.15 μM in serum (2-4). In our studies, p,p′-DDT, at concentrations at, or below, the average level in human serum and adipose tissue, produced substantial effects on RTK tyrosine phosphorylation, while o,p′-DDT failed to effect this signaling process. p,p′-DDT also stimulated signaling events downstream of RTKs, such as Grb2-Sos association, and MAPKs phosphorylation. In addition, p,p′-DDT stimulated STAT1 signaling. These results suggest that the mitogenic effects of p,p′-DDT in MCF-10A cells occur independent of the estrogen receptor, but rather through the activation of RTKs and JAKs-STATS signaling pathways. The studies provide evidence that signaling pathways, other than those associated with the estrogen receptor, constitute viable pathways by which environmental chemicals, such as p,p′-DDT, may effect cell proliferation. Environmental chemicals may also employ the signaling pathways of growth factors and cytokines, and through activation of the protein tyrosine kinases-related signal transduction processes effect cell proliferation. Since RTK and JAKs-STATS path-

ways are the most essential and universal pathways regulating cell proliferation and differentiation in biological systems, the interaction of environmental chemicals with these signaling pathways will affect cell growth and differentiation, and ultimately, disease pathogenesis, especially when lower levels of such chemicals persist over several decades. The differences between the p,p′-DDT and o,p′-DDT isomers in activation of these signaling pathways also serve to illustrate further the unique characteristics and effects associated with environmental chemicals.

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