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Xenobiotic-induced TNF-α expression and apoptosis through the p38 MAPK signaling pathway

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Abstract

Some xenobiotics, such as dichlorodiphenyltrichloroethane (DDT), bind to and activate estrogen receptors (ERs), eliciting estrogenic effects in both wildlife and humans. However, our laboratory and others have demonstrated that DDT and DDT-like compounds target non-ER pathways. In search for a molecular mechanism we recently established that DDT and its metabolites stimulate activator protein-1 (AP-1)-mediated gene expression through the p38 mitogen-activated protein kinase (MAPK) cascade. Here, we determined that DDT-induced p38 activity produces a novel environmental signaling pathway in endometrial Ishikawa and human embryonic kidney (HEK) 293 cells. Xenobiotic exposure stimulates expression of the death ligand, tumor necrosis factor-α (TNF-α) as demonstrated using RT-PCR and reporter gene assays. Furthermore, DDT-induced p38 activity led to the release of cytochrome *c* from the mitochondria and activation of caspase-3/7. Ultimately, DDT-treated cells underwent cell death. Taken together, these data demonstrate DDT induces both the expression of the death ligand TNF-α and apoptosis through a p38 MAPK-dependent mechanism.

Keywords: Apoptosis; p38 MAPK; DDT; HEK 293; Ishikawa; TNF-α

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1. Introduction

Organochlorines represent a class of xenobiotics characterized by a chlorinated hydrocarbon backbone motif. These chemicals, found in pesticides, plastics and industrial wastes are ubiquitous environmental pollutants (Ahlborg et al., 1995; Korach et al., 1997). Dichlorodiphenyltrichloroethane (DDT), one of the most widely used pesticides until 1973, was banned due to its adverse effects on wildlife (Ahlborg et al., 1995; Ware, 2001). However, this chemical is still used in many developing countries today and exists at high concentrations along with its metabolites throughout the world, including the U.S., because of their long half-lives in soil, water and adipose tissue of animals (Cocco et al., 2000; Mitra and Raghu, 1998; U.S. Department of Health and Human Services, 1992; Tyler et al., 1998; Ware, 2001; Wolff et al., 1993).

DDT and some of its metabolites bind the estrogen receptors (ERs) (Klotz et al., 1996; Kuiper et al., 1998) and induce estrogen-like effects in exposed animals and humans (Ahlborg et al., 1995; Gulledge et al., 2001). However, other mechanisms appear to exist to explain the action of DDT but are less clearly defined. For example, previous experiments performed by our laboratory and others using ER knockout mice and ER null cell lines indicate that the effects of DDT-like compounds are not solely through ER-dependent mechanisms (Frigo et al., 2002a; Ghosh et al., 1999). Our laboratory has demonstrated that DDT and its metabolites, dichlorodiphenyldichloroethane (DDD) and dichlorodiphenyldichloroethylene (DDE), can stimulate activator protein-1 (AP-1)-mediated gene expression through activation of the p38 mitogen-activated protein kinase (MAPK) signaling cascade (Frigo et al., 2004). Because p38 is a stress MAPK and stress MAPKs are often implicated in cell death, we explored the action of DDT treatment on pathways leading to cell death as a novel mechanism for this class of xenobiotics.

One mechanism of cell death is the interaction of plasma membrane receptors with their cognate ligands (Schulze-Osthoff et al., 1998). This binding event allows the recruitment of downstream signaling partners, which ultimately results in activation of caspases and subsequent cell death. The most common death ligands known are those of the TNF superfamily, which include tumor necrosis factor-α (TNF-α), FasL and TRAIL. Though TNF-α prompts activities other than cell death, it can function through its receptor, TNF-receptor1 (TNF-R1), to trigger apoptosis (Leong and Karsan, 2000). FasL binds to its receptor, Fas, which was the first member of the TNF receptor superfamily described in terms of its function in apoptosis. TRAIL triggers apoptosis through interaction with its death receptors, DR4 and DR5 (Schulze-Osthoff et al., 1998).

Death ligand/death receptor signaling can involve a mitochondrial-mediated mechanism. Apoptosis occuring through a mitochondrial pathway is triggered by pro-apoptotic members of the Bcl-2 family, such as Bid, Bad, Bak, Bik, Bim and Hrk. In response to environmental cues, these proteins engage another set of pro-apoptotic Bcl-2 members, the Bax subfamily. Bax triggers the sudden release of cytochrome *c* and subsequent activation of caspases, leading to cell death (Fesik, 2000). For example, ligand binding of a death receptor recruits and activates initiator caspases such as caspase-2, -8 or -10. Caspase-8 can then cleave and activates Bid, which translocates to the mitochondria and triggers Bax-subfamily proteins to release cytochrome *c*. The release of cytochrome *c* stimulates the activation of downstream effector caspases such as caspase-3, -6, or -7, which in turn degrade cellular components, resulting in eventual death (Green, 2000).

The p38 MAPK is often involved in stress-mediated signaling, which can lead to cell death (Whitmarsh and Davis, 1996). MAPK kinases (MKK) 3 and 6 phosphorylate/activate p38. Activation of p38 leads to a translocation into the nucleus, where it can then stimulate transcription factors such as AP-1 to increase the expression of various genes. Recently, the promoter of TNF-α has been demonstrated to contain a functional AP-1-like site (Brinkman et al., 1999; Liu and Whisler, 1998; Newell et al., 1994; Tsai et al., 1996), indicating expression of death ligands may be regulated by the p38 MAPK.

Here, we demonstrate that DDT induces expression of TNF-α and ultimately cell death in human embryonic kidney (HEK 293) and human endometrial Ishikawa cells through the p38 MAPK signaling pathway using an inhibitor of p38. In addition, we stimulated the p38 pathway using a constitutively active MKK6 mutant to further validate our findings. Finally, we implicate an apoptotic pathway involving the release of cytochrome *c* from the mitochondria and subsequent effector caspase-3/7 activation. Taken together,

these results demonstrate a novel mechanism of DDT exposure in which stimulated p38 induces death ligand expression and ultimately apoptosis.

2. Materials and methods

2.1. Chemicals

2,2-Bis(*ortho*,*para*-dichlorophenyl)-1, 1, 1-trichloroethane (*o*,*p*′DDT) and 2,2-bis(*para*,*para*-chlorophenyl)-1,1,1-trichloroethane (*p*,*p*′DDT) were purchased from AccuStandard (New Haven, CT). All DDT metabolites were dissolved in dimethylsulfoxide (DMSO). Doxorubicin was purchased from Sigma (St. Louis, MO). SB203580 (p38 inhibitor) was purchased from Calbiochem (San Diego, CA) and dissolved in DMSO. Human TNF-α was purchased from R&D Systems, Inc. (Minneapolis, MN) and reconstituted in 1 × PBS containing 0.1% bovine serum albumin. Purified cytochrome *c* was purchased from Sigma (St. Louis, MO). All other chemicals were reagent grade.

2.2. Cell culture

Ishikawa human endometrial adenocarcinoma cells and HEK 293 cells were grown as previously described (Frigo et al., 2002a,b, 2004). HEK 293 cells were used for assays involving transfection because of their high transfection efficiencies. Both cell types used were estrogen-unresponsive (Frigo et al., 2002a,b, 2004). For experiments, cultures of cells were transferred to phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated FBS (DCC-FBS), BME amino acids, MEM non-essential amino acids, sodium pyruvate and penicillin–streptomycin and plated at a density of 5×10^5 cells/well in 24-well plates (approximately 80% confluency) and maintained for 24 h unless otherwise described.

2.3. RNA isolation and RT-PCR analysis

Ishikawa cells were treated for 0, 1, 2 or 4 h with *p*,*p*′DDT (50 μM) and with or without a 30 min SB203580 (6 μM) pretreatment as indicated. Kinase inhibitor concentrations were chosen based on non-toxic levels, published IC_{50} values from manufacturers, and previous experiments demonstrating inhibition of known MAPK signaling pathways (Alam et al., 2000; Frigo et al., 2004). Total RNA from Ishikawa cells was isolated using TRIzol reagent (GIBCO, Carlsbad, CA). First-strand cDNA was synthesized from 4 μg of total RNA with superscript II (Life Technologies, Rockville, MD). Amplification reactions were carried out in a total volume of 25 μL using Platinum® PCR Supermix according to manufacturer's instructions (Life Technologies, Rockville, MD). The sequences of the primers used were as follows: TNF-α sense, 5′-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3′ and TNF-α antisense, 5′-GCAATGATCCCAAA-break GTAGACCTGCCCAGACT-3′; and (hypoxanthine phosphoribosyl transferase) HPRT sense, 5′-GGCGTCGTGATTAGTGATGATG AACC-3′ and HPRT antisense, 5′-CTTGCGACCTTGACCATC-TTTGGA-3′ as an internal control for densitometry. The HPRT gene is a constitutively expressed housekeeping gene (Pernas-Alonso et al., 1999). HPRT RNA levels are very low, 1–10 molecules per cell (Steen et al., 1990), which makes it suitable as an endogenous mRNA control in RT-PCR for highly sensitive quantification of low copy or rare mRNAs (Rey et al., 2000; Specht et al., 2001). Amplification was performed in the linear range using a Gene Amp PCR System 9700 (PE Applied Biosystems, Foster City, CA) consisting of a 5 min denaturing step at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 66 °C (TNF-α) or 61 °C (HPRT), and 1 min at 72 °C, followed by a final extension of 7 min at 72 °C. The amplified PCR products were separated by 2% agarose gel electrophoresis run at 100 V for 30 min. Products were visualized with UV light after ethidium bromide staining and identified as single bands located at the predicted size locations. All samples were normalized to the PCR signal obtained for the housekeeping gene, HPRT.

2.4. Transient transfection and reporter gene assay

HEK 293 cells were used for transfection assays because of their high transfection efficiencies. For death ligand promoter luciferase assay, HEK 293 cells were transfected for 5 h with 50 ng of pTNF-1185-Luciferase in conjunction with 150 ng of empty pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) or pcDNA3-CA-MKK6 (constitutive active)

(Jiahuai Han (Scripps Research Institute, La Jolla, CA)) using FuGENE 6^{TM} lipofection reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. pTNF-1185-Luciferase was generously provided by James Economou (UCLA, Los Angeles, CA). Cells were then treated overnight with DMSO (vehicle), 10, 25 or 50 μM *o,p′*DDT in the presence or absence of 6 μM SB203580. In our results we have shown the data from treatments using 25–50 μM DDT and its metabolites, which gave significant AP-1 activity as we previously demonstrated (Frigo et al., 2002a). Various reports show DDT metabolite levels commonly in excess of 20 ng/ml in blood, equivalent to 63 μM (Martin et al., 2002; Lopez-Carrillo et al., 2001; Longnecker et al., 2002) and beyond 4 mM in soils throughout North America (Aigner et al., 1998; Falconer et al., 1997; U.S.G.S., 2001). Finally, cells were harvested and luciferase activity was measured as previously described (Frigo et al., 2002a,b, 2004). The data shown are an average of at least three independent experiments with two replicates.

2.5. Crystal violet assay

For crystal violet assays, Ishikawa cells were treated with DMSO (negative control), TNF-α or *o,p′*DDT and allowed to grow for 0, 1, 2, or 3 days. Then, the media was aspirated off and each well was washed with 1 ml of 1 × PBS. Two hundred microliters of 0.5% crystal violet solution was then added to each well for 10 min, followed by two washes with 1 ml of ddH_2O. Plates were allowed to dry upside down after which 600 μl of 1% SDS was added to each well to solubilize the stain. Plates were agitated on an orbital shaker until a uniform color was seen after which the absorbance of each well was read at 570 nm on a Bio-Tek FL600 microplate reader. The data shown are an average of at least three independent experiments with three replicates.

2.6. Cytochrome c release assay

Ishikawa cells (4×10^6) treated overnight with vehicle (DMSO) or *p,p′*DDT in the presence or absence of SB203580 were harvested the following day with 0.5% EDTA–PBS, pooled together with media and washes containing floating cells, and pelleted by centrifugation at 500 × *g* for 3 min at 4 °C. Pellets were resuspended with buffer A, 20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 10 mM benzamidine, 1 mM dithiothreitol, 250 mM sucrose and protease and phosphatase inhibitor cocktails (Sigma), and then lysed by passing the suspension through a 25-gauge needle 15 times. Homogenates were centrifuged at 500 × *g* for 5 min at 4 °C. Supernatants were further centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant from the 10,000 × *g* spin was designated as cytosol. Twenty-five micrograms of protein from the cytosolic fraction was electrophoresed on 15% polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane (Biorad, Hercules, CA), which was blocked with PBS–Tween (0.05%)–5% low-fat dry milk solution at room temperature for 1 h. The membrane was subsequently probed with polyclonal antibodies raised against cytochrome *c* (1:500 dilution, Santa Cruz, CA), cytochrome *c* oxidase (1:1000, Molecular Probes, Eugene, OR) or β-actin (Sigma, St. Louis, MO). After incubation at 4 °C overnight, blots were washed three times in PBS–Tween (0.05%) solution and incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (1:10,000 dilution; Oxford, Oxford, MI) for 2 h at room temperature. After four washes with PBS–Tween solution, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham, Arlington Heights, IL) and recorded by fluorography on Hyperfilm (Amersham) according to the manufacturer's instructions. The assay was performed two times and the results of a typical experiment are shown.

2.7. Caspase-3/7 assay

Cultures of Ishikawa cells were transferred to 5% DCC–FBS containing DMEM media and plated at a density of 15,000 cells/well in 96-well plates and maintained for 24 h. Cells were then pretreated with or without 6 μM SB203580 for 30 min followed by treatment with DMSO (vehicle), 10 μM doxorubicin (positive control), 10, 25 or 50 μM *p,p′*DDT. The Apo-ONE^{TM} Homogeneous Caspase-3/7 assay was then performed according to the manufacturer's manual. Briefly, Apo-ONE^{TM} substrate ((Z-DEVD)2-Rhodamine 110; fluoresces when active caspase-3 or -7 cleave it) was added after 5 h of treatment and the cells were incubated overnight in 5% CO_2 at room temperature while shaking. The fluorescence of each well was measured

the following day at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a Bio-Tek FL600 fluorescent microplate reader. The data shown are an average of four independent experiments with three replicates.

2.8. Statistical analysis

Data were analyzed using Student's *t*-test or one-way ANOVA and post hoc Tukey's multiple comparisons with GraphPad Prism, Version 3.02 (GraphPad Software, Inc.) as indicated in the figures. Statistically significant changes were determined at the $p < 0.05$, $p < 0.01$ or $p < 0.001$ level as indicated for each figure.

3. Results

3.1. DDT stimulates TNF-α expression through a p38-mediated mechanism

We sought to determine (i) whether DDT treatment increases expression of TNF-α given the AP-1 site located in its promoter and (ii) if so, whether the increase is mediated through the p38 MAPK. RT-PCR analysis using primers for TNF-α reveal *p,p′*DDT increases the expression of TNF-α over a 4-h time period (Fig. 1A). Furthermore, pretreatment with the pharmacological p38 inhibitor SB203580 inhibited the expression, indicating DDT increases TNF-α expression through p38. Treatment with SB203580 alone slightly increased TNF-α expression, suggesting p38 basally inhibits the TNF-α gene. This is consistent with our previous findings that the p38α and β isoforms when not stimulated slightly inhibit AP-1-mediated gene expression (Frigo et al., 2004). These findings suggest basal p38 activity may be needed to suppress TNF-α expression to maintain some homeostasis as p38 has been reported to be necessary for other aspects of cell homeostasis (Cao et al., 2004; Startchik et al., 2002). Certainly though, additional work is needed before any conclusions can be drawn regarding the role of basal p38 activity.

To further support our gene expression findings we examined the effects of DDT exposure on the promoter of TNF-α using a luciferase reporter gene construct linked to the promoter of TNF-α. Here, *o,p′*DDT was used because it was found previously that *o,p′*DDT had greater effects in HEK 293 cells, whereas *p,p′*DDT had similar but greater effects in Ishikawa cells (Frigo et al., 2002a, 2004). *o,p′*DDT stimulated the TNF-α promoter in a dose-dependent manner. This stimulation could be blocked with the addition of SB203580. When constitutively active MKK6, a downstream target of DDT that selectively stimulates p38 MAPK (Dent et al., 2003; Frigo et al., 2004; Ichijo, 1999), was cotransfected into the cells in the absence of DDT exposure, a significant increase in luciferase activity was observed indicating that a p38 cascade could stimulate TNF-α expression (Fig. 1B). Taken together, these results indicate that DDT increased TNF-α expression through a p38-dependent mechanism.

3.2. DDT induces cell death

Activation of the TNF-α pathway can lead to cell death. Because DDT increased expression of TNF-α, we tested whether DDT could affect cell viability using the DNA dye crystal violet. Increasing amounts of TNF-α and similarly the previously reported estrogenic DDT metabolite, *o,p′*DDT (Korach et al., 1997; Tyler et al., 1998), here induced cell death over a 3-day period in human endometrial Ishikawa cells (Fig. 2).

3.3. DDT-induced cytochrome c release and caspase-3/7 activity through the p38 MAPK pathway

To better understand the mechanisms underlying DDT-induced cell death, we tested the involvement of cell death components previously shown to be activated by TNF-α, since we demonstrated DDT increases TNF-α expression. Cell death involving a mitochondrial pathway leads to the release of cytochrome *c* into the cytoplasm that can then activate downstream caspases such as caspases-3 or -7. Using antibodies that target cytochrome *c*, we demonstrate that DDT increased cytoplasmic cytochrome *c* levels (Fig. 3). Furthermore, addition of SB203580 negated this increase, indicating DDT signals the mitochondria through the p38 MAPK.

Using a caspase-3/7 substrate that fluoresces when cleaved, we also demonstrate DDT activated the effector caspase-3/7. Increasing concentrations of *p,p′*DDT

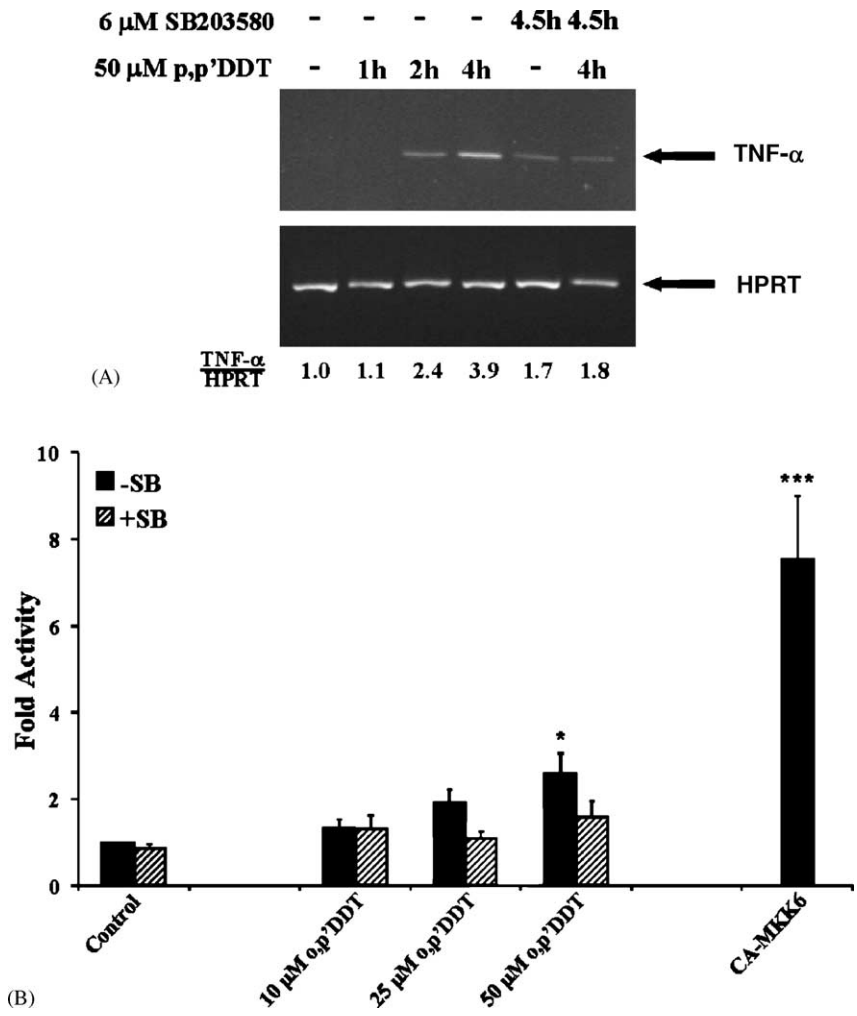

Fig. 1. *p,p′*DDT increases TNF-α mRNA levels through p38 MAPK. (A) Ishikawa cells were treated for 0, 1, 2 or 4 h with *p,p′*DDT (50 μM) with or without a 30 min SB203580 (6 μM) pretreatment as indicated. mRNA was then isolated from the samples followed by RT-PCR using primers for TNF-α or HPRT (loading control). Pictures shown are representative of three independent experiments. (B) HEK 293 cells were cotransfected 5 h with 50 ng of pTNF-1185-Luciferase that contains the promoter region of TNF-α in conjunction with 150 ng of empty expression vector or constitutive active MKK6 expression vector (CA-MKK6). Cells transfected with CA-MKK6 were then treated overnight with vehicle (DMSO) while empty expression vector transfected cells were treated overnight with vehicle, 10, 25 or 50 μM *o,p′*DDT in the presence or absence of 6 μM SB203580. The following day luciferase activity was measured as described in Section 2. Results describe the mean fold activation over vehicle ±S.E. ($n = 4$). $^{*}p < 0.05$, $^{***}p < 0.001$; significant increases from control (ANOVA and Tukey's).

stimulated caspase-3/7 activity in a dose dependent manner (Fig. 4). This stimulation was significantly suppressed, however, when cells were pretreated with SB203580, indicating a role for the p38 MAPK. Collectively, these data demonstrate that DDT stimulates p38-dependent cytochrome *c* release and caspase-3/7 activation, both markers of apoptosis.

4. Discussion

Xenobiotics, such as the organochlorine pesticide DDT, can cause an array of effects on the cell. Much research done on these chemicals focuses on their estrogenic effects because they can act as estrogen mimics by binding to and activating ERs (Tyler et

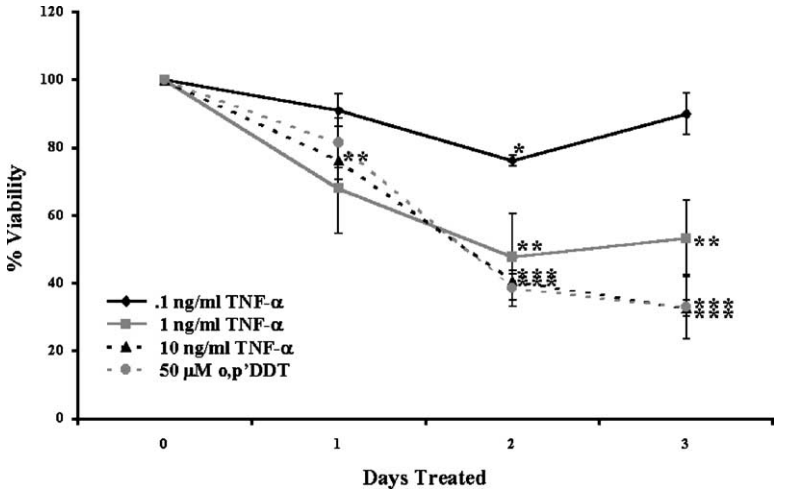

Fig. 2. TNF-α and *o,p′*DDT induce Ishikawa cell death. Ishikawa cells were treated with either TNF-α or *o,p′*DDT for 0–3 days. Cells were then stained with crystal violet and the number of cells/amount of dye present was determined using a microplate reader at an absorbance of 570 nm. Results are expressed as mean percent viability ±S.E. ($n = 3$), where untreated cells are normalized to 100%. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; significant decreases from 100% viability (ANOVA and Tukey's).

al., 1998). Recently, however, our laboratory and others demonstrated that these estrogen-like molecules also function through estrogen-independent pathways (Enan and Matsumura, 1998; Frigo et al., 2002a, 2004; Hatakeyama and Matsumura, 1999; Shen and Novak, 1997a,b; Tessier and Matsumura, 2001). While these reports point to alternative signaling pathways, they fail to address biological endpoints. Here, we demonstrate a novel signaling mechanism of xenobiotics in which DDT induces TNF-α expression and apoptosis through a p38-mediated mechanism.

AP-1-related binding sites are located in the promoter region of TNF-α. These sites have been shown to be critical for death ligand gene expression (Brinkman et al., 1999; Herr et al., 2000; Liu and Whisler, 1998; Newell et al., 1994; Tsai et al., 1996). Since DDT and its metabolites stimulate AP-1 activity through a p38-mediated mechanism (Frigo et al., 2002a, 2004),

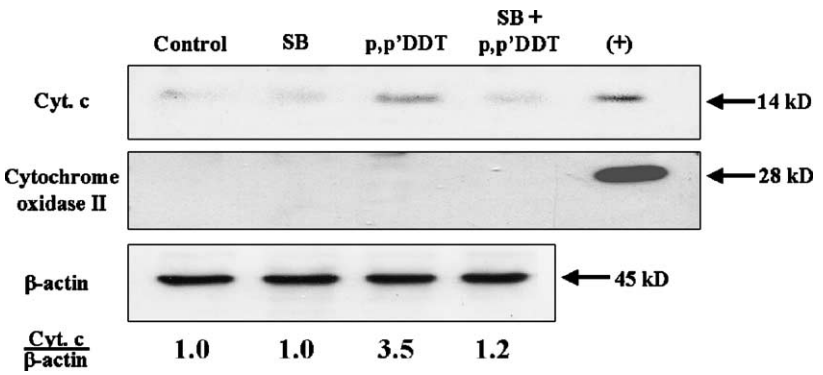

Fig. 3. Treatment with SB203580 prevents release of cytochrome *c* following *p,p′*DDT treatment. Ishikawa cells were treated with DMSO, 6 μM SB203580 or 25 μM *p,p′*DDT for 24 h, or pretreated with 6 μM SB203580 for 1 h followed by 25 μM *p,p′*DDT for 24 h. Cells were fractionated using centrifugation and cytosolic proteins were analyzed by immunoblotting with anti-cytochrome *c*, anti-cytochrome *c* oxidase II or anti-β-actin. Cytochrome *c* oxidase serves as a marker for mitochondrial contamination of cytosolic fractions. Five micrograms of purified cytochrome *c* was used as a positive control for cytochrome *c* staining, 25 μg of a whole cell lysate was used as a positive control for cytochrome oxidase and anti-β-actin was used as a control for protein loading.

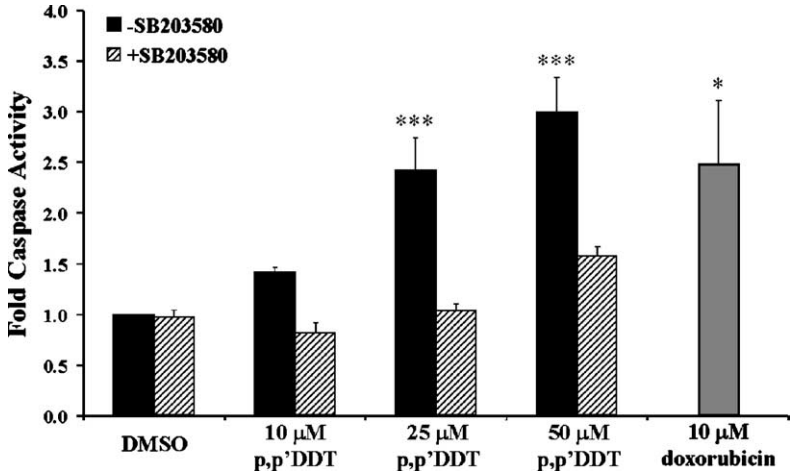

Fig. 4. *p,p′*DDT induces caspase-3/7 activity. Ishikawa cells were pretreated with or without 6 μM SB203580 for 30 min followed by treatment for 5 h with DMSO (vehicle), 10 μM doxorubicin, 10, 25 or 50 μM *p,p′*DDT. Caspase-3/7 substrate was then added to the cells overnight. The fluorescent product was quantitated the following day on a fluorescent microplate reader. Results describe the mean fold caspase-3/7 activation over vehicle ±S.E. ($n = 4$). $^{*}p < 0.05$, $^{***}p < 0.001$; significant increases from control (ANOVA and Tukey's).

we hypothesized that activation of AP-1 by DDT-induced p38 leads to the expression of the death ligand TNF-α, inducing an apoptotic pathway. Through use of molecular and pharmacological techniques, death ligand expression was shown to be p38 MAPK dependent. Using RT-PCR, we demonstrated *p,p′*DDT increased TNF-α mRNA levels over a 4-h period and that the increased expression could be blocked by the p38 inhibitor SB203580. Reporter gene assays were used to further examine the effects of DDT-induced p38 activity on the TNF-α promoter. *o,p′*DDT stimulated the TNF-α promoter in HEK 293 cells through a p38-dependent mechanism. In the experiments done in this study, it is important to note that we varied the DDT metabolite depending upon the cell line used to show the optimal effect. While we have previously shown both DDT conjoiners, *o,p′*DDT and *p,p′*DDT, stimulated p38-mediated activity, *p,p′*DDT did appear to have a more significant effect on Ishikawa cells versus HEK 293 cells, whereas *o,p′*DDT had the greatest effect on HEK 293 cells (Frigo et al., 2002a, 2004). It is unclear at this time why the different cell lines interpret the extracellular signals to different extents but this may be a result of unique cell surface profiles. For example, *o,p′*DDT and *p,p′*DDT have been reported to have different effects on the erbB-2 plasma membrane receptor in breast cell variants (Shen and Novak, 1997b; Tessier and Matsumura, 2001). HEK 293 and Ishikawa cells containing different levels of plasma membrane receptors like erbB-2 could then differentially stimulate the downstream signaling cascade in response to one DDT metabolite over another.

When cells were transfected with a constitutively active MKK6, a p38-selective activating kinase, in the absence of any DDT treatment, there was a significant stimulation of the TNF-α promoter, indicating that p38 induction potentiates the TNF-α promoter. This is in agreement with earlier reports using murine fibroblasts and human monocytes that demonstrated TNF-R1 binding or lipopolysaccharide, respectively, leads to increased TNF-α expression through a p38-dependent pathway (Brinkman et al., 1999; MacKenzie et al., 2002; Mancuso et al., 2002). p38 MAPK signaling has been associated with the induction of cell death in multiple cell types and in response to numerous cellular stresses (Brenner et al., 1997; Ghatan et al., 2000; Schwenger et al., 1997; Xia et al., 1995). However, most attribute p38-induced death to direct activation of the mitochondrial/apoptosome pathway. While this may still be a possibility for DDT-induced signaling, our data demonstrate DDT may stimulate human endometrial and embryonic kidney cell death through death receptor signaling. Our crystal violet studies support the concept that increased levels of TNF-α,

and similarly *o,p′*DDT, kill endometrial Ishikawa cells. This is a novel effect of *o,p′*DDT, given previous studies have focused on *o,p′*DDT's ability to mimic the proliferative hormone estrogen (Korach et al., 1997; Tyler et al., 1998). The increase in cell death at the last time point measured (day 3) still shows a decrease in cell viability, suggesting that much of the cell death we are seeing is not a result of early signaling pathways, rather expression of secondary or even tertiary genes such as TNF-α may be necessary to carry out significant levels of DDT-induced cell death. Additionally, we demonstrated previously that DDT-induced AP-1 activity was in part dependent on a positive feedback loop where selected organochlorines increase the expression of the AP-1 component gene *c-jun* (Frigo et al., 2004). Hence, it may take longer just to get enough AP-1 activity to drive significant death ligand expression.

*p,p′*DDT significantly induced cytochrome *c* release from the mitochondria and caspase-3/7 activity, both markers of apoptosis. However, since crystal violet is only an indicator of cell death, we cannot rule out the possibility that some necrosis may be occurring. In fact, TNF-α itself can lead to necrosis (Leong and Karsan, 2000). Other reports indicate different environmental stresses, such as cobalt chloride, carbon monoxide and UV light, induce cytochrome *c* release and caspase-3/7 activity through a p38-mediated mechanism (Kimura et al., 1998; Zhang et al., 2003; Zou et al., 2002). Consistent with this, when p38 was blocked by SB203580 in Ishikawa cells, no significant cytochrome *c* release or caspase-3/7 activity was observed, indicating that activation of p38 is essential in DDT-induced cell death.

In summary, our results demonstrate the widespread environmental contaminant DDT drives the expression of the death ligand TNF-α through p38 potentiation of AP-1-like factors. DDT-induced p38 MAPK activity caused release of cytochrome *c* from the mitochondria and subsequent activation of the downstream effector caspase-3/7. While a direct link between DDT-stimulated TNF-α expression and cell death has not been made, the similarities in DDT and TNF-α cell death pathways in conjunction with the presence of delayed cell death suggest that increased TNF-α levels

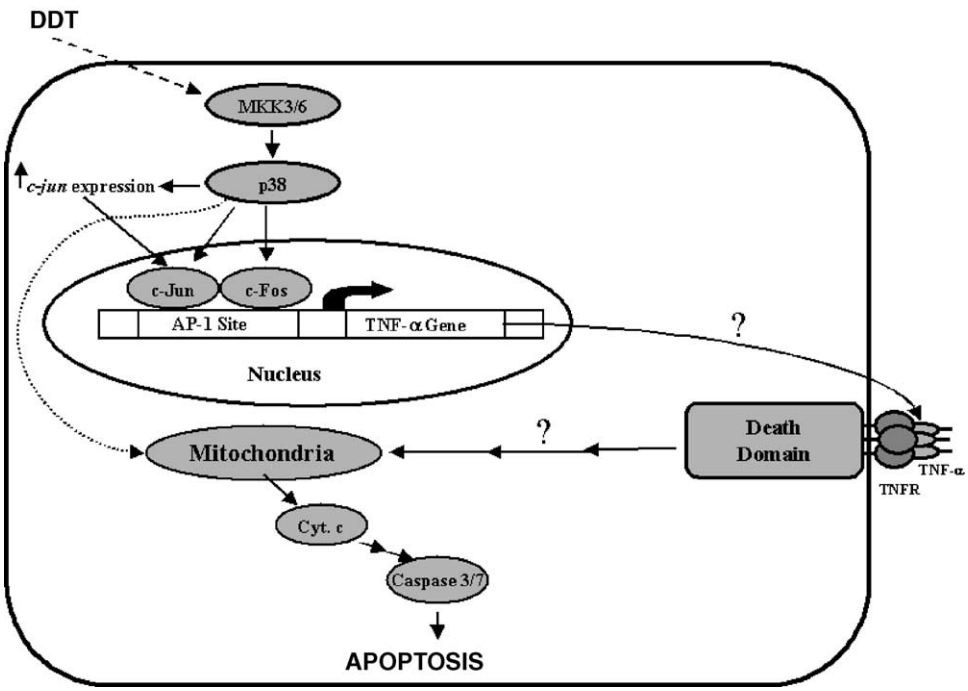

Fig. 5. Potential model for DDT-induced cell death. DDT stimulates AP-1-related transcription factors through a p38-mediated mechanism. The signaled transcription factors can then increase expression of various pro-apoptotic proteins such as TNF-α, which can cause cell death through mitochondrial release of cytochrome *c* and the activation of caspase-3/7.

may in part be responsible for DDT-mediated cell death. The increased TNF-α levels could lead to the ligation of TNF-α to its cognate receptor, prompting signaling to the mitochondria. The release of pro-apoptotic factors such as cytochrome *c* from mitochondria then would activate the effector caspases-3/7. These downstream effector caspases ultimately cleave protein targets that result in the completion of the apoptotic program. A working model is represented in Fig. 5. These data represent an alternative pathway to help understand how xenobiotics affect both wildlife and humans, thus allowing us to interpret and, in turn, build on previous unexplained phenomenon.

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