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Mechanism of AP-1-mediated gene expression by select organochlorines through the p38 MAPK pathway

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Organochlorine compounds have been demonstrated to have detrimental health effects in both wildlife and humans, an effect largely attributed to their ability to mimic the hormone estrogen. Our laboratory has studied cell signaling by environmental chemicals associated with the estrogen receptor (ER) and more recently via ER-independent mechanisms. Here, we show that the organochlorine pesticide dichlorodiphenyltrichloroethane (DDT) and its metabolites induce a stress mitogen-activated protein kinase (MAPK) that leads to AP-1 activation. Through the use of a dominant negative c-Fos mutant, we show that DDT exposure induces the *collagenase* promoter in an AP-1-dependent manner. DDT stimulates an AP-1 complex shift at the DNA to one favoring c-Jun/c-Fos dimers through both increasing c-Jun levels and by post-translational activation of c-Jun and c-Fos in HEK 293 and human endometrial Ishikawa cells. DDT treatment induces phosphorylation of ERK and p38, while JNK phosphorylation levels are slightly decreased. Using pharmacological and molecular inhibitors of the various MAPKs, we implicate the p38 signaling cascade, and to a lesser extent ERK, as necessary pathways for AP-1-mediated gene expression induction by organochlorines. Taken together, these results demonstrate that organochlorines induce the *collagenase* promoter via sequential activation of the p38 kinase cascade and AP-1.

Abbreviations: AP-1, activator protein-1; ATF2, activating transcription factor 2; BMK1, big MAPK 1; DCC-FBS, 5% dextran-coated charcoal-treated fetal bovine serum; p,p′-DDA, 2,2-bis(*p*-chlorophenyl)acetic acid; o,p′-DDD, 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane; p,p′-DDD, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane; o,p′-DDE, 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-phenyl) ethylene; p,p′-DDE, 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene; DDOH, 2,2-bis(*p*-chlorophenyl)ethanol; DDT, dichlorodiphenyltrichloroethane; o,p′-DDT, 2,2-bis(*o*,*p*-dichlorophenyl)-1,1,1-trichloroethane; p,p′-DDT, 2,2-bis(*p*,*p*-chlorophenyl)-1,1,1-trichloroethane; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPKs, mitogen-activated protein kinases; MEF2, myocyte enhancer factor 2; PBS, phosphate-buffered saline; PMA, 12-*O*-tetradecanylphorbol 13-acetate; SRE, serum response element.

Introduction

Activator protein-1 (AP-1) is a generic term used to describe transcription factors that bind specifically to a DNA enhancer sequence [TGA(G/C)TCA] called the 12-*O*-tetradecanylphorbol 13-acetate (PMA)-responsive element, which is also referred to as the AP-1 site. In addition, AP-1 components can bind and potentiate transcription from AP-1-related DNA elements (ex. cAMP response element) (1,2). Members of the Jun and Fos families of proteins dimerize to preferentially bind AP-1 sites with high affinity and, hence, each dimer combination makes up an AP-1 protein complex. Upon stimulation, Jun and Fos proteins recruit p300/CBP co-activators that recruit other co-activators such as the p160s, which can directly bind nuclear hormone receptors like the estrogen receptor (ER) (3). AP-1 is a ubiquitous protein complex that can be induced by multiple stimuli, leading to diverse cellular effects. For example, proliferation, differentiation, cellular stress and death have all been associated with elevated AP-1 activity (2,4–10). Increased AP-1 activity leads to various pathological outcomes, such as carcinogenesis (11–14). Hence, AP-1 appears to play diverse roles in regulation of the cell cycle. Determining what exogenous compounds stimulate AP-1 and how they stimulate AP-1 is central to understanding the role of the environment in pathogenesis. Generally, the final outcome is determined by cell type, promoter context, associative proteins and the type of stimuli.

Environmental stimulation of AP-1 occurs through a combination of signaling events, leading to an increased activity of proteins that directly potentiate Jun and Fos family members or activate transcription factors that regulate expression of *jun* and *fos*. The mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases intimately involved in governing cellular processes such as cell growth, proliferation, differentiation and apoptosis. AP-1 is a major target of the MAPKs. There are three main subfamilies of MAPKs: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. Each MAPK subfamily is phosphorylated/activated by specific MAPK kinases, which are in turn phosphorylated/activated by MAPK kinase kinases that tend to recognize multiple substrates. Currently it is believed that growth and differentiation factors induce an ERK pathway, whereas the JNK and p38 pathways are more involved in stress-mediated signaling (15). However, exceptions to these general concepts exist. For example, increased p38 activity has been implicated in the initiation and progression of carcinogenesis (12,13). To complicate matters, new members of the MAPK family have recently been discovered, such as big MAPK 1 (BMK1), also known as ERK5, that respond to both stress signals and growth factors (16). Ultimately, the given effect a MAPK has on gene expression depends on the cellular and stimulatory context.

Organochlorines represent a class of environmental compounds characterized by a chlorinated hydrocarbon backbone

motif. These chemicals, found in pesticides, plastics and industrial wastes, are ubiquitous environmental pollutants (17,18). Dichlorodiphenyltrichloroethane (DDT), one of the most widely used pesticides until 1973, was banned due to its adverse effects on wildlife (18,19). However, this chemical is still used in many developing countries today and exists at high concentrations along with its metabolites and contaminants throughout the world, including the USA, due to their long half-lives in soil, water and the adipose tissue of animals (19–24).

DDT and some of its metabolites have been shown to bind the ERs (25–27) and induce estrogen-like effects in exposed animals and humans (18,28,29). However, the mechanisms of action of this class of synthetic compounds are still not completely known. Select organochlorine pesticides stimulate early signaling mechanisms, supporting the potential existence of ER-independent pathways (30–36). Additionally, previous experiments performed by our laboratory and others using ER knockout mice and ER null cell lines, in conjunction with potent anti-estrogens, further indicate that the effects of DDT-like compounds are not solely through ER-dependent mechanisms (37,38).

Here, we demonstrate that select DDT metabolites stimulate both artificial and endogenous AP-1-regulated genes. DDT stimulated activation of the endogenous AP-1-regulated promoter of *collagenase*. This stimulation was blocked by transient expression of a dominant negative form of c-Fos. Treatment of Ishikawa cells with p,p′-DDT induces a shift or cycling of the AP-1 complex present at the DNA as demonstrated by supershift analysis to one favoring the incorporation of c-Jun/c-Fos dimers. While c-Jun protein levels increase after organochlorine exposure, other Jun and Fos family member levels are either unchanged or actually decreased. Experiments using GAL4 one-hybrid assays demonstrate that DDT and its metabolites potentiate c-Jun and c-Fos post-translationally, independent of *c-jun* and *c-fos* expression. Through the use of western blots, pharmacological inhibitors and dominant negative mutants our results suggest a necessary role for p38, and to a lesser extent ERK and BMK, but not JNK-mediated pathways, in the induction of AP-1-regulated gene expression. Finally, additional dominant negative studies demonstrate that while p38δ and p38γ isoforms have a greater role than p38α and p38β in PMA-induced AP-1 activity, all p38 isoforms (α, β, δ and γ) were similarly involved in organochlorine-induced AP-1 activity.

Materials and methods

Chemicals

2,2-bis(*o*,*p*-Dichlorophenyl)-1,1,1-trichloroethane (o,p′-DDT), 2,2-bis(*p*,*p*-chlorophenyl)-1,1,1-trichloroethane (p,p′-DDT), 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)ethane (o,p′-DDD), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (p,p′-DDD), 1,1-dichloro-2-(*o*-chlorophenyl)-2-(*p*-phenyl) ethylene (o,p′-DDE), 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (p,p′-DDE) and 2,2-bis(*p*-chlorophenyl)acetic acid (p,p′-DDA) were purchased from AccuStandard (New Haven, CT). 2,2-bis(*p*-Chlorophenyl)ethanol (DDOH) was purchased from Sigma (St Louis, MO). All DDT metabolites were dissolved in dimethylsulfoxide (DMSO). PMA was purchased from Sigma (St Louis, MO) and dissolved in Dulbecco's modified Eagle's medium (DMEM). UO126 (MEK1/2 inhibitor) was purchased from Promega (Madison, WI). SP600125 (JNK inhibitor) was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). SB203580 (p38 inhibitor) was purchased from Calbiochem (San Diego, CA). All pharmacological inhibitors were dissolved in DMSO.

Cell culture, transient transfection, and reporter gene assay

Ishikawa human endometrial adenocarcinoma cells and HEK 293 cells were grown as previously described (37,39). Cultures of cells were transferred to phenol red-free DMEM supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS), BME amino acids, MEM non-essential amino acids, sodium pyruvate and penicillin/streptomycin for 24–48 h prior to plating. Cells were plated at a density of 5×10^5 cells/well in 24-well plates (~80% confluency) and maintained for an additional 24 h in DMEM with 5% DCC-FBS. For AP-1 response assays, cells were then transfected for 5 h with 100 ng of pAP-1(PMA)-luciferase plasmid (Clontech, Palo Alto, CA) using Lipofectamine™ lipofection reagent (Life Technologies, Gaithersburg, MD) or 100 ng of human collagenase promoter upstream of the luciferase reporter gene (hColl-Luc) [kindly provided by Dr Lynn Matrisian, Vanderbilt University (40)] using FuGENE 6™ lipofection reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. For dominant negative experiments, cells were transfected with FuGENE 6™ lipofection reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol for 24 h using 10 ng of pAP-1(PMA)-luciferase plasmid in conjunction with 20, 40, 80 or 160 ng of dominant negative mutant plasmid or 100 ng of hColl-Luc in conjunction with 10, 50, 100 or 150 ng of dominant negative c-Fos (pRc/CMV500-A-Fos, referred to here as DN-c-Fos). Total DNA volume was brought up to 170 ng if necessary using empty pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) or empty pRc/CMV500 expression vector. pRc/CMV500 and pRc/CMV500-A-Fos have been previously described (41,42). MAPK dominant negative mutants were kindly provided by Jiing-Dwan Lee (Scripps Research Institute, La Jolla, CA) (ERK2 and BMK1), Roger Davis (University of Massachusetts Medical School, Worcester, MA) (JNK1 and p38α) and Jiahuai Han (Scripps Research Institute) (p38β, p38δ and p38γ). All dominant negative mutant expression vectors were driven by CMV promoters. For GAL4 one-hybrid assays, 50 ng of pFR-luciferase was transfected with FuGENE 6™ lipofection reagent (Roche) according to the manufacturer's protocol for 5 h in combination with 25 ng of either pFA2-c-Jun or pFA-c-Fos (Stratagene, La Jolla, CA). For all luciferase assays, cells were then incubated for 18–24 h in DMEM with 5% DCC-FBS in the presence of vehicle or various chemicals as previously described (37). Where indicated, kinase inhibitors were added 1 h prior to the addition of PMA or DDT metabolites and maintained during the remainder of the incubation period. 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 (43) and unpublished data. PMA was used as a positive control as we have previously shown that 20 ng/ml PMA gives significant activation of protein kinase C, downstream MAPKs and AP-1 (44). In our results we show the data from treatments using 10–50 μM DDT and its metabolites, which gave significant AP-1 activity, as previously demonstrated (37). Various reports show DDT metabolite levels commonly in excess of 20 ng/ml in blood [equivalent to 63 μM (45–47)] and >4 mM in soils throughout North America (48–50). Finally, cells were harvested and luciferase activity was measured using 30 μl of cell extract and 100 μl of Luciferase Assay Substrate (Promega, Madison, WI) in a Berthold AutoLumat Plus luminometer. The data shown are an average of at least three independent experiments with two replicates.

Western blot analysis

Ishikawa cells were plated in 100 × 20 mm cell culture dishes at 50–80% confluency overnight in DMEM containing 5% DCC-FBS. The following day cells were switched to 0% DCC-FBS for 1 h prior to treatment. Cells were then either not treated or treated with 50 μM p,p′-DDT for 15, 30, 60, 120 and 240 min for MAPK expression or 1, 2, 4 and 6 h for c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2 expression. Cells were then harvested in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 3% SDS, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride) on ice for 10 min. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). An aliquot of 100 μg of protein was added to 10 μl of loading buffer (62.5 mM Tris–HCl, pH 6.8, 1.5% w/v SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue) and then boiled for 5 min and electrophoresed on a 12% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk solution in phosphate-buffered saline (PBS), 0.05% Tween for 1 h. The membrane was subsequently incubated with rabbit antibodies to phosphorylated or unphosphorylated ERK, JNK, p38 (Cell Signaling Technology, Beverly, MA) diluted 1:1000 in PBS + 0.05% Tween, c-Jun (H-79) diluted 1:10 000, JunB (210) diluted 1:500, JunD (329) diluted 1:1000, c-Fos (4) diluted 1:20 000, FosB (102) diluted 1:500, Fra-1 (R-20) diluted 1:200, Fra-2 (L-15) diluted 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (Sigma, St Louis, MO) diluted 1:500, overnight at 4°C. The next day, blots were washed in PBS, 0.05%Tween and incubated with goat

anti-rabbit antibodies conjugated to horseradish peroxidase (1:2000 dilution; Cell Signaling Technology) for 60 min at room temperature. Following three washes with PBS + Tween solution and one wash with PBS alone, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ) and recorded by fluorography on Hyperfilm (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Fluorograms were quantitated by image densitometry using the Fuji MacBas Program, Version 2.5 (Fujifilm, Japan).

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts of Ishikawa cells exposed to 50 μM p,p′-DDT or PMA for 0, 1, 2 or 4 h were prepared using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce, Rockford, IL) supplemented with 1% protease inhibitor cocktail (Sigma) according to the manufacturer's protocol. Oligonucleotides (Promega) with the sequences 5′-CGCTTGATGAGTCAGCCGGAA-3′ and 3′-GCGAACTACTCAGTCGGCCTT-5′ for AP-1 assays and 5′-ATTCGATCGGGGCGGGGCGAGC-3′ and 3′-TAAGCTAGCCCCGCCCCGCTCG-5′ for Sp-1 assays were used. Probes were radiolabeled at the 5′-end with [γ-^{32}P]ATP using T4 polynucleotide kinase. Two micrograms of nuclear extract was used in each binding reaction. AP-1 and Sp-1 binding were assayed using the Gel Shift Assay System (Promega). For gel shift assays, nuclear extracts were incubated with probe DNA in a reaction buffer containing 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl (pH 7.5) and 0.25 mg/ml poly(dI·dC) at room temperature for 20 min. For competition assays, nuclear extracts were incubated with unlabeled competitor oligonucleotides at a 100-fold molar excess for 10 min prior to radiolabeled probe addition. For supershift assays, 2 μg of c-Jun(H-79), c-Fos(4) or GAL4(DBD) antibody (Santa Cruz Biotechnology) was incubated with nuclear extract 30 min prior to probe addition. One microliter of Novex® TBE Hi-Density Sample Buffer was then added to each sample. Competitors were added in 100-fold molar excess 10 min prior to the addition of radiolabeled probe. The protein–DNA complex was separated in a 6% Novex® DNA Retardation Gel (Invitrogen, Carlsbad, CA) at 100 V for ~1 h 15 min or 2 h 15 min for supershift assays, dried on Whatman filter paper and visualized by autoradiography (Kodax BIOMAX film).

Statistical analysis

Parametric data were analyzed using one-way ANOVA and *post hoc* Tukey's multiple comparisons while non-parametric data (Table I, HEK 293 data) were analyzed using Kruskal–Wallis and *post hoc* Dunn's multiple comparisons due to the large differences in activation. All data were analyzed with GraphPad Prism, Version 3.02 (GraphPad Software Inc.). Statistically significant changes were determined at the $P < 0.05$, $P < 0.01$ or $P < 0.001$ level as indicated for each figure or table.

Results

DDT and its metabolites stimulate both synthetic and endogenous AP-1-mediated gene expression in HEK 293 and Ishikawa cells

Previously, we demonstrated that select DDT metabolites potentiate AP-1-mediated activity through an ER-independent mechanism in HEK 293 and Ishikawa stable cell lines (37). Discrepancies have been reported indicating differences in responses in transiently versus stably transfected cells (51,52). In accordance with our stable cell line findings, transiently transfected Ishikawa cells treated with different DDT metabolites potentiate a consensus AP-1 response element linked to a luciferase reporter gene (Table I). DDT, DDD and DDE all stimulated AP-1-responsive gene expression in a dose-dependent manner at environmentally relevant concentrations, 25 and 50 μM (45–50). Metabolites reported to have negligible ER binding capacity stimulated AP-1 activity, indicating the presence of an ER-independent mechanism (37). While p,p′-DDT (50 μM) gave the greatest activity in Ishikawa cells, inducing luciferase activity 17 ± 4-fold, o,p′-DDT (50 μM) was most potent in the HEK 293 cells, inducing luciferase activity 380 ± 60-fold. p,p′-DDA and p,p′-DDOH, two metabolites reported to have no effect, again did not demonstrate significant AP-1 activity.

Table I. Effects of DDT and its metabolites on AP-1-mediated gene expression in HEK 293 and Ishikawa cells

	HEK 293	Ishikawa
DMSO	1.0 ± 0[a]	1.0 ± 0
PMA 20 ng/ml	560 ± 70[b]	23 ± 4[b]
o,p′-DDT 25 μM	4.1 ± 0.8[c]	2.0 ± 0.7
o,p′-DDT 50 μM	380 ± 60[b]	7.8 ± 1
p,p′-DDT 25 μM	1.9 ± 0.3	3.4 ± 1
p,p′-DDT 50 μM	380 ± 40[b]	17 ± 4[b]
o,p′-DDD 25 μM	1.9 ± 0.2	N/A[d]
o,p′-DDD 50 μM	120 ± 20[b]	N/A
p,p′-DDD 25 μM	N/A	8.7 ± 3
p,p′-DDD 50 μM	N/A	20 ± 3[b]
o,p′-DDE 25 μM	N/A	1.8 ± 0.3
o,p′-DDE 50 μM	N/A	5.2 ± 1
p,p′-DDA 25 μM	0.87 ± 0.1	1.7 ± 0.5
p,p′-DDA 50 μM	1.3 ± 0.1	1.2 ± 0.5
p,p′-DDOH 25 μM	1.6 ± 0.2	0.96 ± 0.4
p,p′-DDOH 50 μM	1.3 ± .1	1.1 ± .3

[a]Data are expressed as fold activity with vehicle/DMSO expressed as 1.00. Values are shown as means ± SE ($n = 5$). HEK 293 data are non-parametric due to large fold activations.
[b,c]Statistically significant when compared between control (DMSO) and various treatments calculated using Kruskal–Wallis and Dunn's tests (HEK 293) or ANOVA and Tukey's test (Ishikawa): [b]$P < 0.001$; [c]$P < 0.05$.
[d]Data not available.

To demonstrate the effects of DDT and its conjoiners on an endogenously regulated AP-1 gene, we tested the expression of the AP-1-responsive gene promoter of *collagenase*. Overnight treatment with o,p′-DDT of HEK 293 cells transiently transfected with the intact *collagenase* promoter, which contains an AP-1-binding site, linked to a luciferase reporter gene gave a dose-dependent increase in luciferase activity. This potentiation was blocked by co-expression of a dominant negative c-Fos mutant (which inhibits AP-1 activity) (unpublished data). These results, in conjunction with our previous findings, demonstrate that DDT and its metabolites increase AP-1 activity in both transiently and stably transfected cells. In addition, DDT stimulates both synthetic and endogenous AP-1-mediated gene expression.

DDT treatment induces AP-1 protein complex cycling

We determined the effects of DDT treatment on AP-1 complex–DNA binding using EMSA (Figure 1). To demonstrate probe specificity, we took nuclear extracts of Ishikawa cells treated for 4 h with 20 ng/ml PMA (positive control) and were able to compete off radiolabelled AP-1 probe with cold AP-1, but not Sp-1 probe (Figure 1A, lanes 1–6). Surprisingly, cells treated over a 4 h time course with 50 μM p,p′-DDT demonstrated high basal AP-1 protein–DNA binding activity, with no significant change over time upon p,p′-DDT treatment (Figure 1A, lanes 7–10). AP-1 is comprised of members of the Jun and Fos families of proteins, of which c-Jun/c-Fos heterodimers most often account for the majority of AP-1 activity. To see if there was a shift in the AP-1 complex present upon DDT treatment we used supershift analysis using antibodies that disrupt the specific protein–DNA complexes to demonstrate a significant increase ($P < 0.01$ and $P < 0.05$, respectively) in c-Jun (40.7 ± 10.2%) and c-Fos (11 ± 0.47%) involvement at the AP-1 DNA binding site after 4 h of p,p′-DDT treatment as determined by densitometry (Figure 1B). As a check for antibody specificity, we also demonstrate that addition of a non-specific GAL4(DBD) antibody did not affect the AP-1–DNA complex (Figure 1B, lanes 4 + 8).

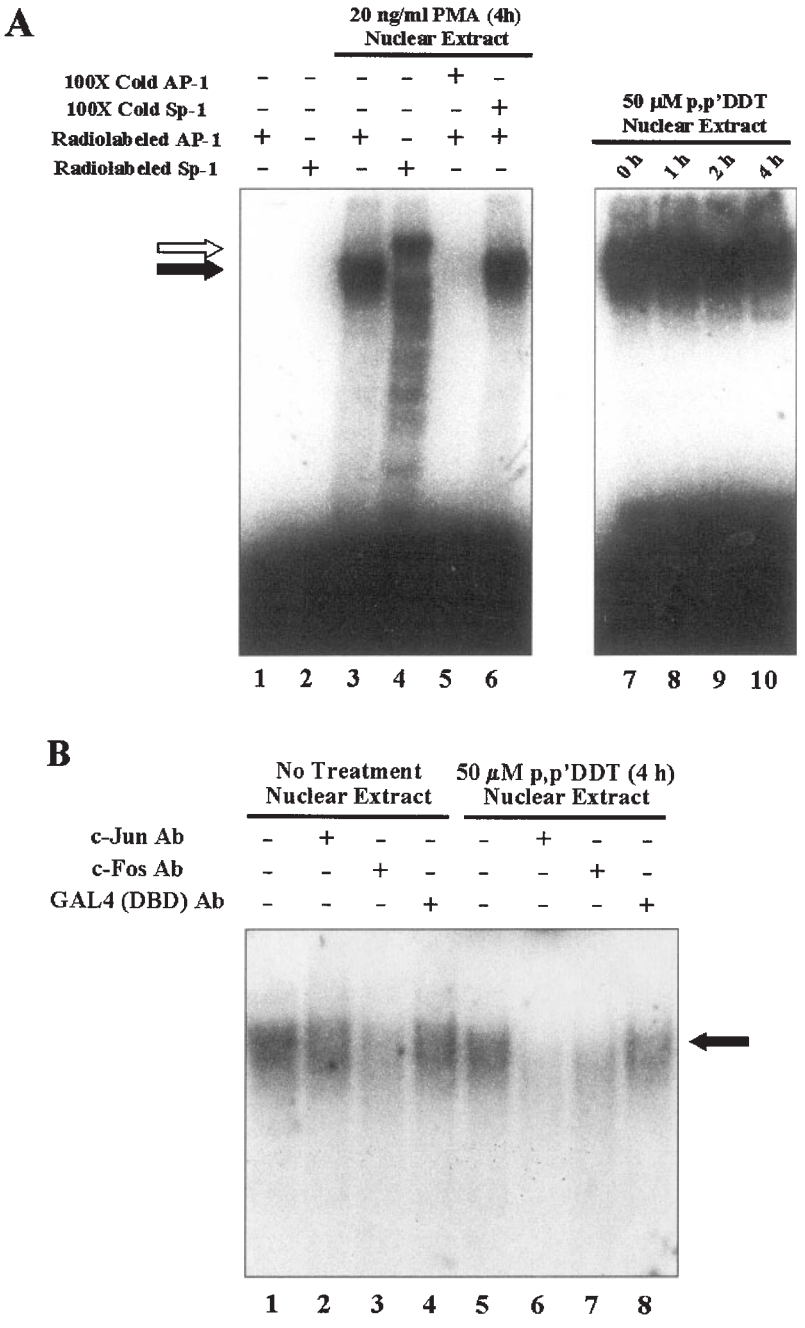

Fig. 1. DDT treatment induces a change in the DNA-bound AP-1 complex. Radiolabeled AP-1 or Sp-1 probe was generated as described in Materials and methods. (**A**) Ishikawa cells were treated for 4 h with 20 ng/ml PMA (lanes 3–6) or 50 µM p,p′-DDT for 0, 1, 2 or 4 h (lanes 7–10, respectively) followed by nuclear extract isolation as described in Materials and methods. Radiolabeled AP-1 or Sp-1 probe was then combined (lanes 3–10) or not (lanes 1 and 2) with nuclear extracts. Unlabeled AP-1 (lane 5) and Sp-1 (lane 6) competitor oligonucleotides were present at 100-fold molar excess. (**B**) For supershift assays Ishikawa cells were either untreated (lanes 1–4) or treated (lanes 5–8) for 4 h with 50 µM p,p′-DDT followed by nuclear extract isolation. Extracts were additionally incubated with no antibody, c-Jun supershift antibody, c-Fos supershift antibody or GAL4(DBD) supershift antibody as indicated. The specific protein–DNA complexes are marked by a white arrow (Sp-1–DNA) or a black arrow (AP-1–DNA). EMSA gels were exposed to X-ray film for 16 h. Similar results were obtained in three independent experiments.

p,p′-DDT increases c-Jun protein levels, but not other Jun and Fos family members

Given our EMSA data, we decided to determine the effects of DDT treatment on the expression of the Jun and Fos family members to help begin to understand why c-Jun and c-Fos are recruited to the DNA following DDT treatment (Figure 2). p,p′-DDT treatment increases c-Jun protein expression 2–4 h after exposure. JunD, JunB, c-Fos, FosB and Fra-1 expression, however, demonstrated no significant change over the high basal level of expression. In fact, JunD and Fra-1 levels decreased over time. We were unable to detect the Fos family member Fra-2 in Ishikawa cells. Hence, DDT treatment

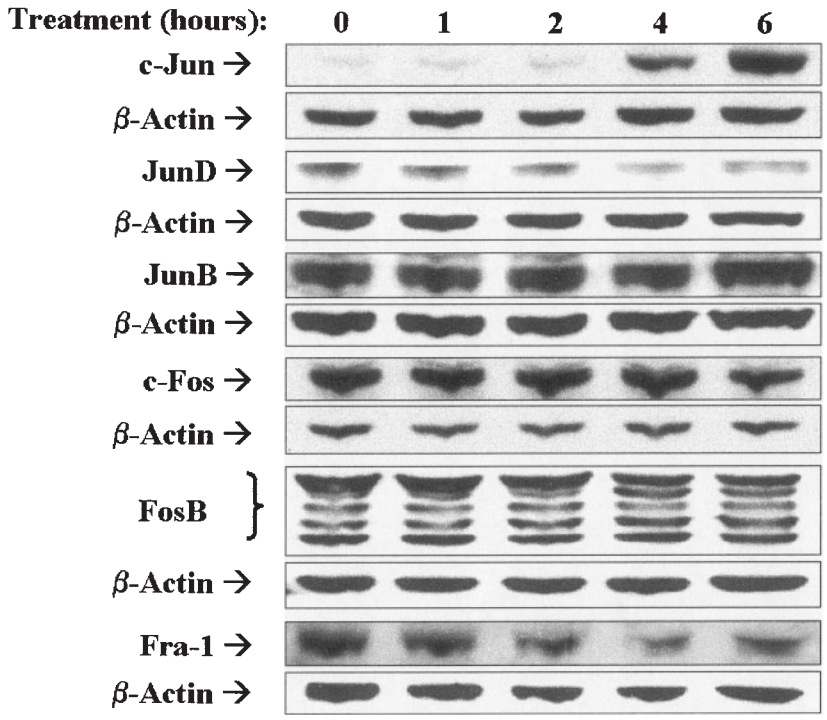

Fig. 2. c-Jun, but not c-Fos, protein levels are increased after p,p′-DDT exposure. Two 100 mm dishes of Ishikawa cells were plated at 80% confluency in DMEM with 5% DCC-FBS overnight. The following day, cells were switched to serum-free medium and not treated (lane 1) or exposed to 50 μM p,p′-DDT for 1, 2, 4 or 6 h (lanes 2–5, respectively). Preparation of cell extracts, gel electrophoresis and western blot analysis were carried out as described in Materials and methods. Jun or Fos family members were initially detected and then membranes were stripped and probed with antibodies for β-actin (loading control). Similar results were obtained in three independent experiments.

appears to cause a shift or cycling of the AP-1 complex present to one that favors a conformation containing c-Jun (a more active AP-1 component), potentially explaining why there is a large increase in AP-1 activity despite no increase in AP-1–DNA binding.

DDT and its metabolites potentiate both the c-Jun and c-Fos transcription factors

c-Jun and c-Fos belong to the bZIP family of transcription factors, well-established targets of various signaling pathways (53,54). We examined regulation of the c-Jun and c-Fos transcription factors using a GAL4 one-hybrid assay. In this assay, the activation domains of either c-Jun or c-Fos were linked to a GAL4 DNA-binding domain. Activation of this hybrid protein was measured through a co-expressed luciferase reporter construct containing five GAL4 binding sites. Consistent with our AP-1 results, the active DDT metabolites tested (o,p′-DDT, p,p′-DDT and o,p′-DDD) all stimulated the GAL4–c-Jun and GAL4–c-Fos hybrids, while inactive metabolites (p,p′-DDA and DDOH) had no effect (Figure 3A and B), indicating that active metabolites can stimulate c-Jun and c-Fos without a corresponding increase in c-Jun or c-Fos protein levels. Collectively, DDT and its metabolites potentiate AP-1 not only through expression of *c-jun*, but also through the activation of both c-Jun and c-Fos at the post-translational level.

DDT activates ERK and p38 MAPKs

The major MAPKs (ERK, JNK and p38) potently regulate AP-1 activity in various cellular contexts (4). To determine a role, if any, of the MAPKs in DDT metabolite-induced AP-1 activity, we first examined the effect of p,p′-DDT on MAPK activity. Stimulated MAPKs are dually phosphorylated on threonine and tyrosine residues located on conserved sequences within the kinases (55). The phosphorylated/activated MAPKs can be detected by antibodies directed against the phosphorylated peptides containing these residues. Ishikawa cells were treated with 50 μM p,p′-DDT for up to 4 h and cell extracts were analyzed for phosphorylated and total MAPK protein by western blotting. Phosphorylated ERK1 (p44) and ERK2 (p42) levels slowly increased over a 4 h period following treatment with p,p′-DDT (Figure 4, top). Phosphorylated p38 was not detected in untreated cells but greatly increased in a time-dependent manner to the last time point examined (4 h) (Figure 4, lane 6). Interestingly, phosphorylated JNK1 (p46) and JNK2 (p54) were detected in untreated Ishikawa cells, however, upon p,p′-DDT treatment phosphorylated JNK levels decreased over time. Cells treated with vehicle (DMSO) showed no change in phosphorylated or unphosphorylated ERK, JNK or p38 levels (unpublished data). Additionally, the increase in levels of phosphorylated MAPKs was not due to an increase in total MAPK levels.

SB203580 inhibits AP-1-mediated gene expression by DDT and its metabolites in both Ishikawa and HEK 293 cells

To determine which MAPK signaling pathways are necessary for DDT metabolite-induced activation of AP-1, we examined the effects of UO126, an ERK pathway inhibitor, SP600125, a JNK selective inhibitor, and SB203580, a p38(α/β) selective inhibitor. PMA-induced AP-1 activity, known to be predominantly mediated by the ERK pathway, was severely inhibited in both Ishikawa and HEK 293 cells treated with 1 μM UO126 (Table II). However, UO126 had no significant effect on DDT metabolite-induced luciferase activity. Treatment of HEK 293 cells with the JNK inhibitor did not lead to a reduction in

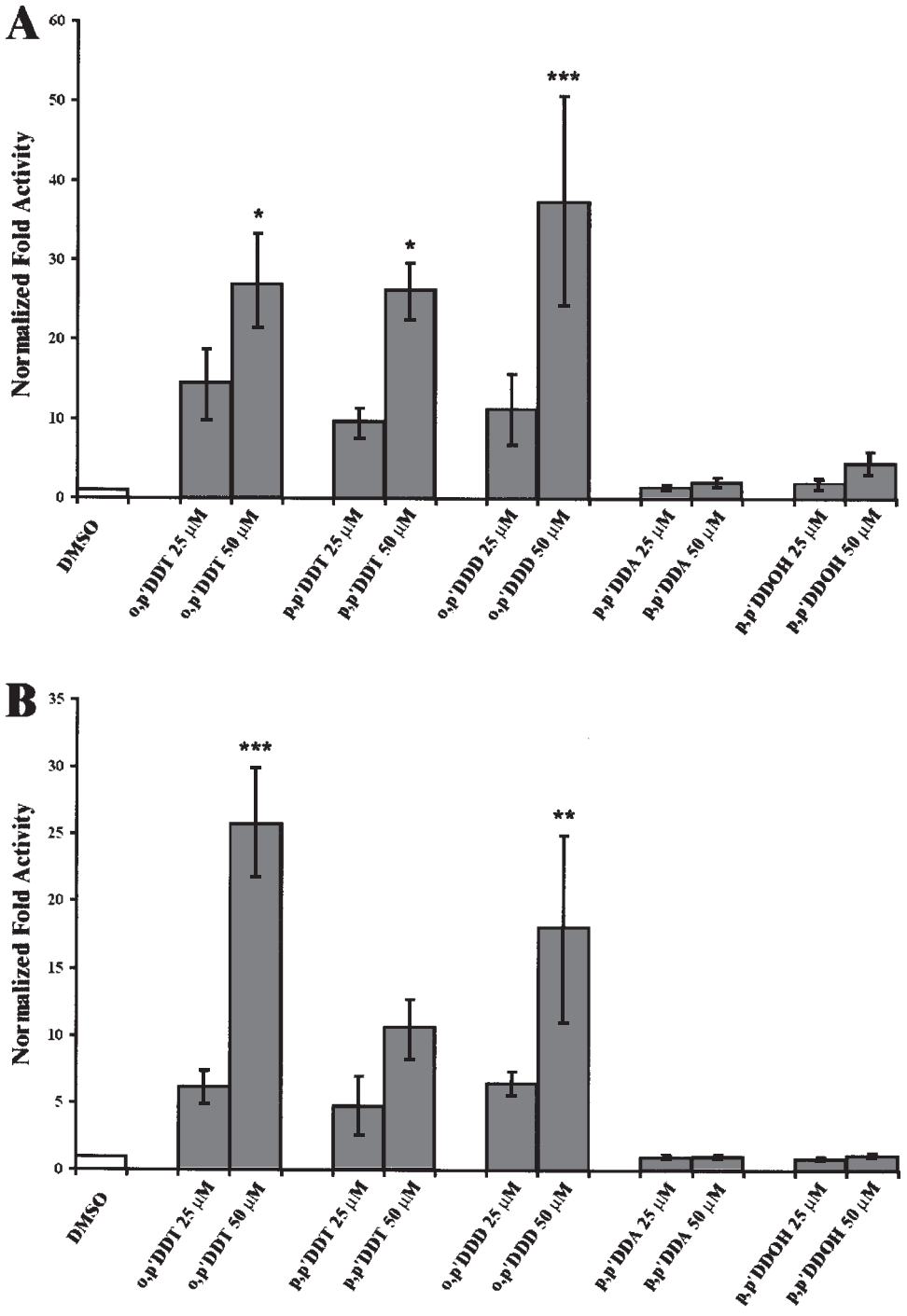

Fig. 3. DDT metabolites stimulate both c-Jun and c-Fos post-translationally. Approximately 1×10^5 HEK 293 cells were plated overnight in DMEM with 5% DCC-FBS. The following day GAL4–c-Jun (**A**) or GAL4–c-Fos (**B**) (25 ng) was co-transfected into the HEK 293 cells along with a GAL4–luciferase reporter (50 ng). Cells were then treated 5 h later with vehicle/DMSO or DDT metabolite. The following day luciferase activity was assayed. Results describe the mean fold activation over vehicle ± SE ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; significant increases from control (ANOVA and Tukey's test).

luciferase activity greater than that seen in vehicle (DMSO)-treated cells. Treatment of either Ishikawa or HEK 293 cells with SB203580, however, strongly inhibited luciferase activity by the active DDT metabolites (o,p′-DDT, p,p′-DDT and o,p′-DDD) (Figure 5 and Table II). Conversely, SB203580 treatment either had no effect or even potentiated vehicle, PMA or the inactive DDT metabolite p,p′-DDA effects, suggesting that p38 may inhibit basal AP-1 activity. Together, these data implicate p38, but not the ERK or JNK pathways, in DDT metabolite-induced AP-1 activity.

Dominant negative p38 inhibits DDT metabolite expression of AP-1-luciferase

Phosphorylation site dominant negative mutants were used to further examine the role of the MAPKs because of the potential non-specific effects of pharmacological inhibitors.

Dominant negative ERK2 and BMK1 gave the greatest inhibition of PMA-induced AP-1 activity of the four dominant negative MAPKs, decreasing activity 40–50% (Figure 6A). ERK also appears to play a role in p,p′-DDT-induced activation of AP-1–luciferase expression, decreasing activity 40% (Figure 6B).

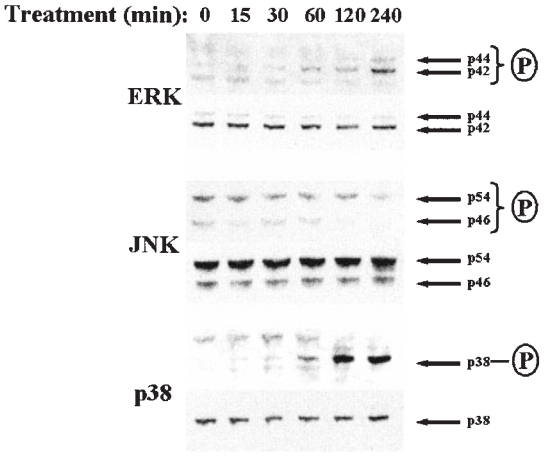

Fig. 4. Activation of MAPKs by p,p′-DDT in Ishikawa cells. Two 100 mm dishes of Ishikawa cells were plated at 80% confluency in DMEM with 5% DCC-FBS overnight. The following day, cells were switched to serum-free medium and not treated (lane 1) or exposed to 50 μM p,p′-DDT for 15, 30, 60, 120 or 240 min (lanes 2–6, respectively). Preparation of cell extracts, gel electrophoresis and western blot analysis were carried out as described in Materials and methods. Phosphorylated (P) MAPKs were initially detected and then membranes were stripped and probed with antibodies that detect total MAPK proteins. Individual MAPKs are identified by their size (kDa). Similar results were obtained in three or four independent experiments.

Consistent with the western blot and pharmacological inhibitor data, JNK does not appear to play a role in either PMA- or DDT metabolite-induced AP-1 activity (Figure 6A and B). In agreement with the results obtained with the pharmacological inhibitors above, dominant negative p38 decreased DDT-induced AP-1 activity the most. Dominant negative p38α reduced p,p′-DDT-stimulated luciferase activity >80% (Figure 6B), whereas PMA-induced activity was decreased only 30% (Figure 6A). Inhibition was significantly different ($P < 0.05$) than vehicle/DMSO-treated cells (unpublished data).

Different dominant negative p38 isoform mutants do not affect DDT metabolite-induced AP-1 activation

Dominant negative p38α, p38β, p38δ and p38γ constructs were used to further determine p38 effects on AP-1–luciferase expression. DDT metabolite activation of AP-1 did not appear to differ when blocked by various p38 dominant negative mutants, as all reduced AP-1 activity 70–80% (Figure 6D). However, PMA-induced AP-1 activity was inhibited more by p38δ and p38γ (Figure 6C). In fact, at lower transfected concentrations of dominant negative plasmid, inhibition of p38α and p38β actually appeared to potentiate PMA-induced activity, consistent with our pharmacological inhibitor data in treated Ishikawa cells.

Discussion

The transcription factor AP-1 acts as an environmental sensor, detecting changes in the extracellular milieu through the use of multiple signaling cascades. Environmental carcinogens such as cadmium, dioxin and silica have been reported to stimulate AP-1 activity through kinase signaling pathways (12,56,57). Here, we demonstrate that members of the organochlorine

Table II. Effects of pharmacological MAPK pathway inhibitors on AP-1-mediated gene expression in HEK 293 and Ishikawa cells

	HEK 293	Ishikawa
DMSO	1.0 ± 0[a] (100 ± 0)[b]	1.0 ± 0 (100 ± 0)
UO 126 1 μM	0.81 ± 0.1 (81 ± 6)	0.90 ± 0.2 (90 ± 20)
SP 600125 1 μM	0.66 ± 0.2 (66 ± 20)	N/A[c]
SB 203580 6 μM	2.4 ± 0.6 (240 ± 60)	1.5 ± 0.2 (150 ± 20)[d]
PMA 20 ng/ml	690 ± 100 (100 ± 0)	25 ± 4 (100 ± 0)
UO 126 1 μM + PMA 20 ng/ml	100 ± 20 (15 ± 1)	10 ± 2 (41 ± 2)[e]
SP 600125 1 μM + PMA 20 ng/ml	570 ± 100 (83 ± 5)	N/A
SB 203580 6 μM + PMA 20 ng/ml	490 ± 300 (77 ± 40)	48 ± 9 (190 ± 10)[e]
o,p′-DDT 50 μM	72 ± 5 (100 ± 0)	4.0 ± 0.9 (100 ± 0)
UO 126 1 μM + o,p′-DDT 50 μM	55 ± 8 (76 ± 10)	3.3 ± 0.6 (96 ± 20)
SP 600125 1 μM + o,p′-DDT 50 μM	50 ± 4 (70 ± 8)	N/A
SB 203580 6 μM + o,p′-DDT 50 μM	9.6 ± 5 (14 ± 8)[e]	1.9 ± 0.5 (57 ± 20)
p,p′-DDT 50 μM	52 ± 6 (100 ± 0)	6.3 ± 1 (100 ± 0)
UO 126 1 μM + p,p′-DDT 50 μM	34 ± 6 (65 ± 4)	5.9 ± 1 (110 ± 20)
SP 600125 1 μM + p,p′-DDT 50 μM	39 ± 8 (75 ± 8)	N/A
SB 203580 6 μM + p,p′-DDT 50 μM	18 ± 10 (33 ± 20)[f]	3.0 ± 0.5 (53 ± 8)[d]
o,p′-DDD 50 μM	8.7 ± 0.5 (100 ± 0)	11 ± 1 (100 ± 0)
UO 126 1 μM + o,p′-DDD 25 μM	8.4 ± 2 (100 ± 30)	8.1 ± 2 (74 ± 9)d
SP 600125 1 μM + o,p′-DDD 25 μM	7.1 ± 0.1 (86 ± 4)	N/A
SB 203580 6 μM + o,p′-DDD 25 μM	0.25 ± 0.1 (3.1 ± 2)[d]	4.4 ± 1 (42 ± 7)[e]
p,p′-DDA 50 μM	1.2 ± 0.1 (100 ± 0)	1.8 ± 0.4 (100 ± 0)
UO 126 1 μM + p,p′-DDA 50 μM	1.2 ± 0.1 (100 ± 10)	1.3 ± 0.3 (82 ± 20)
SP 600125 1 μM + p,p′-DDA 50 μM	0.78 ± 0.1 (69 ± 10)	N/A
SB 203580 6 μM + p,p′-DDA 50 μM	0.96 ± 0.5 (91 ± 50)	1.4 ± 0.2 (96 ± 20)

[a]Data are expressed as fold activity with vehicle/DMSO expressed as 1.0. Values are shown as means ± SE ($n = 4$).
[b]Data are alternatively expressed as percent activity relative to vehicle/DMSO, 50 μM o,p′-DDT, 50 μM p,p′-DDT, 50 μM o,p′-DDD or 50 μM p,p′-DDA where treatment without inhibitor was normalized to 100%. Values are shown as means ± SE ($n = 4$).
[c]Data not available.
[d–f]Statistically significant pharmacological inhibitor effects when compared to vehicle/DMSO, 50 μM o,p′-DDT, 50 μM p,p′-DDT, 50 μM o,p′-DDD or 50 μM p,p′-DDA treatment alone calculated using ANOVA and Tukey's test: [d]$P < 0.05$; [e]$P < 0.001$; [f]$P < 0.01$.

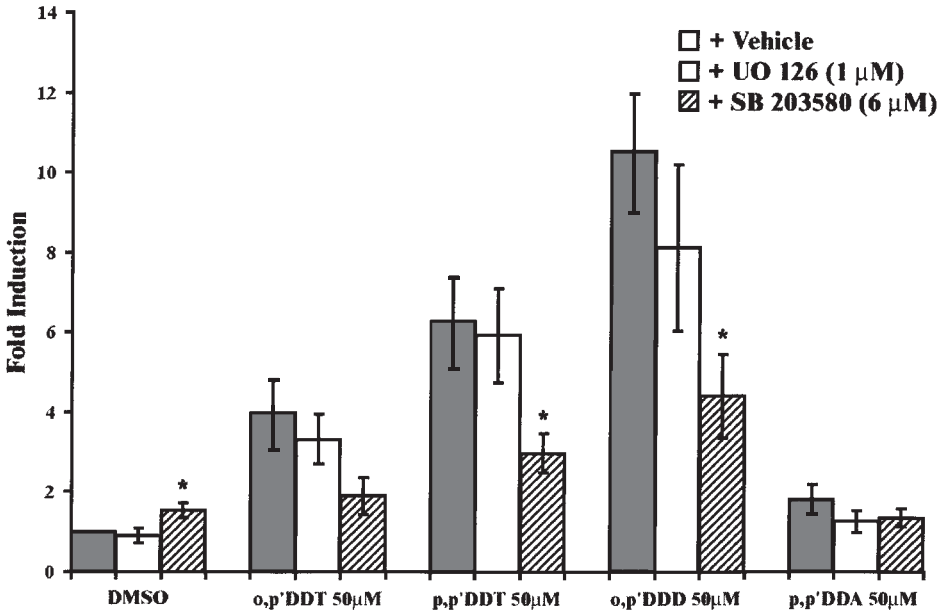

Fig. 5. SB203580 inhibits induction of AP-1-regulated genes by DDT and its conjoiners. Ishikawa cells were plated as for transfections. Cells were transfected for 5 h with 100 ng pAP-1(PMA)–luciferase. After this period, the indicated concentration of kinase inhibitor was added, followed 30 min later by the addition of vehicle or DDT metabolite (50 μM). The following day luciferase activity was assayed. Results describe the mean fold activation over vehicle ± SE ($n = 8$). $^{*}P < 0.05$; significant differences from vehicle or DDT metabolite treatment without inhibitor (ANOVA and Tukey's test).

class of compounds stimulate AP-1-mediated gene expression through activation of the p38 MAPK. Previous work describing DDT attribute most of the harmful effects to the ability of DDT to mimic the proliferative hormone estrogen (17). Our work demonstrates that another signaling pathway exists, one involving the stress kinase p38.

In agreement with our previous studies in stably transfected cell lines (37), transiently transfected HEK 293 and Ishikawa cells treated with DDT, DDD or DDE showed increased AP-1 activity, while p,p′-DDA and DDOH, two metabolites found in humans, had no effect. Interestingly, o,p′-DDT had the greatest effect in HEK 293 cells while p,p′-DDT treatment had the largest effect in Ishikawa cells. HEK 293 and Ishikawa cells may interpret extracellular signals differently due to unique cell surface profiles. For example, o,p′-DDT and p,p′-DDT have been previously reported to have different effects on the erbB-2 plasma membrane receptor in breast cell variants (30,35). It is possible that HEK 293 and Ishikawa cells may have different levels of plasma membrane receptors, like erbB-2, which preferentially induce a greater kinase signaling cascade from one DDT metabolite over another.

To further confirm what we had found using synthetic AP-1-regulated genes, we decided to examine the effects of the organochlorines on an endogenously AP-1-regulated gene, *collagenase*. Collagenase is a member of a larger family of extracellular matrix remodeling proteins known as matrix metalloproteinases. Increased expression of *collagenase* can lead to tumor progression and metastasis. Thus, *collagenase* expression may represent a possible mechanism through which AP-1 stimulates carcinogenesis. o,p′-DDT treatment significantly increased *collagenase* promoter activity. This increase can be blocked by inhibition of AP-1.

Surprisingly, DDT treatment did not increase AP-1–DNA binding and, in fact, may have slightly decreased it over time. This is probably because there is a high basal level of DNA binding in the cells we tested. Supershift analysis, however, revealed that while total AP-1 complex levels did not change, DDT treatment induced a shift or cycling of the AP-1 complex present to one that favors c-Jun and, to a lesser extent, c-Fos (Figure 7), explaining the increase in AP-1 activity, as c-Jun and c-Fos most often account for the majority of AP-1 activity. The AP-1 shift appears to be caused by two major mechanisms: (i) an increase in *c-jun* expression; and (ii) post-translational modification of both c-Jun and c-Fos.

Organochlorine treatment led to an increase in c-Jun protein expression as expected. The promoter of *c-jun* contains a necessary AP-1 response element and hence promotes a positive feedback to enhance AP-1 activity (58). In addition, a myocyte enhancer factor 2 (MEF2) site is located within the *c-jun* promoter. This site is regulated by the p38γ isoform and, hence, may represent a mechanism through which p38 increases c-Jun levels. Results from our dominant negative studies suggested that BMK1 may also play a role in DDT-induced AP-1 activity. Marinissen *et al*. demonstrated that the MEF2 site is BMK1 responsive, indicating a potential alternative organochlorine-regulated pathway (59). Finally, an additional AP-1-like element located in the *c-jun* promoter can bind transcription factor complexes such as c-Jun/activating transcription factor 2 (ATF2) dimers. ATF2 can be phosphorylated/activated by p38α, p38β and p38δ, introducing another mechanism of p38-induced *c-jun* expression (60,61).

c-fos, which is often regulated by kinase signaling pathways that converge at a serum response element (SRE) located in its promoter region, surprisingly was not significantly affected by DDT exposure. This could in part be due to a high basal expression level of *c-fos* in Ishikawa cells, as may have been the case for *junD*, *junB*, *fosB* and *fra-1*, which were also unresponsive to DDT treatment. Expression of the AP-1 components *c-jun* and *c-fos* represents an interesting dichotomy. On one side, stress signaling pathways such as DDT-induced p38 activity strongly promote the expression of *c-jun* alone, whereas, conversely, a proliferative signal such as

PMA-induced ERK activity initially increases *c-fos* expression through targeting the SRE (62). Thus, understanding the temporal expression of individual AP-1 genes may give insight into how a cell ultimately responds (i.e. proliferation, differentiation, death, etc.) to extracellular stimuli (Figure 8).

The effects on AP-1 were further assessed using the GAL4 one-hybrid assay. DDT and DDD induction of both c-Jun and c-Fos activity indicate that AP-1 can be stimulated post-translationally. While c-Jun is a well-known target of MAPK signaling pathways, the identity of c-Fos-regulating kinases remains a mystery (4).

Given the role of MAPK signaling in AP-1 activity, we sought to examine the effects of DDT metabolites on MAPK pathways. A combination of western blots and reporter gene assays using pharmacological and molecular inhibitors of the MAPK pathways implied a significant role for the p38 MAPK and, to a lesser extent, ERK in DDT-induced AP-1 activity. Interestingly, experiments done using the p38α and p38β isoform selective pharmacological inhibitor SB203580 (63,64), as well as specific p38 dominant negative mutants, revealed that p38α and p38β can inhibit both basal and PMA-stimulated AP-1 activity (Table II and Figure 6). The temporal

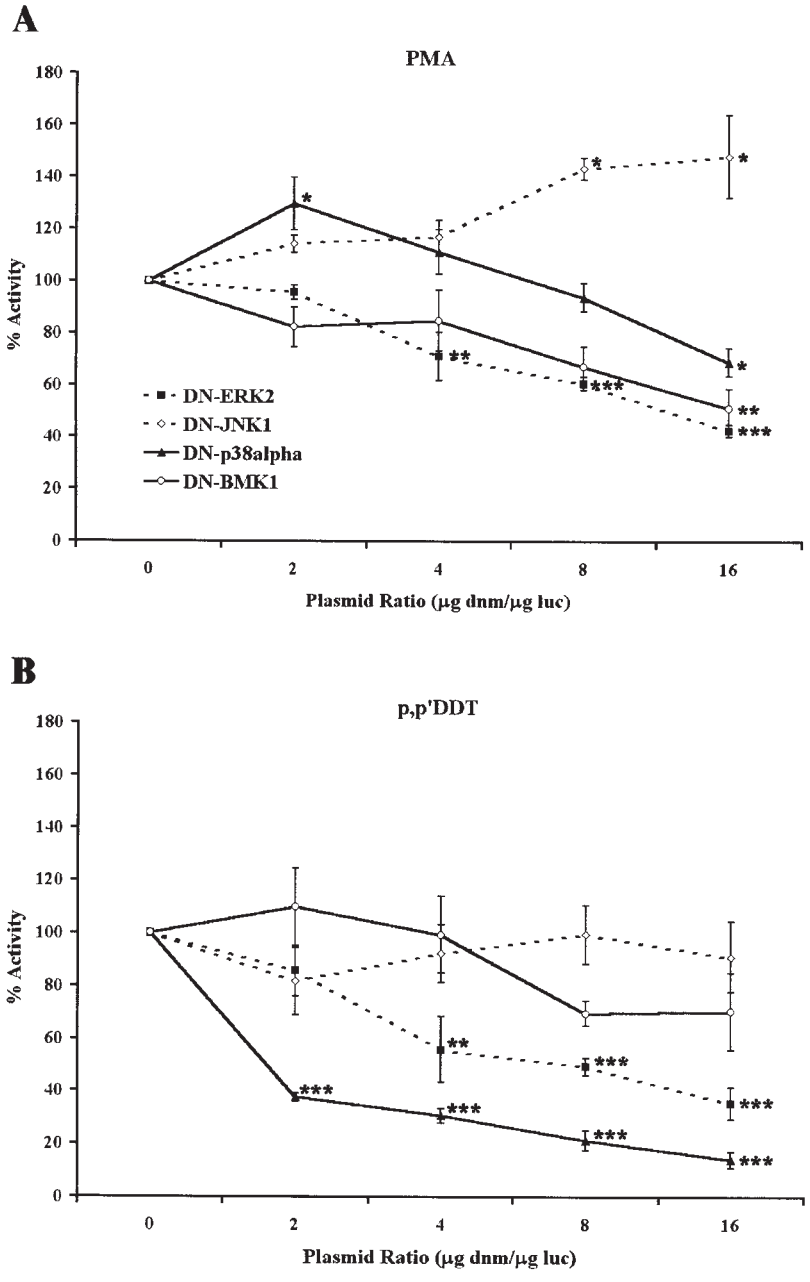

Fig. 6. Co-expression of dominant negative mutants of p38 inhibit DDT metabolite-induced AP-1-regulated gene expression. HEK 293 cells were plated as for transfections. The following day cells were transfected with 10 ng pAP-1(PMA)–luciferase and 0, 20, 40, 80 or 160 ng of the indicated MAPK dominant negative mutant (dnm); either ERK2, JNK1, p38α and BMK1 (**A** and **B**) or p38α, p38β, p38δ and p38γ (**C** and **D**). Total DNA was equalized with an empty mammalian expression vector containing the same CMV promoter. The following day cells were treated with vehicle (data not shown), 20 ng/ml PMA (A and C) (control) or 50 μM DDT metabolite (B and D). Luciferase analysis was performed the following day as described in Materials and methods. Each data point, presented as a percentage of luciferase activity in the absence of MAPK mutants, represents the mean ± SE ($n = 4$–6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; significant increases from control (ANOVA and Tukey's test).

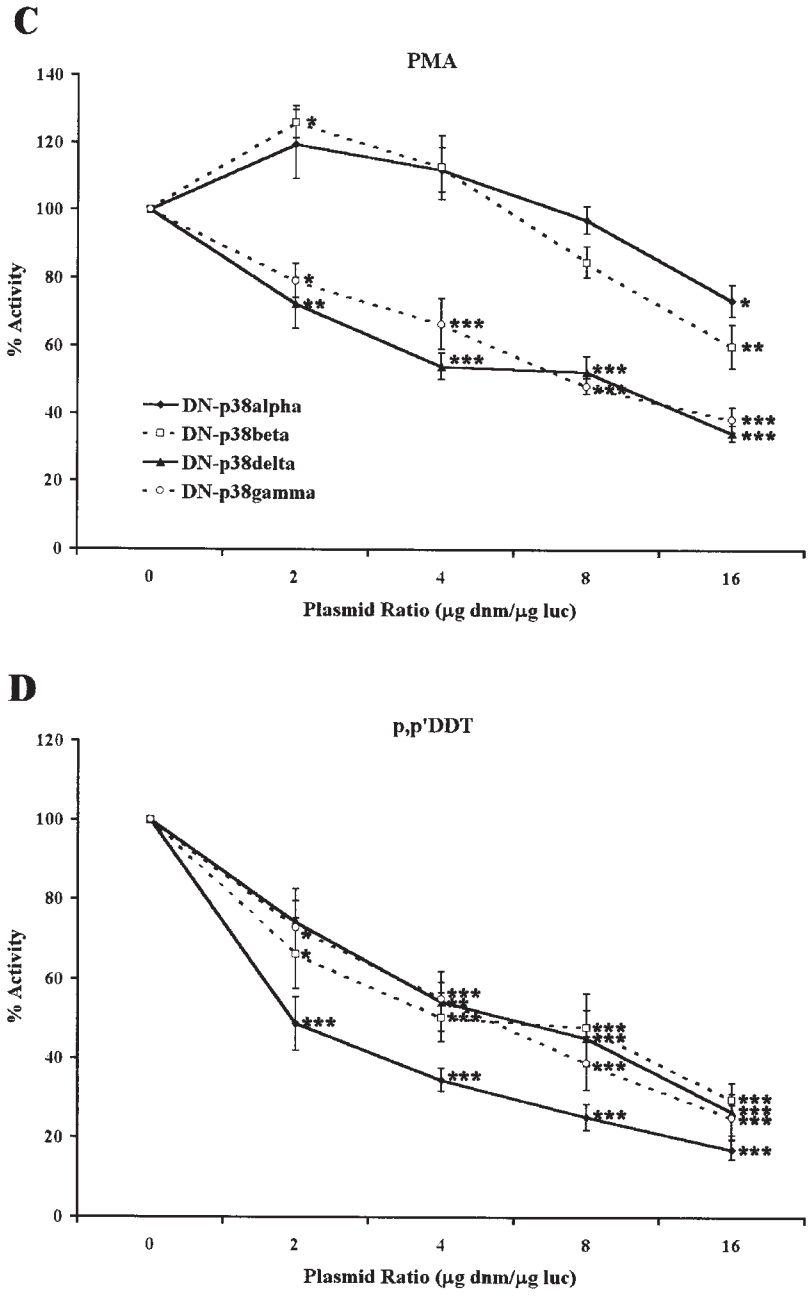

Fig. 6. *Continued*

phosphorylation/activation of p38 agrees with expression of the AP-1 regulated gene *c-jun*, as a strong p38 signal is detected ~2–3 h before c-Jun protein levels increase (Figures 2 and 4). The use of the p38 dominant negative mutants indicated that all isoforms contribute to DDT-induced AP-1 activity (Figure 6). This is in contrast to a recent report that found opposite effects of p38β versus p38δ and p38γ on regulation of AP-1-dependent activities by the p38 activators MKK6 and/or arsenite in human breast cancer cells (65). The explanation for this phenomenon, however, may lie in the differences in stimuli and cell type which heavily dictate p38 effects (64).

The observation of organochlorines mediating signaling for both c-Jun and c-Fos may be an indication that p38 is not necessarily targeting c-Jun and c-Fos directly, but possibly targeting another protein that in turn leads to increased c-Jun and c-Fos activity. Recently, it has been reported that kinase signaling pathways can directly phosphorylate and potentiate co-activators such as p300, SRC-1,GRIP1 and AIB1 that can form complexes with AP-1 (66–70). This would then drive gene expression by making direct interactions with the core RNA polymerase machinery and unraveling chromatin using intrinsic HAT activity, allowing the RNA polymerase to access the DNA and start transcription. Since these proteins interact with a number of transcription factors, it is possible that stimulation of co-activators will lead to diverse effects on gene expression through the multiple sites that can be targeted.

In summary, our data demonstrate that DDT and its metabolites stimulate the *collagenase* promoter through a p38 MAPK and AP-1-dependent mechanism. AP-1 activity is up-regulated in two ways: (i) through an increase in *c-jun* expression; and (ii) post-translationally stimulating the activity of both c-Jun and c-Fos. While the MAPK ERK appears to play

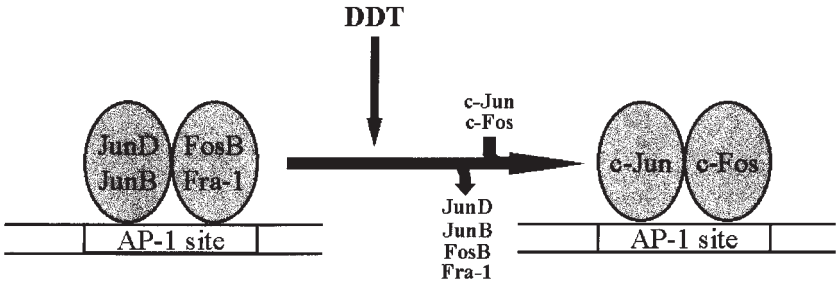

Fig. 7. Working model of DDT-induced cycling of AP-1 complex.

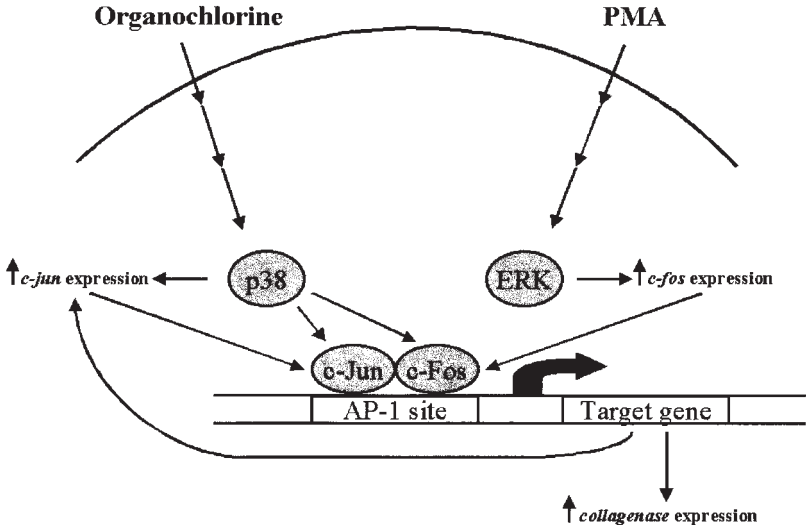

Fig. 8. A schematic for PMA- and organochlorine-induced AP-1 activity. PMA signals the ERK cascade, which in turn targets the SRE site of *c-fos*. Alternatively, select organochlorines signal a p38-mediated mechanism that up-regulates AP-1 activity by (i) targeting c-Jun and c-Fos post-translationally and (ii) increasing *c-jun* expression. Both pathways thus converge at the level of AP-1, which can then bind to various promoters and stimulate the expression of genes such as *collagenase* and *c-jun* itself.

a role, the MAPK p38 is essential for DDT induction of AP-1 in both HEK 293 and Ishikawa cells. Environmental compounds have been well documented to cause tumor formation and growth by mimicking aspects of the cell molecular endocrine system. Likewise, our findings demonstrate that these same compounds can affect the induction of other known carcinogenic signaling pathways. This work is a novel finding for a widespread class of compounds that are rarely found individually, but rather are present as a mixture of compounds. The fact that multiple organochlorine metabolites signal via a common pathway suggests that more profound effects may be found in the environment, where concentrations of individual pollutants may not have to be high enough to cause significant effects since a collective mixture of lower concentration metabolites could have the same impact. Determining what dictates the final cellular outcome following pesticide activation of the intertwined signaling pathways remains the focus of this laboratory.

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