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Endosulfan upregulates AP-1 binding and ARE-mediated transcription via ERK1/2 and p38 activation in HepG2 cells

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Abstract

Endosulfan is an organochlorine insecticide and has been implicated in neurotoxicity, hepatotoxicity, immunosuppression and teratogenicity. However, the molecular mechanism of endosulfan toxicity is not yet clear. Recent studies demonstrated that oxidative stress induced by endosulfan is involved in its toxicity and accumulating evidence suggests that endosulfan can modulate the activities of stress-responsive signal transduction pathways including extracellular signal regulated kinases (ERK) 1/2. However, none of the previous studies investigated the ability of endosulfan to modulate activating protein-1 (AP-1) binding and antioxidant response element (ARE)-mediated transcription as an underlying mechanism of endosulfan toxicity. In this report, we show that treatment of HepG2 cells with endosulfan significantly increased oxidative stress-responsive transcription via AP-1 activation. In addition, endosulfan-induced transcription was enhanced in cells depleted of glutathione by buthionine sulfoximine (BSO) treatment. Exposure to endosulfan resulted in a significant increase in the activities of MAPKs, ERK1/2 and p38. Endosulfan-induced increases in enzymatic activities of these MAPKs were consistent with MAPK phosphorylation. Endosulfan exposure also caused an increase in c-Jun phosphorylation. These results suggest a model for endosulfan toxicity in which endosulfan increases ERK1/2 and p38 activities and these activated MAPKs then increase c-Jun phosphorylation. Phosphorylated c-Jun, in turn, increases AP-1 activity, which results in activation of ARE-mediated transcription.

Keywords

Endosulfan; AP-1; ARE; ERK; p38; HepG2

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

1. Introduction

Endosulfan is an organochlorine insecticide belonging to the cyclodiene group. Once one of the most commonly used pesticides in agriculture and horticulture worldwide, a global ban on the manufacture and use of endosulfan was negotiated under the Stockholm Convention in April 2011 due to threats to human health and the environment. More than 80 countries had already banned it or announced phase outs by the time the Stockholm Convention ban was agreed upon. However, it is still used extensively in India, China, and several other countries (Becker et al., 2011; Desalegn et al., 2011). US Environmental Protection Agency (EPA) has currently listed endosulfan as a priority pollutant.

Endosulfan is absorbed through the skin as well as by respiratory and oral routes, and stored in fat (Ceron et al., 1995). Endosulfan has been reported to affect a variety of organ systems and physiological functions including the nervous system (Agarwal et al., 1978; Hincal et al., 1995; Kamijima and Casida, 2000; Srikanth et al., 1989), the immune function (Banerjee and Hussain, 1986; Kannan et al., 2000), the hepatic system (Gupta and Gupta, 1979), and the reproductive system (Dalsenter et al., 1999; Sinha et al., 1995). In addition, endosulfan has been shown to induce teratogenic effects in rats (Singh et al., 2007). However, the cellular and molecular mechanism underlying endosulfan toxicity has not been clearly elucidated.

Recent studies suggest that mitochondrial dysfunction and oxidative stress caused by endosulfan have a role in its toxicity (Kannan et al., 2000; Kannan and Jain, 2003; Sohn et al., 2004). There has been accumulating evidence in support of a role for reactive oxygen species as second messengers and critical modulators of gene transcription and stress-responsive signal transduction cascades (Dalton et al., 1999; Torres and Forman, 2003), including the mitogen-activated protein kinases (MAPK) pathways. MAPK cascades are the most conserved signal transduction systems in all eukaryotes and have been shown to participate in cell differentiation, cell division, cell movement, and cell death (Schaeffer and Weber, 1999). MAPKs comprise a group of protein serine/threonine kinases such as extracellular signal regulated kinases (ERKs), Jun N-terminal kinases (JNKs)/stress-activated protein kinases (SAPK) and p38, which are activated in response to extracellular stimuli through dual phosphorylation at conserved threonine and tyrosine residues. MAPKs are important intracellular signaling mediators, controlling gene expression through phosphorylation of transcription factors and modulation of the function of these factors (Davis, 1995). Recently, several studies have reported the ability of endosulfan to modulate the activities of MAPKs, especially ERKs using clonal rat prolactinoma cell line (Bulayeva and Watson, 2004), MCF-7 cells (Li et al., 2006), and human HaCaT keratinocytes (Antherieu et al., 2007; Ledirac et al., 2005). However, none of the previous studies examined the ability of endosulfan to modulate AP-1 activity and ARE-mediated transcription via MAPKs as an underlying mechanism of endosulfan toxicity. In the present study, we investigated the mechanism of endosulfan toxicity in relation to intracellular oxidative stress and the changes in AP-1 binding and oxidative stress-responsive transcription.

Because the liver is the main organ involved in the metabolism and the toxicity of xenobiotics, hepatocytes have been increasingly used in recent years as *in vitro* models to investigate the metabolism and action of xenobiotics (Bursztyka et al., 2008; Suolinna, 1982). Primary cultures of human hepatocytes and hepatoma cell line HepG2 are frequently used to evaluate the hepatic disposition of drugs and other xenobiotics (Wilkening and Bader, 2003). Therefore, in the present study, we used HepG2 cells to investigate whether the mechanism of endosulfan toxicity was related to intracellular oxidative stress and changes in MAPK signaling in human hepatocytes.

In this report, our findings showed that endosulfan was capable of increasing ERK and p38 enzymatic activities by phosphorylation, and activated ERK and p38 in turn increased c-Jun phosphorylation. Phosphorylated c-Jun induced AP-1 activation, which led to activation of ARE-mediated transcription. These results provided new insights into the molecular mechanism underlying endosulfan toxicity in human hepatocytes.

2. Materials and methods

2.1. Reagents

Endosulfan was a mixture of alpha- and beta-isomers mixed at a ratio of 61.4: 38.6, and was obtained from Chem Service (West Chester, PA, USA). The stock solution of endosulfan was prepared in absolute ethanol. Hydrogen peroxide was purchased from Sigma (St. Louis, MO, USA) and used as a positive control for western blots and kinase activity assays.

2.2. Cell culture and Cytotoxicity Assay

HepG2 cells (Human hepatoma cell line, ATCC# HB-8065) were grown in MEM (Gibco) supplemented with 10 % fetal bovine serum, 100 μ M nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μ g/ml streptomycin (all from Gibco). Cells were maintained in a humidified incubator at 37 °C with 5 % CO₂. Cytotoxicity assays were performed as previously described (Shokri et al., 2000). Briefly, each well of 48-well tissue culture plates were seeded with 4×10^4 cells in 200 μ l of complete MEM media and incubated for 24 h. Cells were then dosed with various concentrations of endosulfan and grown for an additional 24 h. Each treatment was performed in triplicates. To determine cell viability, supernatants were aspirated and cells were rinsed once with 1 $\times$ phosphate-buffered saline (PBS) and incubated with 200 μ L of freshly prepared, neutral red (Life Technologies) solution (40 μ g/ml) in complete MEM. After 3-h incubation, the solution was removed and the cells were washed with PBS (1 $\times$) and then fixed with CaCl₂ (1 %, w/v) in formaldehyde (0.5 %, v/v). Cells were then lysed and the dye was extracted with acetic acid (1 %, v/v) in 50 % ethanol at room temperature for 10 min. Lysates were thoroughly mixed on an orbital shaker for 2–3 min. Optical density (OD) was measured at 540 nm using a HTS 7000 microplate reader (Perkin-Elmer Corp., Norwalk, CT). The level of cytotoxicity was determined using the following calculation:

$$\% \text{ Cytotoxicity} = (1 - \text{mean OD}_{540} \text{ treated cells} / \text{mean OD}_{540} \text{ negative control}) \times 100.$$

All experiments were repeated at least three times.

2.3. Transient Transfection and Reporter Gene Assay

HepG2 cells were seeded onto 24-well plates 24 h before transfection at a density of 2×10^4 cells/well. Cells were transfected with a rat NAD(P)H: quinone oxidoreductase 1 (NQO1) - based chloramphenicol acetyl transferase (CAT) reporter plasmid (QR-ARE) using lipofectamine (Life Technologies) according to the manufacturer's protocol. M1 is QR-ARE with a point mutation at the promotor region and was co-transfected as a negative control. pSV- β Gal plasmid (Promega) was co-transfected as an internal control for transfection efficiency. The CAT reporter genes containing the promotor region of rat NAD(P)H: quinone oxidoreductase-1 (QR-ARE, M1-ARE) were provided by Dr. Glen Andrews (University of Kansas Medical Center). After 4–5 h incubation, the transfection mixture was replaced with complete medium and cells were incubated for an additional 24 h before treatment with endosulfan. For transient transfection assays with L-buthionine-[S, R]-sulfoximine (BSO, Sigma), BSO (500 μ M) was added at least 16 h prior to endosulfan treatment. After 24 h treatment with endosulfan, the CAT assay was performed using a commercially available ELISA kit according to the manufacturer's protocol (Roche Biotechnology). All assays were performed in triplicate and all CAT activities were

normalized against β -galactosidase activity. The fold induction of the treated sample was calculated over that of vehicle control.

2.4. Nuclear Extract Preparation

Nuclear extract was prepared as previously described (Taylor and Khachigian, 2000). Briefly, after 24-h exposure to 10 μ M and 30 μ M of endosulfan, cells were washed with 1 $\times$ PBS twice and harvested in 4 ml PBS. Hydrogen peroxide was used as a positive control because it is well known that AP-1 is induced by hydrogen peroxide [reviewed in (Dalton et al., 1999)]. Cells were centrifuged at 1300 rpm (Eppendorf, F45-24-11) for 15 min at 4 $^{\circ}$ C, resuspended in 200 μ l of ice-cold solution A (10 mM Hepes, pH. 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 % nonidet P-40, 1 mM DTT, 0.5 mM PMSF, 4 μ g/ml aprotinin, 10 μ g/ml leupeptin), and incubated on ice for 5 min. The suspension was centrifuged at 13,000 rpm (Eppendorf, F45-24-11) for 5 min, and the supernatant was removed. The nuclei pellet was resuspended and lysed in 40 μ l of ice-cold solution C (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 4 μ g/ml aprotinin, 10 μ g/ml leupeptin) by gentle shaking for 20 min at 4 $^{\circ}$ C. The nuclei lysate was centrifuged at 13,000 rpm (Eppendorf, F45-24-11) for 5 min and the supernatant was transferred to clean tubes. Nuclear extract was combined with the same volume of ice-cold solution D (20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20 % glycerol, 1 mM DTT, 0.5 mM PMSF, 4 μ g/ml aprotinin, 10 μ g/ml leupeptin) and kept at -80° C.

2.5. Electrophoretic Mobility Shift Assay (EMSA) with AP-1 Probe

AP-1 binding was measured by EMSA with 5'- 32 P-end-labeled double-stranded oligonucleotides. The oligomer for the AP-1 consensus sequence, 5'-CGCTGATGACTCAGCCGGAA-3' was obtained from a commercial source (Santa Cruz Biotechnology). AP-1 probe was end-labeled with T4 polynucleotide kinase (New England Biolabs). Binding reactions were performed in 25 μ l final volume containing 10 μ g of nuclear protein, binding buffer (10 mM Tris, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4 % glycerol, 1 μ g poly dI-dC, 60 μ g/ μ l BSA), and 32 P-end-labeled AP-1 probe (50,000 cpm/reaction). For competition studies, the binding reaction was done with 25-fold molar excess cold AP-1 probe. After incubation at 4 $^{\circ}$ C for 15 min and then at room temperature for 30 min, 2 μ l of bromophenol blue/glycerol (0.1 % / 70 %) solution was added to each reaction and the mixture was loaded onto 6 % polyacrylamide gels and electrophoresed at 100 V for 2 h. Densitometry was performed on the autoradiographs using ImageQuant software (Molecular Dynamics). Each experiment was repeated at least three times.

2.6. Western Blotting

HepG2 cells were grown in 6-well plates and treated with 10 and 30 μ M endosulfan for 4 and 24 hours. Hydrogen peroxide was used as a positive control because it is well known that exposure to hydrogen peroxide leads to activation of the MAPKs [reviewed in (Torres and Forman, 2003)]. Cell lysates were prepared in 1 $\times$ SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2 % (w/v) SDS, 10 % glycerol, 50 mM DTT, and 0.1 % (w/v) bromophenol blue. Cell lysates were sonicated for 10–15 sec to shear DNA and reduce sample viscosity, and then heated at 95–100 $^{\circ}$ C for 5 min. After cooling on ice, cell lysates were spun down for 5 min at 14,000 rpm (Eppendorf, F45-24-11). Protein samples were separated on SDS-polyacrylamide gel and electrotransferred to Immobilon-P transfer membrane (Millipore). Immunoblotting was performed using monoclonal or polyclonal antibodies against nonphosphorylated MAPKs and phosphorylated MAPKs, phospho-p44/42 MAPK (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), and phospho-SAPK/JNK (Thr183/Tyr185) (Cell Signaling Technology), and HPR-conjugated secondary antibodies (Amersham). For c-Jun, two antibodies specific to phosphorylated c-Jun at serine 63 and 73 respectively were used (Cell Signaling Technology). Each antibody specific to

each phosphorylated and nonphosphorylated MAPKs do not cross-react with other MAPK family members. To control for loading differences between samples, the blots were probed with an anti-mouse polyclonal antibody for β -actin (Abcam) or anti-rabbit monoclonal antibody for GAPDH (Cell Signaling Technology). The blots were developed using LumiGLO (Cell Signaling Technology) or ECL (Amersham) and exposed to x-ray film. Densitometry was performed on the radiographs using Scion Image software (Scion Corporation). The data are expressed as fold-induction of treated samples over that of the vehicle control. Each experiment was repeated at least three times.

2.7. Immunoprecipitation and Kinase activity assay (radioactive)

For radioactive kinase activity assays, immunoprecipitation and kinase reactions were performed using commercial nonradioactive kinase assay kits (Cell Signaling Technology). Manufacturer's instructions were followed for all procedures except the kinase reaction. Briefly, after exposure to endosulfan at 30 μ M for 4 and 24 hours, cells were lysed in 1 $\times$ lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM β -Glycerolphosphate, 1 mM Na_3VO_4 , 1 μ g/ml Leupeptin, 1 mM PMSF). After 4-time sonication for 5 seconds each, cell lysates were centrifuged for 10 min at 14,000 rpm (Eppendorf, F45-24-11) and supernatants were transferred into new tubes. The kinase of interest was then immunoprecipitated using fusion protein beads overnight. For JNK, N-terminal c-Jun (1–89) fusion protein bound to glutathione sepharose beads was used to selectively “pull down” JNK from cell lysates. For ERK kinase, immobilized phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibody crosslinked to agarose hydrazide beads was used to selectively immunoprecipitate active ERK from cell lysates. A monoclonal phospho-specific antibody to p38 MAP Kinase (Thr180/Tyr182) was used to selectively immunoprecipitate active p38 from the cell lysates. After immunoprecipitation, beads were washed with 1 $\times$ lysis buffer twice and then with 1 $\times$ kinase buffer (25 mM Tris, pH 7.5, 5 mM β -glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4 , 10 mM MgCl_2) twice. Kinase reaction was done with 250 μ g of protein in 50 μ l 1 $\times$ kinase buffer, 10 μ Ci of [γ - 32 P]-ATP and 25 μ M of cold ATP. Reaction mixtures were incubated at 30 $^{\circ}\text{C}$ for 45 min with gentle rotation. For ERK, kinase buffer was supplemented with 2 μ g Elk-1 fusion protein per reaction and for p38, 2 μ g ATF-2 fusion protein. The kinase reaction was terminated with 25 μ l of 3 $\times$ SDS sample buffer (187.5 mM Tris-HCl pH 6.8, 6 % w/v SDS, 30 % glycerol, 150 mM DTT, 0.3 % w/v bromophenol blue). Samples were boiled at 95–100 $^{\circ}\text{C}$ for 5 min. After centrifugation of samples at 14,000 rpm (Eppendorf, F45-24-11) for 2 min, 30 μ l of supernatant was loaded on a 10 or 12 % SDS-PAGE gel. Gels were dried and autoradiographs prepared with both PhosphorImager and X-ray films. For autoradiographs from PhosphorImager, densitometry was performed using ImageQuant software (Molecular Dynamics). The data are expressed as fold-induction of treated samples over that of vehicle controls. Each experiment was repeated at least three times.

2.8. Immunoprecipitation and Kinase activity assay (nonradioactive)

Immunoprecipitation and kinase activity assays for JNK are performed using commercial nonradioactive kinase assay kits according to the manufacturer's instructions (Cell Signaling Technology). All procedures were the same as those for radioactive kinase assays except the kinase reaction step. The kinase reaction was performed with 250 μ g of protein in 50 μ l 1 $\times$ kinase buffer supplemented with 100 μ M ATP for 45 min at 30 $^{\circ}\text{C}$ with gentle rotation. The kinase reaction was terminated with 25 μ l of 3 $\times$ SDS sample buffer (187.5 mM Tris-HCl pH 6.8, 6 % w/v SDS, 30 % glycerol, 150 mM DTT, 0.3 % w/v bromophenol blue). Samples were boiled at 95–100 $^{\circ}\text{C}$ for 5 min. After centrifugation of samples at 14,000 rpm (Eppendorf, F45-24-11) for 2 min, 30 μ l of supernatant was loaded on a 12 or 15 % SDS-PAGE gel. Separated protein samples were electrotransferred to Immobilon-P transfer

membrane (Millipore). Immunoblotting was performed using polyclonal antibodies against phosphorylated c-Jun at Ser 63 (Cell Signaling Technology), and HRP-conjugated secondary antibodies (Amersham). That is, JNK activity was measured by quantitation of JNK-induced phosphorylation of c-Jun at Ser 63. The blots were developed using LumiGLO (Cell Signaling Technology) or ECL (Amersham) and exposed to x-ray film. Densitometry was performed on the radiographs using Scion Image software (Scion Corporation). The data are expressed as fold-induction of treated samples over that of the vehicle controls. Each experiment was repeated at least three times.

2.9. Statistical Analysis

All statistical analyses were performed using StatView software (SAS Institute Inc., Cary, NC). The results are presented as the mean $\pm$ SEM of three separate experiments. The significance of mean differences was detected by analysis of variance (ANOVA) followed by Fisher's Protected Least Squares Differences *post hoc* test for individual comparisons. The criterion for statistical significance was set at $p < 0.05$.

3. Results

3.1. Cytotoxicity of Endosulfan

For cytotoxicity studies, HepG2 cells were exposed to serial concentrations of endosulfan between 1 μ M and 100 μ M for 24 h. Cytotoxicity increased sigmoidally in a dose-dependent manner and reached 98–99 % at 100 μ M endosulfan. The LD50 was determined to be 40 μ M (Fig. 1). Based on the cytotoxicity results, endosulfan concentrations were chosen at 20 and 40 μ M for CAT assays, and 10 and 30 μ M for other experiments.

3.2. Effect of Endosulfan on ARE-mediated Transcription

Transient transfection analyses were performed with rat NQO1 - based CAT reporter plasmids, pQR-ARE and pM1, to investigate the change in transcriptional response of HepG2 cells after 24-h exposure to endosulfan. Endosulfan significantly increased CAT activity 1.21-fold over that of the vehicle control at 40 μ M and 1.17-fold at 20 μ M ($p < 0.0001$ and $p=0.0001$, respectively) (Fig. 2). These results suggest that endosulfan can affect ARE-mediated transcription through an oxidative stress response mechanism.

We also performed the CAT assay with BSO to examine if depletion of glutathione (GSH) would affect the level of ARE-mediated transcription induced by endosulfan. The depletion of GSH by pretreatment with BSO significantly enhanced endosulfan-inducible transcriptional activation ($p < 0.0001$ for treatments with and without BSO), and increased CAT activity 1.93-fold at 40 μ M and 1.77-fold at 20 μ M over that of the vehicle control, which were 1.6-fold and 1.52-fold increase over those of the samples treated with 40 μ M and 20 μ M endosulfan, respectively. Treatment of BSO alone also increased CAT activity 1.58-fold over that of untreated sample in ARE-mediated transcription ($p < 0.0001$). Our results also showed that BSO could enhance endosulfan-inducible activation of ARE-mediated transcription; however, the effect of glutathione depletion was additive to that of endosulfan rather than synergistic. These results suggest that coexposure with toxicants that lower cells' capacity to respond to oxidative stress could enhance endosulfan-mediated stress.

3.3. Effect of Endosulfan on AP-1 Binding

EMSA's were performed with a probe containing the AP-1 consensus binding sequence. Sequence specificity of nuclear protein binding to AP-1 was confirmed by competition studies with 25-fold molar excess cold AP-1 probe (data not shown). Endosulfan treatment

(30 μ M) resulted in a significant increase in the level of AP-1 binding over that of vehicle control (Et-OH) by 1.21-fold ($p = 0.0388$) (Fig. 3).

3.4. Effect of Endosulfan on c-Jun Phosphorylation

The results from the reporter gene experiment and AP-1 binding assay indicated that endosulfan affected ARE-mediated transcription. The level of AP-1 is known to be regulated posttranscriptionally by the level of c-Jun phosphorylation. Thus, we quantitated c-Jun phosphorylation on both ser73 and ser63 following 24-h exposure to endosulfan (Fig. 4). At 30 μ M endosulfan, c-Jun phosphorylation significantly increased at both ser73 and ser63: by 5.50-fold on ser73 ($p = 0.0258$) and by 2.90-fold on ser63 ($p = 0.0258$) over those of vehicle control. At 10 μ M endosulfan, c-Jun phosphorylation did not change on ser73 and increased by 1.96-fold on ser63, which was not statistically significant. We also examined c-Jun phosphorylation on ser63 after 4-h exposure to endosulfan (Fig. 10). At 30 μ M endosulfan, c-Jun phosphorylation significantly increased by 2.78-fold ($p = 0.019$). At 10 μ M, endosulfan increased c-Jun phosphorylation by 1.54-fold, which was not statistically significant (Fig. 12D). Our data demonstrated that exposure to 30 μ M endosulfan significantly increased c-Jun phosphorylation after both 4 and 24 h.

3.5. Effect of Endosulfan on MAPK Phosphorylation

To determine the effect of endosulfan exposure on MAPK activation, we investigated the changes in the levels of phosphorylation of MAPKs after 4- and 24-h exposure to endosulfan. After 24-h exposure, endosulfan increased phosphorylation by 1.81-fold for JNK2 and by 1.93-fold for JNK1 over those of vehicle control at 30 μ M (Fig. 5). At 10 μ M, endosulfan resulted in 1.85-fold increase for JNK2 and 1.34-fold increase for JNK1 phosphorylation. However, none of these changes were statistically significant. After 4-h exposure to 10 μ M endosulfan, the levels of JNK1 and JNK2 phosphorylation were similar to those of vehicle controls. At 30 μ M, endosulfan resulted in 1.27-fold increase for JNK2 and 2.07-fold increase for JNK1 phosphorylation, which were not statistically significant (Figs. 11 and 12A). Thus, our data revealed that exposure to endosulfan did not cause significant changes in the levels of JNK1 and JNK2 phosphorylation.

Exposure to 30 μ M endosulfan resulted in significant increases in the levels of ERK1 and ERK2 phosphorylation (Fig. 6) after 24 h: 7.88-fold increase ($p = 0.0017$) in ERK1 (p44) and 2.27-fold increase ($p = 0.0225$) in ERK2 (p42) phosphorylation. At 10 μ M endosulfan, ERK1 phosphorylation increased by 2.87-fold, and ERK2 phosphorylation by 1.53-fold, which were not statistically significant. After 4 h, there was a significant 2.36-fold increase in ERK2 phosphorylation by 30 μ M endosulfan ($p = 0.023$). For ERK1 phosphorylation, endosulfan caused non-significant increases: 1.44-fold at 10 μ M and 2.84-fold at 30 μ M (Figs. 11 and 12B). Thus, our data showed that 30 μ M endosulfan resulted in significant increases in ERK2 phosphorylation after both 4- and 24-h exposure and in ERK1 phosphorylation only after 24 h.

For p38, 24-h exposure to endosulfan significantly increased p38 phosphorylation by 2.54-fold at 30 μ M ($p = 0.024$) over that of vehicle control, and by 1.85-fold at 10 μ M, which was not significant (Fig. 7). After 4-h exposure, endosulfan caused a significant increase in p38 phosphorylation by 1.51-fold ($p = 0.0038$) at 30 μ M. At 10 μ M endosulfan, the level of p38 phosphorylation was similar to that of vehicle control. Put them together, 30 μ M endosulfan resulted in significant increases in p38 phosphorylation after both 4- and 24-h exposure.

Our results demonstrated that, while endosulfan had the potential to change the levels of phosphorylation of all three MAPKs, significant increases were observed only in ERK1/2 and p38 phosphorylation, to a greater extent with ERK1/2.

We also probed the blots with each antibody for nonphosphorylated MAPKs. Our results demonstrated that the activation of JNKs, ERKs and p38 resulted from the phosphorylation of pre-existing MAPKs rather than *de novo* protein synthesis because JNK, ERK and p38 did not show any significant dose-dependent increases or decreases in the amount of each nonphosphorylated form (Figs. 5–7 and 11). Yu and colleagues also showed that induction of ERK2 activity resulted from the phosphorylation of pre-existing ERK2 (Yu et al., 1999).

3.6. Effect of Endosulfan on MAPK Activity

The effect of endosulfan exposure on MAPK activities in HepG2 cells was examined following 4- and 24-h exposure to 30 μ M endosulfan through radioactive kinase activity assay. Endosulfan exposure did not cause a significant change in the activity of JNK1/2 (data not shown). Endosulfan (30 μ M) significantly increased the enzymatic activity of ERK by 2.69-fold after 4-h exposure ($p = 0.0126$), and by 1.99-fold after 24-h exposure (Fig. 8). Endosulfan also significantly increased p38 activity only after 4-h exposure by 1.90-fold ($p = 0.0061$) (Fig. 9). After 24 h, p38 activity was similar to that of vehicle control. These results demonstrated that endosulfan could significantly increase ERK and p38 activities after the shorter exposure time such as 4 h rather than 24 h.

We also performed nonradioactive kinase activity assay for JNK. Our results from the nonradioactive kinase enzymatic activity assays were consistent with those of radioactive one, and showed endosulfan was not capable of increasing JNK activity significantly (data not shown).

The present kinase activity assay results for ERK and p38 agreed with those of the phosphorylation assays for these MAPKs after 4-h exposure to 30 μ M endosulfan. These results supported that ERK and p38 are the primary target MAPKs of endosulfan.

After 24-h exposure to 30 μ M endosulfan, the enzymatic activity of p38 decreased to the level of the vehicle control, while ERK activity was still higher than that of vehicle control by 1.99-fold even though it was not statistically significant. However, the levels of phosphorylation of ERK1/2 and p38 were significantly higher than those of vehicle control after 24-h exposure to 30 μ M endosulfan. Furthermore, c-Jun phosphorylation significantly increased at both ser73 and ser63 after 24-h exposure to 30 μ M endosulfan. This implied that ERK1/2 and p38 phosphorylation have been sustained up to 24 h and these MAPKs were still active enough to phosphorylate c-Jun significantly.

4. Discussion

Endosulfan is one of the most commonly used pesticides in agriculture and has been ubiquitously detected in food chains and environmental matrices (Ceron et al., 1995; Desalegn et al., 2011; Sethunathan et al., 2002; Turner et al., 1997). There have been a lot of reports and case studies on endosulfan toxicity (Hack et al., 1995; Karatas et al., 2006; Sutherland et al., 2004; Yavuz et al., 2007). However, the molecular mechanism of endosulfan toxicity still remains unclear.

Several studies have suggested the involvement of endosulfan-induced oxidative stress in its toxicity (Kannan et al., 2000; Kannan and Jain, 2003; Sohn et al., 2004). In addition, endosulfan can deplete glutathione (GSH), and increase lipid peroxidation in the liver, kidney and brain in rats (Agarwal et al., 1978; Hincal et al., 1995; Singh and Pandey, 1989; Zeiger et al., 2001).

It is well known that oxidative stress can increase mRNA and protein levels of the proto-oncogenes, c-fos and c-Jun, two components of activating protein-1 (AP-1) (Dalton et al.,

1999), and enhance AP-1 binding (Dalton et al., 1999; Tharappel et al., 2002; Turpaev, 2002). The antioxidant response element (ARE) is a *cis*-acting DNA response element originally characterized in the promoters of the genes coding for rat and mouse glutathione S-transferase Ya subunits (GST-Ya) (Rushmore et al., 1991). AREs are present in the promoters of genes that detoxify carcinogens (namely genes encoding phase II detoxifying enzymes) (Dalton et al., 1999). AREs mediate the expression and coordinated induction of the NQO1 (Favreau, 1991) and GST Ya subunit genes in response to antioxidants, xenobiotics, oxidants, and hydrogen peroxide (Dalton et al., 1999; Jeyapaul and Jaiswal, 2000; Rushmore et al., 1991). The core sequence of ARE (GTGACnnnGC) is similar to the AP-1 binding site (TGACTCA). Therefore, it has been suggested that AP-1 might be the activator of ARE (Bergelson et al., 1994; Jaiswal, 1994) and a variety of inducers that act through ARE have also been shown to induce AP-1 activity (Dalton et al., 1999).

Because no studies on endosulfan in relation to AP-1 activation have been reported, we investigated the ability of endosulfan to modulate AP-1 binding activity using EMSA's with a probe that contained the AP-1 consensus binding sequence. Our results showed that endosulfan exposure significantly increased the level of AP-1 binding over that of vehicle control. We also confirmed the sequence specificity of AP-1 binding by competition studies.

We performed a reporter gene assay with pretreatment of BSO to investigate if depletion of glutathione (GSH) would affect the level of ARE-mediated transcription induced by endosulfan. GSH is a central player in a series of critical cell functions including antioxidant defense, detoxification of electrophiles by conjugation, maintenance of thiol-disulfide status and modulation of redox-sensitive signal transduction (Meister, 1991; Otto et al., 1999; Sen and Packer, 1996). Tissue GSH metabolism can be manipulated by the use of BSO, a specific inhibitor of glutathione biosynthesis (Meister, 1991). The present study showed that endosulfan significantly increased ARE-mediated transcription after pretreatment with BSO. However, the effect of BSO on endosulfan-induced ARE-mediated transcriptional activation was additive to that of endosulfan rather than synergistic. All together, our data suggest that endosulfan affects ARE-mediated transcription through an oxidative stress responsive mechanism and BSO enhances endosulfan-induced activation of ARE-mediated transcription by GSH depletion.

The AP-1 family of transcription factors consists of homodimers and heterodimers of Jun, Fos or ATF2 bZIP proteins that bind to a common DNA site, the AP-1 binding site. In addition to regulation by heterodimerization between Jun, Fos and ATF proteins, AP-1 activity is regulated through interactions with specific protein kinases and a variety of transcriptional coactivators (Karin et al., 1997). The role of MAPKs in regulation of AP-1 activity has been investigated and it has been reported that several different types of MAPKs, ERKs, JNKs, FRK, and p38 contribute to induction of AP-1 activity in response to a diverse array of extracellular stimuli (Karin, 1995; Karin et al., 1997; Shaulian and Karin, 2002). MAPKs contribute to induction of AP-1 activity by upregulation of c-Jun synthesis as well as the increase of c-Jun phosphorylation as a posttranscriptional regulation. Phosphorylation of c-Jun stimulates its ability to activate transcription (Karin, 1995). The activated JNKs phosphorylate c-Jun on ser73 and ser63 in the amino terminal A1 transactivation domain, increasing the stability of c-Jun (Karin et al., 1997) and potentiating c-Jun's ability to activate transcription as either a homodimer or a heterodimer with the other transcription factors (Pulverer et al., 1991; Smeal et al., 1992; Smeal et al., 1991). The present study showed that endosulfan significantly increased c-Jun phosphorylation on both ser73 and ser63 residues following 24-h exposure to 30 μ M (Figs. 4 A and B). After 4-h exposure to 30 μ M endosulfan, we checked c-Jun phosphorylation on ser63 only, and it was significantly increased ($p = 0.019$) (Figs. 10 and 12D). JNK has been known to be the most efficient protein kinase to phosphorylate the N-terminal sites of c-Jun (Karin, 1995).

However, a recent study suggested that additional cell-type-specific factors could determine which MAPK module linked to c-Jun (Schaeffer and Weber, 1999), and there have been accumulating studies to demonstrate that c-Jun can also be activated by other MAPKs, ERK1/2 and p38. ERKs activated c-Jun during PC12 cell differentiation (Leppa et al., 1998), in oncogenic transformation via epidermal growth factor-mediated activation (Schnidar et al., 2009), and during iron chelator-induced IL-8 generation in human epithelial cells (Choi et al., 2007). c-Jun can also act as an effector of both the JNK and ERK pathways during development of *Drosophila* (Kockel et al., 1997; Peverali et al., 1996). Organochlorines induced the collagenase promoter via sequential activation of the p38 kinase cascade and AP-1(c-jun/c-fos) (Frigo et al., 2003), and mulberry anthocyanin induced apoptosis through an activation of the p38/c-jun pathway (Huang et al., 2011). Therefore, c-Jun is a common downstream effector of JNK, ERK and p38 (Frigo et al., 2003; Junttila et al., 2008; Ravingerova et al., 2003; Waskiewicz and Cooper, 1995).

Recently, there have been several studies on the ability of endosulfan to modulate the activities of stress-responsive signal transduction pathways. Endosulfan activated the ERK1/2 pathway in the pituitary tumor cell line GH₃/B6/F10 (Bulayeva and Watson, 2004), in MCF-7 cells (Li et al., 2006), and in human HaCaT keratinocytes (Antherieu et al., 2007; Ledirac et al., 2005). On the contrary, Kang et al. (2001) reported endosulfan decreased ERK2 activity in neural stem cells through the inhibition of gap junctional intercellular communication (GJIC) (Kang et al., 2001). Recent studies on MAPK pathways indicate that in mammalian systems physiologic responses associated with a certain MAPK module can be cell-type specific and dependent on the timing of kinase activation (Schaeffer and Weber, 1999). This might be an explanation for the discrepancy between the results of Kang et al. (2001) and other studies including the present study.

Considering that the liver is the main organ involved in the metabolism and toxicity of xenobiotics and previous studies on the activation of MAPKs by endosulfan have used *in vitro* model systems other than hepatocytes, we used HepG2 cells to determine whether and which MAPKs pathways are related to endosulfan toxicity in human hepatocytes. The HepG2 cell line has been well characterized and known to retain many cellular functions found in normal hepatocytes (Roe et al., 1993). It has been routinely used in toxicological studies involving biotransformation, since the major biotransformation reactions are intact (Dehn et al., 2005).

We investigated the changes in the levels of phosphorylation of MAPKs in HepG2 cells after 4- and 24 h-exposure to endosulfan. MAPKs are activated through dual phosphorylation at conserved threonine and tyrosine residues in the activation domain (Cobb and Goldsmith, 1995; Su and Karin, 1996; Torres and Forman, 2003). We found that endosulfan significantly increased ERK1 phosphorylation after 24-h exposure and ERK2 phosphorylation following both 4- and 24-h exposure to 30 μ M (Figs. 6A-B, 11, and 12B). The present results are consistent with the previous studies that showed the induced phosphorylation of ERK1/2 following 1-h exposure to 25 μ M endosulfan (Ledirac et al., 2005), and the sustained phosphorylation of ERK1/2 up to 6 h by 25 μ M endosulfan (Antherieu et al., 2007) in HaCaT cells. Endosulfan also significantly increased p38 phosphorylation after both 4- and 24-h treatment at 30 μ M, to a lesser extent than that of ERK1/2 phosphorylation (Figs. 7A-B, 11 and 12C). Endosulfan slightly increased JNK phosphorylation, although not statistically significantly (Figs. 5A-B, 11 and 12B). In addition, our radioactive and nonradioactive kinase activity assays demonstrated that endosulfan significantly activated ERK1/2 and p38 after 4-h (Figs. 8–9). Endosulfan was capable of increasing JNK activity slightly but not significantly. Our kinase activity assay results were consistent with the results of MAPK phosphorylation assays after 4-h exposure to 30 μ M endosulfan. All together, our findings clearly indicate that MAPKs were

implicated in endosulfan toxicity and ERK and p38 were major protein kinases to phosphorylate c-Jun in response to endosulfan exposure in HepG2 cells.

5. Conclusions

In summary, our results suggest a model where endosulfan increases the ERK and p38 activities and activated ERK and p38 in turn increase c-Jun phosphorylation. Phosphorylated c-Jun upregulates AP-1 activity, which results in activation of ARE-mediated transcription. This was the first study to demonstrate that endosulfan significantly increased oxidative-stress-responsive transcription via AP-1 activation in HepG2 cells.

Highlights

- Endosulfan increased ERK1/2 and p38 activities in HepG2 cells.
- Endosulfan-induced increase in c-Jun phosphorylation upregulated AP-1 activity.
- Endosulfan activated ARE-mediated transcription.

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Fig. 1.

Cytotoxicity of endosulfan on HepG2 cells. Cells were treated with various concentrations of endosulfan (1 ~ 100 μM) and grown for 24 h. Each treatment was performed in triplicate. To determine cell viability, the cells were incubated with 200 μM of neutral red solution (40 $\mu\text{g/ml}$) in complete MEM. Optical density (OD) was measured at 540 nm. Ethanol was used as a vehicle control at $\leq 0.5\%$ final concentration. The data represent the mean $\pm$ SEM of three separate experiments.

Fig. 2.

Effect of endosulfan on ARE-mediated transcription in HepG2 cells. Cells were transfected with rat NQO1 - based CAT reporter plasmids: pQR-ARE (QR, closed bars) or pM1 (M1, open bars). BSO (500 μ M) was added after transfection and ~16 h prior to endosulfan treatment. Hydrogen peroxide (HP) was used as the positive control. After 24-h exposure to endosulfan, CAT concentrations were determined and then normalized to the level of β -galactosidase activity. All of the assays were performed in triplicate and each CAT activity was normalized against β -galactosidase activity. The data represent the mean $\pm$ SEM of three separate experiments. Asterisks (*) indicate a significant change from vehicle control (Et-OH) and double asterisks (**) from untreated control ($p < 0.05$). (a: untreated control for hydrogen peroxide and BSO only treatments; b: vehicle control for endosulfan treatments; c: positive control)

Fig. 3.

Effect of endosulfan on AP-1 binding. AP-1 binding was measured by EMSA with 5'-³²P-end-labeled double-stranded oligonucleotides after 24-h exposure to endosulfan (Endo). AP-1 probe was end-labeled with T4 polynucleotide kinase. For competition studies, a binding reaction was done with 25-fold molar excess cold AP-1 probe. The upper panel shows a representative image of multiple experiments. The bar graph shows the results of the densitometric analyses of multiple experiments. Each experiment was repeated at least three times. Hydrogen peroxide (HP) was used as the positive control and ethanol (Et-OH) as the vehicle. The data represent the mean $\pm$ SEM of three independent experiments. Asterisks (*) indicate a significant change from vehicle control ($p < 0.05$).

(B) **Fig. 4.**

Effect of endosulfan on c-Jun phosphorylation after 24 h. HepG2 cells were treated with 10 and 30 μ M endosulfan for 24 hours. (A) Western immunoblotting was performed using polyclonal antibodies against phosphorylated and nonphosphorylated c-Jun. For phospho-c-Jun, two antibodies specific to phospho-c-Jun at serine 63 and 73 respectively were used. The levels of phospho-c-Jun were normalized to the level of β -actin. A representative example of the three experiments is presented. Hydrogen peroxide (HP) was used as the positive control and ethanol (Et-OH) as the vehicle. (B) Results of the densitometric analyses of multiple experiments. The data represent the mean $\pm$ SEM of three independent experiments. Asterisks (*) indicate a significant change from vehicle control ($p < 0.05$). (HP: hydrogen peroxide; Et-OH: ethanol; Endo: endosulfan)

(A)

(B) **Fig. 5.**

Effect of endosulfan on JNK phosphorylation after 24 h. HepG2 cells were treated with 10 and 30 μ M endosulfan for 24 hours. (A) Western immunoblotting was performed using polyclonal antibodies against phosphorylated and nonphosphorylated JNKs. The levels of phospho-JNK were normalized to the level of β -actin. A representative result of three experiments is presented. Hydrogen peroxide (HP) was used as the positive control and ethanol (Et-OH) as the vehicle. (B) Results of the densitometric analyses of multiple experiments. The data represent the mean $\pm$ SEM of three independent experiments. (HP: hydrogen peroxide; Et-OH: ethanol; Endo: endosulfan)

(B) **Fig. 6.**

Effect of endosulfan on ERK phosphorylation after 24 h. HepG2 cells were treated with 10 and 30 μ M endosulfan for 24 hours. (A) Western immunoblotting was performed using polyclonal antibodies against phosphorylated and nonphosphorylated ERKs. The levels of phospho-ERK were normalized to the level of β -actin. This figure is a representative of three experiments. Hydrogen peroxide (HP) was used as the positive control and ethanol (Et-OH) as the vehicle. (B) Results of the densitometric analyses of multiple experiments. The data represent the mean $\pm$ SEM of three independent experiments. Asterisks (*) indicate a significant change from vehicle control ($p < 0.05$). (HP: hydrogen peroxide; Et-OH: ethanol; Endo: endosulfan)

(B) **Fig. 7.**

Effect of endosulfan on p38 phosphorylation after 24 h. HepG2 cells were treated with 10 and 30 μ M endosulfan for 24 hours. (A) Western immunoblotting was performed using polyclonal antibodies against phosphorylated and nonphosphorylated p38. The levels of phospho-p38 were normalized to the level of β -actin. This figure is a representative of three experiments. Hydrogen peroxide (HP) was used as the positive control and ethanol (Et-OH) was vehicle. (B) Bar graph shows the result of the densitometric analyses of the multiple experiments. The data represent the mean $\pm$ SEM of three independent experiments. Asterisk (*) indicates a significant change from vehicle control ($p < 0.05$). (HP: hydrogen peroxide; Et-OH: ethanol; Endo: endosulfan)

Fig. 8.

Effect of endosulfan on ERK enzymatic activity. HepG2 cells were treated with 30 μ M endosulfan for 4 and 24 hours. To “pull down” active ERK from cell lysates, immobilized phospho-p44/42 MAPK (Thr202/Tyr204) monoclonal antibody crosslinked to agarose hydrazide beads was used. The kinase reactions were performed using Elk-1 fusion protein as the substrate (2 μ g per reaction). This figure is a representative of three independent experiments. The bar graph shows the result of the densitometric analyses of multiple experiments. The data represent the mean $\pm$ SEM of three independent experiments. Hydrogen peroxide (HP) was used as the positive control and ethanol (Et-OH) as the vehicle. Asterisks (*) indicate a significant change from vehicle control ($p < 0.05$).

Fig. 9.

Effect of endosulfan on p38 enzymatic activity. HepG2 cells were treated with 30 μ M endosulfan for 4 and 24 hours. To “pull down” active p38 from cell lysates, monoclonal phospho-specific antibody to p38 MAP Kinase (Thr180/Tyr182) crosslinked to agarose hydrazide beads was used. The kinase reactions were performed using ATF-2 fusion protein as the substrate (2 μ g per reaction). This figure is a representative of three independent experiments. The data represent the mean $\pm$ SEM of three independent experiments. Hydrogen peroxide (HP) was used as the positive control and ethanol (Et-OH) as the vehicle. Asterisks (*) indicate a significant change from vehicle control ($p < 0.05$).

Fig. 10.

Effect of endosulfan on c-Jun phosphorylation after 4 h. HepG2 cells were treated with 10 and 30 μM endosulfan for 4 hours. Western immunoblotting was performed using polyclonal antibodies against phosphorylated and nonphosphorylated c-Jun. For phospho-c-Jun, antibody specific to phospho-c-Jun at serine 63 was used. The level of phospho-c-Jun was normalized to the level of GAPDH. A representative example of the three independent experiments is presented. Hydrogen peroxide (H₂O₂) was used as the positive control and ethanol (Et-OH) as the vehicle.

Fig. 11.

Effect of endosulfan on MAPKs phosphorylation after 4 h. HepG2 cells were treated with 10 and 30 μM endosulfan for 4 hours. Western immunoblotting was performed using polyclonal antibodies against phosphorylated and nonphosphorylated ERK1/2, JNK1/2 and p38. The levels of phosphorylated MAPKs were normalized to the level of GAPDH. This figure is a representative of three independent experiments. Hydrogen peroxide (H₂O₂) was used as the positive control and ethanol (Et-OH) as the vehicle.

(A)

(B)

(C)

(D)

Fig. 12.

Effect of endosulfan on MAPKs and c-Jun phosphorylation after 4 h. Bar graphs show the results of the densitometric analyses of western immunoblotting for JNK1/2 (A), ERK1/2 (B), p38 (C) and c-Jun (D). The data represent the mean $\pm$ SEM of three independent experiments. Asterisk (*) indicates a significant change from vehicle control ($p < 0.05$) and # indicates a significant change from untreated control ($p < 0.05$). (HP: hydrogen peroxide; Et-OH: ethanol; Endo: endosulfan)