Effects of Organochlorine Insecticides on MAP Kinase Pathways in Human HaCaT Keratinocytes: Key Role of Reactive Oxygen Species

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Organochlorine pesticides (OCs) are reported as potential carcinogens in humans. The aim of this study was to investigate the effects of four OCs (dieldrin, endosulfan, heptachlor, and lindane) on mitogen-activated protein kinase (MAPK) cascades and more specifically to identify the mechanism underlying OC-induced ERK1/2 activation. Organochlorine pesticides increased phosphorylated Raf, MEK1/2, ERK1/2, and c-Jun in human HaCaT cells, but they had no effect on p38 MAPK activation. Moreover, blockade of Raf, MEK1/2, or PKC activation with geldanamycin, U0126, or calphostin C inhibited ERK1/2 phosphorylation, demonstrating a PKC–Raf–MEK1/2 pathway. We also showed that these insecticides induced the production of reactive oxygen species (ROS). Pre-treatment with the antioxidant molecule N-acetyl cysteine sharply decreased the level of phospho-ERK1/2 and had no effect on Raf and MEK1/2 activation, suggesting a Raf-independent mechanism. This study indicates that OCs strongly activate the ERK1/2 pathway, and it identifies a critical role of ROS in OC-induced ERK activation, probably by stabilizing its phosphorylation.

Key Words: ERK1/2; keratinocytes; mitogen-activated protein kinases; organochlorines; reactive oxygen species; signal transduction.

INTRODUCTION

Organochlorine pesticides (OCs) are organic compounds that persist in the environment, bioaccumulate through the food chain, and pose a risk of causing adverse effects to human health and the environment. These pesticides, characterized by their cyclic structure, number of chlorine atoms, and low volatility, can be divided into four groups: dichlorodiphenyl-ethanes (such as DDT), cyclodiene (such as dieldrin, endosulfan, and heptachlor), chlorinated benzenes (such as hexachlorobenzene), and cyclohexanes (such as lindane). Although these chemicals were widely used until the mid-1970s, most of them are now banned from use in developed countries. However, they are still being produced and used in other countries. Furthermore, one of these insecticides, endosulfan, is still in widespread use throughout the world despite its known adverse effect on humans as an endocrine-disrupting compound (Andersen et al., 2002; Scippo et al., 2004). Most OCs are also considered persistent organic pollutants (POPs), a category of chemicals that includes nine OCs (aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, and toxaphen), targeted by the Stockholm Convention in May 2001, which aimed to eliminate their production and restrict or ban their use throughout the world (http://www.pops.int/).

Many human epidemiologic and animal studies have shown that exposure to OCs is positively correlated with endocrine disruption (Lemaire et al., 2004), reproductive and immune dysfunctions (Ayub et al., 2003; Reed et al., 2004; Saiyed et al., 2003), and cancers (e.g., breast cancer [Kalantzi et al., 2004; Zou and Matsumura; 2003]). Human exposure occurs mainly by ingestion (from eating contaminated foods), inhalation, absorption through skin, and often during pest control operations both at home and in resort areas. Therefore, to investigate the toxicological effects of OCs after cutaneous exposure, we used the spontaneously immortalized human keratinocyte cell line, HaCaT (Boukamp et al., 1988). This well-characterized cell line is still able to proliferate and differentiate, and it has been shown to be a good model in toxicology (Delescluse et al., 1998; Ledirac et al., 1997). Moreover, these cells show good predictive results in comparison with keratinocyte and skin models, and they therefore constitute an appropriate model for studying the molecular mechanisms regulating keratinocyte growth and differentiation.

The mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that transduce signals from the plasma membrane to the cell nucleus. They play a critical role in controlling cell survival, proliferation, and differentiation (Chang and Karin, 2001). In epithelial cells, in particular keratinocytes, deregulation of MAPK signaling pathways can lead to hyperproliferation and altered differentiation, which in turn contribute to photoaging, psoriatic epidermis, and
chlor, like endosulfan, induces apoptosis in human lymphocytes. However, more recent studies have demonstrated that heptachlor (Chuang and Chuang, 1998), a finding that led to the suggestion of phosphorylation and by the inhibition of apoptosis (Okoumassoun et al., 2003). Heptachlor has also been shown to increase the amount of phosphorylated ERK1/2 in human lymphocytes (Chuang and Chuang, 1998), a finding that led to the suggestion that heptachlor could be considered a potent human mitogen. However, more recent studies have demonstrated that heptachlor, like endosulfan, induces apoptosis in human lymphocytes (Kannan et al., 2000; Rought et al., 2000). In fact, in contrast with the proliferating effects observed in animals, in human lymphocytes heptachlor provokes alterations of cell cycle progression and induction of programmed cell death (Chuang et al., 1999).

The aim of this study was to gain a better understanding of the cellular events leading to OC-mediated toxicity and more particularly to investigate whether various compounds belonging to the OCs family, such as dieldrin, endosulfan, heptachlor, and lindane (Fig. 1), can activate MAPKs and thus influence important cellular processes. In another respect, OCs such as dieldrin and more recently endosulfan, have been reported to induce reactive oxygen species (ROS) production in human liver. Therefore, the induction of ROS production by these molecules and the involvement of ROS production in MAPK signaling pathways were also investigated.

**MATERIALS AND METHODS**

**Chemicals.** Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, sodium pyruvate, Eagle’s non-essential amino-acids were from BioWhittaker (Cambrex company, Walkersville, MD). Anti-phospho-p38, anti-phospho-c-Jun, anti-phospho-MEK1/2, anti-phospho-Raf, anti-c-Jun, anti-p38, anti-MEK2, and MEK1/2 inhibitor U0126 were purchased from Cell Signaling (Beverly, MA). Anti-ERK2 was from Santa Cruz (Santa Cruz, CA). Anti-phospho ERK1/2 and N-acetyl cysteine (NAC) were from Sigma-Aldrich (St. Louis, MO). Raf inhibitor geldanamycin and PKC inhibitor calphostin C were purchased from Alexis Biochemicals. Organochlorine pesticides were from ChemService (West Chester, PA).

**Cell culture.** HaCaT cells (a generous gift from Prof. N. E. Fuesing, German Cancer Research Institute, Heidelberg, Germany) were derived from a spontaneously immortalized human keratinocyte but are a non-tumorigenic epidermal cell line that exhibits many of the morphological and functional properties of normal human keratinocytes. HaCaT cells were cultured in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mM), and non-essential amino acids (0.1 mM). Cultures were incubated at 37°C in a humidified atmosphere with 95% air and 5% CO2.

**Cell viability.** The cytotoxicity of OCs was evaluated after 24 h of exposure by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay, according to the procedure of Fautrel et al. (1991). Briefly, HaCaT cells were seeded in 96-well plates and grown to confluence. Then cells were treated with various concentrations of the tested pesticides for 24 h. The next day, medium was removed and 100 μL of serum-free DMEM containing MTT (0.5 mg/ml) was added to each well and incubated for 2 h at 37°C. Finally, solutions were removed, the water-insoluble formazan was dissolved in 100 μl dimethyl sulfoxide (DMSO), and absorbance was measured at 550 nm.

**ROS measurement.** Intracellular ROS generation was assessed by using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) from Molecular Probes (Eugene, OR). HaCaT cells were seeded in 12-well plates and grown to confluence. Then cells were treated for 90 min at 37°C with OCs in the presence of 100 μM H2DCFDA (the stock solution was made in ethanol, so that the final concentration in the medium was 0.33%). After the incubation time, cells were washed twice with cold phosphate buffered saline (PBS), and scraped in potassium buffer (10 mM HEPES [pH 7.4] / methanol (v/v)) completed with Triton X-100 (0.1%). An aliquot of 100 μl was incubated in a black 96-well plate, and relative fluorescence intensity was determined by spectrofluorimetry ($\lambda_{ex} \sim 488$ nm, $\lambda_{em} = 520$ nm).

**Western blot analysis.** HaCaT cells were plated in six-well plates and grown to confluence. The confluent cells were serum starved for 24 h to establish quiescence (except for p38 MAPK), and then stimulated with OCs for the indicated periods and at the indicated concentrations. After treatments, cells were scraped and resuspended in buffer A containing the protease inhibitor cocktail (25 mM HEPES [pH 7.5], 5 mM MgCl2, 5 mM EDTA, 5 mM DT T, 2 mM PMSE, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin). The protein concentration in each cell lysate was measured by a commercial method (BCA Protein Assay Kit), using bovine serum albumin (BSA) as the standard. Thirty micrograms of total protein were resolved by 11% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and blotted on polyvinyliden difluoride (PVDF) membranes. Membranes were immunoblotted with anti-phospho-c-Jun (1:1000), anti-phospho-p38 (1:2000), anti-phospho-Raf

**FIG. 1.** Chemical structure of the organochlorine insecticides (OCs) selected.
(1:1000), anti-phospho-MEK1/2 (1:1000), anti-c-Jun (1:1000), anti-p38 (1:1000), or anti-MEK2 (1:1000) antibodies overnight at 4°C, or with anti-ERK2 (1:5000), anti-phospho-ERK1/2 (1:5000) antibodies for 1 h at room temperature. Membranes were then reacted with horseradish peroxidase–conjugated secondary antibodies (anti-mouse or anti-rabbit immunoglobulin G) for 1 h at room temperature. After washing, blots were reacted using an ECL detection kit (Amersham Biosciences, Piscataway, NJ).

**Western blot densitometric quantitation.** Films were scanned and quantitated with the Chemi Genus Bio Imaging System (SynGene, Sunnyvale, CA). The amount of phosphorylated protein detected was quantified, and values from multiple experiments were averaged and graphed. The Y-axis was presented as arbitrary units. Error bars in each of the figures represent the standard deviation of the mean.

**Statistical analysis.** The statistical differences between different treatment groups were determined by Student’s *t*-test, and probability levels were noted as *(* *p* < 0.05) and **(* *p* < 0.001). Data are expressed as means ± standard deviations (SD) for at least three independent determinations for each experimental point.

### RESULTS

**Cytotoxicity**

Cytotoxicity studies of four OCs were performed by using two different endpoints – the MTT reduction and neutral red (data not shown) tests. As shown in Table 1, significant differences were observed between the tested compounds. Indeed, endosulfan and heptachlor exerted a cytotoxic effect with an IC₅₀ ranging from ≈20–40 to 100 µM, depending on the presence or absence of serum in the medium, whereas dieldrin and lindane did not lead to cellular cell death, regardless of concentration and culture conditions. It should be pointed out, however, that because of poor solubility (< 200 µM) of the two latter molecules in the culture medium, their IC₅₀ values could not be determined precisely. Nevertheless these experiments allowed us to optimize experimental concentrations for the induction studies. Figure 2 illustrates the dose-dependent toxicity of the two most toxic insecticides after 24 h of exposure in complete medium, and it shows the absence of any relevant cytotoxicity at 25 µM, according to the cytotoxicity tests used. We therefore used the subtoxic concentration 25 µM to study the early events leading to the toxic response.

**OCs Induce ERK1/2 and JNK but Not p38 MAPK Activation**

To determine the effects of OCs on MAPK cascades, we studied the ability of these molecules to activate the phosphorylation of ERK1/2, c-Jun, and p38 MAPK after 1 h of exposure on HaCaT cells. As shown in Figure 3, OCs activate both ERK1/2 and c-Jun, but they have no induction effect on p38 MAPK phosphorylation, compared with that obtained with sorbitol-induced osmotic stress (Fig. 3, line So). Endosulfan and heptachlor induce a strong activation of ERK1/2, above that produced by FBS, whereas c-Jun is more strongly activated by dieldrin and endosulfan, and to a lesser extent by heptachlor and lindane. As expected, no effect was obtained on the inactive forms of ERK, c-Jun, and p38 MAPK. These results are in agreement with those previously obtained for ERK1/2 activation by 50 µM heptachlor in human lymphocytes (Chuang and Chuang, 1998; Chuang et al., 1999), and they clearly indicate that OCs—endosulfan in particular—are able to strongly activate the ERK1/2 and JNK signaling pathways, and therefore interfere with cellular functions.

**Raf and MEK1/2 Are Required for OC-Induced ERK1/2 Phosphorylation**

Figure 4 shows the dose-dependent increase in ERK1/2 activation by the four OCs. The maximal induction effect was

### TABLE 1

Comparative In Vitro Cytotoxicity of Organochlorine Insecticides in HaCaT Cells

<table>
<thead>
<tr>
<th>Insecticides</th>
<th>Serum +</th>
<th>Serum –</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dieldrin</td>
<td>&gt;200.0</td>
<td>&gt;200.0</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>92.10 ± 2.59</td>
<td>39.60 ± 0.69</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>109.6 ± 8.98</td>
<td>17.4 ± 0.57</td>
</tr>
<tr>
<td>Lindane</td>
<td>&gt;200.0</td>
<td>&gt;200.0</td>
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*In vitro* cytotoxicity of insecticides was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test. Cells were treated for 24 h with various concentrations of insecticides in the presence or absence of serum in the medium. Insecticide solutions were prepared in DMSO so that the final concentration in the medium was 0.25%. IC₅₀ was the concentration that decreased viability to 50%. Values are expressed as mean ± SE from at least three independent experiments.

![FIG. 2. In vitro cytotoxicity of endosulfan and heptachlor. HaCaT cells were incubated for 24 h with different concentrations (0–200 µM) of heptachlor or (0–150 µM) endosulfan, and cytotoxicity was measured by the MTT test. Each point is the mean ± SD of three independent experiments replicated five times.](image-url)
observed for all tested compounds at concentration of 25 µM, with a strong effect of endosulfan at levels as low as 10 µM. The phosphorylation of both MEK1/2 and Raf was also induced by OCs, with a lesser effect of lindane, a less potent activator than other OCs (Fig. 5A). These results suggest the involvement of Raf and MEK1/2 in ERK1/2 activation by OCs, and they clearly indicate that OCs of the cyclodiene group are the more potent activators, a difference that may be explained by their different chemical structures. Experiments with DDT, a dichlorodiphenylethane, have produced similar results to those we obtained with cyclodienes (data not shown), and they have confirmed the existence of a relationship between the chemical structure (polycyclic structures by comparison with that of lindane) and the level of ERK1/2-induced phosphorylation.

To further confirm the upstream mechanism of OC-induced ERK1/2 activation, we analyzed the effects on induced-ERK1/2 phosphorylation of U0126, a specific inhibitor of MEK1/2, and geldanamycin, which destabilizes Raf-1 and disrupts the Raf-MEK-ERK pathway (Schulte et al., 1996). As shown in Figure 5B, U0126 completely blocked ERK1/2 activation. Surprisingly, although geldanamycin completely blocked any activation of Raf phosphorylation by OCs (data not shown), ERK1/2 was still weakly activated by dieldrin, endosulfan, and heptachlor. These results suggest that OCs activate ERK1/2 through distinct pathways including the Raf-MEK signaling cascade and a Raf-independent mechanism.

Involvement of PKC in OC-Induced ERK1/2 Phosphorylation

Protein kinase C (PKC) is involved in many cellular responses and in particular in Raf-1 stimulation. In addition, OCs were previously described as stimulating PKC activity both in vitro and in vivo (Bagchi et al., 1997; Moser and Smart, 1989). Therefore, to examine the role of PKC in OC-induced ERK1/2 activation, experiments were performed with calphostin C, a highly specific inhibitor of PKC (Fig. 5B). Pretreatment of HaCaT cells for 4 h with calphostin C entirely blocked OC-induced ERK1/2 phosphorylation, suggesting a key role of PKC. We also noted that the amounts of phosphorylated Raf-1 were similar to those obtained by treatment with geldanamycin (data not shown), suggesting a direct PKC-MEK1/2 mechanism for the residual ERK1/2 activation observed with geldanamycin. Taken together, these data clearly indicate that OCs can activate ERK1/2 via PKC–Raf-MEK1/2-dependent and PKC–MEK1/2-dependent pathways (Schönwasser et al., 1998). This direct activation of MEK by PKC, such as PKC-ζ, has already been demonstrated for insulin-induced and lipopolysaccharide (LPS)-induced ERK1/2 phosphorylation (Monick et al., 2000; Sajan et al., 1999).
OC-Induced Intracellular ROS Generation

Many studies have shown that oxidative stresses induced JNK and p38 MAPK signaling cascades, and to a lesser extent the ERK1/2 pathway. Although OCs have been described to increase ROS formation, particularly in the liver, few studies have been conducted that involved other human tissue or cell types (Kannan and Jain, 2003). To determine whether OC treatments are associated with changes in intracellular ROS levels in HaCaT cells, we measured oxidation of (H2DCF) diacetate (H2DCFDA). Intracellular esterases convert cell-permeable H2DCFDA to HDCF, which is subsequently oxidized by ROS to fluorescent dichlorodihydro-fluorescein (DCF) (Myhre et al., 2003). Primaquine, a known pro-oxidant compound (Magwere et al., 1997), was chosen as a positive control. Figure 6 illustrates DCF fluorescence in response to two insecticide concentrations (25 and 50 μM), and shows dose-dependent increase in ROS generation with dieldrin, endosulfan, and heptachlor, without dose-dependence with lindane. At 25 μM, all the tested compounds induce a significant increase in DCF fluorescence: a 1.6-fold increase with lindane, a 2-fold increase with dieldrin and endosulfan, and a 2.5-fold increase with heptachlor. Maximum effect is observed at 50 μM heptachlor (3.4-fold over DMSO control).

Effect of Oxidative Stress on ERK and JNK Signaling Pathways

To determine the role of OC-induced ROS generation on both ERK and JNK activation, we studied the effects of the antioxidant molecule N-acetyl cysteine (NAC). To determine the optimal concentration of NAC that inhibits OC-induced oxidative stress, HaCaT cells were pre-treated for 24 h with various concentrations of NAC (1–10 mM). Figure 7 shows a dose-dependent decrease of OC-induced oxidative stress after NAC pre-treatment, and it demonstrates that the oxidative stress induced by lindane and dieldrin was completely blocked with 5 mM of NAC, whereas 10 mM of NAC was necessary to inhibit ROS production induced by endosulfan and heptachlor.

We next examined the effects of NAC on ERK1/2 activation by OCs. HaCaT cells were pre-treated for 24 h with 10 mM NAC, and then stimulated for 1 h with the four insecticides. As indicated in Figure 8A, NAC pre-treatment markedly decreases the OC-induced ERK1/2 activation, whereas the corresponding level of phospho-MEK1/2 remains unchanged. Moreover, NAC pre-treatment has no effect on serum-induced ERK1/2 activation, as expected, because this activation is ROS-independent and has no significant effect on basal ERK phosphorylation. These results indicate clearly that ROS contribute to increase the level of OC-induced ERK1/2 phosphorylation, but they do not lead to activation of the upstream MAPKs. These data...
provide strong support of a crucial role for ROS in OC-induced ERK1/2 phosphorylation, probably via a Raf–MEK-independent mechanism. Oxidative modifications of other molecules, among them, ERK-specific phosphatases, may also contribute to an increase in ERK1/2 phosphorylation level (Levinthal and Defranco, 2005). Results presented in Figure 8B indicate that pre-treatment with NAC partially prevents OC-induced c-Jun phosphorylation, suggesting the possibility of a similar mechanism in OC-induced JNK activation.

**DISCUSSION**

The association between the levels of exposure to organochlorine pesticides and cancers is still a matter for significant debate. Indeed, in addition to their known estrogenic characteristics, OCs have been especially implicated as risk factors for breast cancers. Although OCs such as lindane and DDT have been shown to increase cell proliferation of MCF-7 cells (Steinmetz et al., 1996; Zou and Matsumura, 2003), no mechanism has been clearly established yet, and available data do not support the hypothesis that these chemicals increase the risk of breast cancer. Although the toxicological effects of OCs have been studied extensively in animals, and several studies have revealed that they provoke a vast range of disorders, they remain poorly documented in humans. Dietary contribution is probably the most significant route of human exposure. Maximum levels were found in vegetables, specifically in Egypt; plants like potato tubers or strawberries could contain high levels of lindane residues (850 µg/kg) and dieldrine (220 µg/kg), respectively (Monsour, 2004). Although dietary intake has decreased since 1970, tissue bioaccumulation, which is a common feature of insecticides, should not be ruled out. This could lead to unexpectedly high intracellular OC concentrations at hepatic and epidermal sites. Furthermore, during the application period, OCs may be found in surface water and reservoirs. The general population can also be exposed to OCs during pest control operations, both at home and in recreational areas. Finally, the highest dermal and inhalation exposures documented were reported in farm workers involved in the spraying of these insecticides. The mean dermal exposures to mixers and sprayers of endosulfan via a tractor-mounted boom sprayer and highboy were 16.18 and 8.06 mg/kg/day, respectively (Lonsway et al., 1997). The authors concluded that not using protective measures when spaying endosulfan could lead to poisoning. The present study was therefore designed to assess the toxicological effects of OCs in humans after acute dermal exposure by using the HaCaT cell line, the most widely studied keratinocyte cell line and to try to better understand the effects of OCs on a population chronically exposed by dermal contact.

Because MAPKs are key enzymes in signal transduction, we determined whether OCs had an inducing effect on MAPK cascades, and we provided data supporting the existence of an additional ROS-dependent mechanism in MAPK activation by OCs. In this report we demonstrate that multiple members of the MAPK family are stimulated by OCs and that ERK1/2 is
strongly activated in HaCaT cells. Organochlorine-induced ERK1/2 activation is significantly reduced or completely blocked by inhibitors of MEK1/2 (U0126), Raf-1 (geldanamycin), and PKC (calphostin C). In addition, our data indicate that OCs induce Raf and MEK1/2 phosphorylation, and our findings thus demonstrate that these pesticides induce ERK1/2 phosphorylation via successive activations of PKC, Raf-1, and MEK1/2. Our results are consistent with a previous study that reported ERK1/2 activation by 50 μM heptachlor in human lymphocytes (Chuang and Chuang, 1998); they also clearly demonstrate that insecticides of the organochlorine family induce the same MAPK activation profile in HaCaT cells, namely ERK and JNK, but not p38 MAPK activation.

Furthermore OCs have been shown to induce oxidative stress in certain mammalian species (Bayoumi et al., 2000), and the ROS generation has been described to interfere with various signaling pathways, including MAPKs. Indeed all MAPK cascades are known to be activated in response to oxidant injury (Gupta et al., 1999; Martindale and Holbrook, 2002), and they can therefore have an impact on cell survival and cell death. A recent study has reported that exposure to 10–100 μM endosulfan levels induced apoptosis in human T cells via a bcl-2-independent mechanism and suggested that endosulfan-induced apoptosis may be linked to excessive ROS production (Kannan et al., 2000). However, the mechanisms that mediate MAPK activation by oxidants and the variability of mammalian sensitivity to oxidant injury remain to be understood. Moreover, no data are available on OC-induced oxidative stress in human keratinocytes, or on the impact of this oxidant injury on cell signaling.

Under our experimental conditions, the four OC tested enhanced the production of ROS in a dose-dependent manner. When cells were pre-treated with increasing concentrations of the antioxidant agent NAC, ROS induction was reduced, and the phosphorylation of both ERK1/2 and c-Jun was partially decreased. Interestingly, MEK and Raf phosphorylation was not affected by NAC pre-treatment, suggesting that OCs-induced ERK1/2 phosphorylation is dependent in part on a ROS-dependent mechanism downstream of the MEK1/2 level. Recent studies have demonstrated the role of oxidative stress in the inactivation of phosphatase activity, and have precisely described the mechanism of ROS regulation (Lee and Esselman, 2002; Persson et al., 2004). Indeed, protein tyrosine phosphatases (PTPs) were shown to be reversibly oxidized and inactivated after H2O2 treatment in various cellular systems, upon the oxidation of cysteine thiols. Moreover, inactivation of phosphatase activity by H2O2 has been demonstrated to contribute to MAPK activation, which could explain the ROS-dependent increase of ERK and JNK phosphorylation by OCs. More recently, a study on phosphatase inhibition during oxidative stress in murine neuronal cells has shown that glutamate-induced oxidative stress specifically inhibited the phosphatase activity regulating ERK1/2 (PP2A, MKPs), whereas other phosphatases, such as that regulating JNK, were not affected (Levinthal and Defranco, 2005). In contrast, our results clearly indicate that ROS-dependent events induced by OCs contribute to both ERK and JNK activation.

Therefore, to identify the cellular events leading to ROS-induced MAPK phosphorylation after OCs exposure, further experiments are in progress to investigate whether ERK and JNK-directed phosphatases are inactivated. Nevertheless OC-induced ERK activation is completely blocked in the presence of U0126, indicating that inactivation of ERK phosphatases would not be sufficient by itself to drive the increase in phosphorylated-ERK1/2. Furthermore, the results presented here demonstrate that OC-induced ERK activation requires sequential activation of PKC, Raf, and MEK1/2. Taken together, these findings may contribute to better understanding of the mechanism underlying OC-induced alterations suspected to play a role in carcinogenesis.

In summary, we have shown that OCs activate ERK1/2 and JNK cascades, but have no effect on p38 MAPK. This activation of ERK1/2 by OCs results in Raf and MEK1/2 activation, as well as activation of PKC. The results presented here also reveal that all the pesticides tested stimulate ROS generation in HaCaT cells, and that this increase in ROS is involved to some degree in ERK and JNK activation. However, MEK1/2 phosphorylation is not affected by this oxidative stress. Thus we provide evidence that OC-induced MAPK activation involves both the classical MAPK signaling pathways, such as PKC and Raf-MEK activation process for ERK1/2, and an additional ROS-dependent mechanism (Figure 9) that should be further investigated.

![FIG. 9. Summary of the proposed mechanism for mitogen-activated protein kinase (MAPK) activation by OCs. Exposure of HaCaT cells to OCs results in the activation of ERK1/2 and JNK signaling pathways. In addition, these molecules induce the generation of intracellular ROS, which appears to be critical for the OC-induced MAPK activation. Our study shows that OCs induce MAPK activation via PKC and Raf-MEK activation processes, but it also demonstrates that the oxidative stress induced by these chemicals contributes in part to the increase in ERK1/2 phosphorylation in a MEK-independent and Raf-independent manner.](image-url)
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