Research Article

Exposure to \( p,p' \)-DDE Induces Morphological Changes and Activation of the PKC\( \alpha \)-p38-C/EBP\( \beta \) Pathway in Human Promyelocytic HL-60 Cells

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Received 24 May 2016; Revised 26 August 2016; Accepted 31 August 2016

Academic Editor: Susan A. Rotenberg

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Dichlorodiphenyldichloroethylene (\( p,p' \)-DDE), the most persistent metabolite of dichlorodiphenyltrichloroethane (DDT), is still present in the human population. Both are present in the bone marrow of patients with bone marrow disorders, but thus far there are no studies that assess the capability of \( p,p' \)-DDE to affect myeloid cells. The aim of this study was to determine the effect of \( p,p' \)-DDE on promyelocytic cell differentiation and intracellular pathways related to this event. \( p,p' \)-DDE induced morphological changes compatible with promyelocytic differentiation in a concentration-dependent manner. The \( p,p' \)-DDE effect on [\( \text{Ca}^{2+} \)]\(_i\), C/EBP\( \beta \) protein levels, PKC\( \alpha \) and p38 activation, and the role of oxidative stress or PLA2 was assayed. Exposure to 1.9 \( \mu \)g/mL of \( p,p' \)-DDE increased [\( \text{Ca}^{2+} \)]\(_i\), PKC\( \alpha \), p38, and C/EBP\( \beta \) protein levels; the increase of nuclear C/EBP\( \beta \) protein was dependent on p38. PKC\( \alpha \) phosphorylation was dependent on PLA2 and \( p,p' \)-DDE-induced oxidative stress. p38 phosphorylation induced by \( p,p' \)-DDE was dependent on PLA2, PKC activation, and oxidative stress. These effects of \( p,p' \)-DDE at concentrations found in human bone marrow may induce alterations in immature myeloid cells and could affect their cellular homeostasis. In order to establish the risk from exposure to \( p,p' \)-DDE on the development of bone marrow disorders in humans, these effects deserve further study.

1. Introduction

Dichlorodiphenyltrichloroethane (DDT) is a persistent organic pollutant that was used until the end of the twentieth century as a pesticide and to control vectors of dengue and malaria worldwide [1]. Because of the high persistence of DDT in the environment and its slow elimination from the body, younger generations in several countries are currently still exposed to DDT and its most persistent metabolite, dichlorodiphenyldichloroethylene (\( p,p' \)-DDE) [2–4]. \( p,p' \)-DDE has been found in several human tissues including bone marrow (ranging from 0.46 to 1.6 ppm) [5–7]. Pesticide exposure alters cell differentiation in bone marrow resulting in noncompetent cells or haematological disorders [8–12]. DDT and \( p,p' \)-DDE exposure are associated with a risk of developing bone marrow disorders such as aplastic anaemia, acute myeloid leukaemia (AML), and non-Hodgkin lymphoma [13–19]. Epidemiological data has associated home pesticide exposure during a child’s early years with acute lymphoblastic leukaemia (ALL) and exposure before birth with AML [20]. Although epidemiological studies demonstrate the adverse effects of DDT on haematological or immunological parameters [21–25], currently there are no data available on the association between \( p,p' \)-DDE exposure...
and haematopoietic cell differentiation that could explain either haematological disorders observed in the population or the molecular mechanism underlying DDT toxicity in these cells.

Myeloid differentiation is a process in which committed myeloid progenitors undergo growth arrest and subsequently terminal differentiation into mature granulocytes or monocytes [26]. During myeloid differentiation, cells suffer a characteristic change in their morphology including nuclear restructuring and changes in the size of myeloid precursors [27, 28]. The differentiation of myeloid precursors is accomplished by signalling cascades which are not well understood. CCAAT/enhancer binding protein (C/EBP) is involved in gene expression and regulates differentiation in haematopoietic cells [29]. C/EBP expression is induced in myeloid progenitors when emergency granulopoiesis is required and during monocyte differentiation [30–32]. Its function is regulated by posttranslational modifications including phosphorylation [33]. The C/EBP levels of the cytoplasmic protein (inactive form) and nuclear protein (active form) are controlled by the mitogen-activated protein kinases (MAPK) pathways including extracellular signal-regulated kinases (ERK1/2) (activated by growth factors and survival factors), c-Jun N-terminal kinases (JNK), and p38 (activated by stress stimuli) [34, 35]. It has been shown that protein kinase C (PKC) plays a critical role in MAPK activation [36–38].

The few in vitro experiments that have been performed demonstrate that DDT induces adipogenic and osteogenic differentiation of mesenchymal stem cells [39] and adipocyte differentiation from preadipocyte 3T3-L1 cells and increases the nuclear levels of C/EBP [40]. DDT negatively affects haematoecic cells and their immunocompetence, but its effect on differentiation of immune cells and the mechanisms underlying these effects remain to be defined. Previous studies in nonmyeloid cells and monocytes have shown that p,p'-DDE and/or p,p'-DDE exert different effects on the signalling molecules that participate in myeloid cell differentiation including intracellular calcium levels (Ca2+), reactive oxygen species (ROS) production, activation of MAPK and PKC family, and phospholipase A2 (PLA2) stimulation [41–44]. However, these effects have not yet been evaluated in myelocytic cells.

Considering the impact that DDT exposure could have on haematopoietic differentiation, the goal of this study was to examine the effects of its more persistent metabolite, p,p'-DDE, on differentiation in the promyelocytic HL-60 cell line and to elucidate which molecular mechanisms implicated in myeloid cell differentiation are altered by p,p'-DDE exposure. Specifically, this study evaluated the capability of p,p'-DDE to induce C/EBP activation through MAPK activation as well as the participation of PKC activation and ROS, both of which are involved in myeloid differentiation.

2. Materials and Methods

2.1. Cell Culture. The HL-60 cell line was maintained in RPMI 1640 medium (Sigma-Aldrich Chem. Co., St. Louis, USA) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% L-glutamine (Gibco-BRL, Grand Island, NY). The culture was incubated at 37°C in an atmosphere containing 95% humidity and 5% CO2. For all experiments, cells were resuspended in fresh medium and treated with p,p'-DDE, MAPK inhibitors, or the antioxidant Trolox (Sigma-Aldrich Chem. Co., St. Louis, USA) as indicated. The p,p'-DDE stock solution was prepared using 1% ethanol as the vehicle, and 5.0–20 μL of the stock solution was added to the cultures to achieve final concentrations of 0.019, 0.19, or 1.9 μg/mL of p,p'-DDE.

2.2. Cell Treatments. Cells were plated in complete medium at a density of 2 × 105 cells/mL. Cells were given 12 h to stabilize before treatments were added; control cells had no treatment and experimental samples received 0.019, 0.19, or 1.9 μg/mL of p,p'-DDE. MAPK inhibitors (PD98059, SP600125, and SD203508 for ERK1/2, JNK, and p38, resp., Sigma-Aldrich Chem. Co., St. Louis, USA) were added 1 h before treatment with p,p'-DDE. Inhibitors for PLA (cPLA2, Calbiochem, USA) or PKC (Miy PKC, Promega, USA) were added 1 h before treatment with p,p'-DDE. Five micrograms of BAPTA-AM (a Ca2+ chelator) was added 30 min before treatment with p,p'-DDE. Trolox (Sigma-Aldrich Chem. Co., St. Louis, USA) was added to reach a final concentration of 50 μM 1 h before treatment with p,p'-DDE.

2.3. Cell Viability Assay. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) assay was used for cell viability assay [45]. After 12 h of stabilization, HL-60 cells were treated with 0.019, 0.19, or 1.9 μg/mL of p,p'-DDE for 24, 48, 72, 96, and 120 h. For 1.9 μg/mL of p,p'-DDE cell viability assay was performed also at 144 and 168 h. Absorbance at 590 nm was measured with a spectrophotometer microplate reader (SpectraMax 250, Molecular Devices, Sunnyvale, CA) and the percentage of viable cells was calculated as the absorbance of each experimental condition relative to the absorbance of the control group. Three independent experiments were performed in triplicate.

2.4. Cell Proliferation Assay. Cell proliferation was measured by a [3H]-thymidine incorporation assay after 24, 48, 72, 96, and 120 h of exposure to p,p'-DDE. Twelve hours before harvesting, 0.5 μCi/well of [3H]-thymidine (Amersham Life Science) was added, and cells were harvested using an automatic Skatron combi cell harvester (Skatron Inst., Lier, Norway) onto fiberglass filters. The incorporation of [3H]-thymidine was determined using a beta-plate scintillation counter (Beckman, LS 6500). Three independent experiments were performed in triplicate.

2.5. Cytochemistry. For morphological analyses, HL-60 cells grown under different experimental conditions were harvested and centrifuged onto cyto spin slides (Shandon CytoSpin 4, Thermo Fisher Scientific, Waltham, MA). According to the manufacturer's instructions, slides were stained with

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Wright staining solution for 20 min and then observed under a light microscope. Morphological changes of cells were evaluated as cell size (big or small) and nucleus shape (bean or band). Four randomly selected fields containing at least 100 cells were counted. The percentage of cells with morphological changes was calculated from three independent experiments. Monocyte/macrophage differentiation was assayed for nonspecific esterase (NSE) presence using a nonspecific α-naphthyl-acetate staining kit (Sigma-Aldrich Chem. Co., St. Louis, USA) according to the manufacturer’s recommended protocol. Positive cells to NSE activity were stained reddish-brown colouration.

2.6. Determination of CD14 and CD16 by Flow Cytometry Analysis. The expression of cell surface antigens (CD14 and CD16) was determined after 24, 48, 72, 96, and 120h of exposure to p,p′-DDE by measuring the binding of fluorescein isothiocyanate-CD14 or phycoerythrin-CD14 conjugated antibodies (Abcam, Hong Kong) by flow cytometry. Cells exposed to p,p′-DDE concentrations were harvested at a density of 10⁶ cells/mL and then incubated with appropriate dilutions of the antibodies according to the manufacturer’s instructions. Excess antibody was removed by washing the cells once with PBS (0.01 M phosphate buffered saline and 0.15 M NaCl). Specific antibody binding was measured regarding total fluorescence of the cell population with a four-colour FACS Calibur (Becton Dickinson). The fluorescence was compared to unstained controls with 10,000 events recorded. All data was analysed using FlowJo v7/8 (Tree Star Inc., Ashland, OR).

2.7. Measurement of [Ca²⁺]i by Flow Cytometry. [Ca²⁺]i was measured by fluorescence-activated cell sorting (FACS) using Fluo-3-loaded cells (Fluo-3AM, Molecular Probes, Oregon). HL-60 cells were resuspended to a density of 2.5 × 10⁷/mL in PBS containing 13 μM Fluo-3AM and 8% pluronuc acid F-127 (Molecular Probes, Oregon, USA) and were incubated for 25 min at 37°C. For treatments with BAPTA-AM (Molecular Probes, Oregon), cells were incubated for 30 min with the Ca²⁺ chelator before incubation with Fluo-3AM. Extracellular Fluo-3AM was removed by washing the cells twice. Finally, the cells were adjusted to a density of 6.25 × 10⁴/mL and resuspended in Ca²⁺-free buffer to evaluate the role of [Ca²⁺]i. Ca²⁺ signal was measured with the flow cytometer for 20 seconds to establish a baseline Ca²⁺ level. Subsequently, cells were exposed to different experimental conditions, and the Ca²⁺ signal was measured for up to 3 min. Ionomycin (0.1 μg/μL) and phorbol myristate acetate (PMA) (10 nM) (Sigma-Aldrich Chem. Co., St. Louis, USA) were used to verify the specificity of the signal. Assessment of [Ca²⁺]i was performed at room temperature by flow cytometry (FACS Calibur, Becton Dickinson) using the CellQuestPro software (BD Bioscience) and analysed with FlowJo v7/8 (Tree Star Inc., Ashland, OR).

2.8. Western Blot Analysis. For nuclear and cytosolic extracts, cells were washed and harvested with cold TDE buffer (12 mM Tris-Base, 68 mM NaCl, 2.5 mM KCl, and 0.4 mM monobasic sodium phosphate, pH 7.4) and pelleted by centrifugation at 1900 rpm at 4°C for 5 min. The pellet was resuspended in two volumes of cold buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSE, 10 μg/mL leupeptin, 5 μg/mL antipain, 5 μg/mL chymostatin, 5 μg/mL benzamidine, and 1 μg/mL pepstatin (Sigma Chem., Co., St. Louis, MO)). After incubation for 15 min at 4°C, 0.5 volumes of 10% NP-40 were added and the samples were mixed for 1 min and centrifuged at 14,000 rpm for 30 s. Cytosolic extracts corresponding to the soluble fraction were retained, and aliquots were stored at −87°C until use. Protein concentrations for total, nuclear, and cytosolic fractions were determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). From each sample, a constant amount of protein (30 μg of total protein or 20 μg of nuclear or cytosolic protein) was separated by 10% SDS-PAGE at 80 V. Proteins were electrotransferred to PVDF membranes (Millipore Corporation, Bedford, MA, USA) in a Hoefer semidy unit (Amershams Biosciences, Buckinghamshire, UK). Blots were blocked for 1 h with 5% fat-free milk in PBS and incubated overnight at 4°C with specific antibodies in PBS containing 3% fat-free milk and 0.1% Tween 20. Specific antibodies were used against phosphorylated PKCα and phosphorylated p38 (Santa Cruz Biotechnology, CA, USA), anti-C/EBPβ (Abcam, Cambridge, USA), anti-histone 4 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (kindly donated by Dr. Manuel Hernández’s Laboratory, Cinvestav). β-Actin and histone 4 were used as loading controls for cytosolic and nuclear proteins, respectively; these proteins were detected on the same membrane after stripping it. Membranes were incubated with anti-rabbit, anti-goat, or anti-mouse biotinylated antibody (Zymed Laboratories Inc., CA, USA), bands were developed using Luminata Forte (Millipore, MA, USA) and subsequently visualised using the Odyssey infrared imaging system (LI-COR Bioscience, Lincoln, USA). At least three independent experiments were performed.

2.9. Data Analysis. Data were analysed using a one-way ANOVA followed by Dunnett’s post hoc test or Bonferroni’s multiple comparison tests to evaluate cell viability differences over time. All values of p < 0.05 were taken to indicate statistical significance. Data analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Viability and Proliferation of HL-60 Cells Exposed to p,p′-DDE. Viability assays showed a significant decrease in MTT reduction at 72 and 96h in cells exposed to 1.9 μg/mL of
3.2. p,p’-DDE Exposure Induces Changes in Cell Morphology. The morphology of promyelocytic cells was monitored for 120 h to identify any changes induced by 0.019, 0.19, or 1.9 μg/mL of p,p’-DDE. Over the course of 120 h, p,p’-DDE exposure induced morphological changes in a concentration-dependent manner. While control cells presented a circular nucleus (their typical morphology in promyelocytic cells), those treated with 1.9 μg/mL of p,p’-DDE exhibited a kidney-shaped nucleus and had a reduced nuclear/cytoplasmic ratio (Figures 2(a)–2(d)). Morphological changes in cells exposed to 0.19 and 1.9 μg/mL of p,p’-DDE began to appear after only 48 h, and by 120 h the percentage of cells with differentiation changes was three- to fourfold higher than the control (Figure 2(e)).

3.3. p,p’-DDE Exposure Does Not Induce Lineage-Specific Markers in Promyelocytic HL-60 Cells. Due to the morphological changes observed in cells treated with p,p’-DDE, we assessed the presence of NSE as indicative of monocytic differentiation, as well as the expression of lineage-specific surface markers. In Figure 3(a) is shown that cells exposed to 0.019, 0.19, or 1.9 μg/mL of p,p’-DDE for five days were negative for NSE. In HL-60 cells treated with p,p’-DDE (0.019, 0.19, and 1.9 μg/mL), the CD16 and CD14 associated with terminal differentiation of granulocytes and monocytes, respectively, in all experimental conditions were negative to these markers from 24 to 120 h of treatment. In Figures 3(b) and 3(c) are presented results from flow cytometry at 120 h.

3.4. p,p’-DDE Exposure Activates p38 Leading to an Increase in C/EBPβ Protein Levels. To examine whether the morphological changes induced by p,p’-DDE exposure are concomitant with activation of the C/EBPβ transcription factor (associated with myelocytic differentiation), nuclear protein levels were evaluated after 12 h of p,p’-DDE (0.019, 0.19, and 1.9 μg/mL) exposure. Nuclear C/EBPβ levels increased only upon exposure to 1.9 μg/mL p,p’-DDE (Figure 4(a)).

It is known that C/EBPβ activation is regulated by MAP kinases such as ERK, JNK, and p38 [46]. Therefore, we assessed the role of these kinases in C/EBPβ activation induced by exposure to 1.9 μg/mL p,p’-DDE using the MAPK inhibitors iERK, iJNK, and ip38 (for ERK, JNK, and p38, resp.). Only p38 inhibition significantly reduced the C/EBPβ nuclear protein activation induced by p,p’-DDE exposure (Figure 4(b)).

3.5. p,p’-DDE Exposure Increases [Ca^{2+}]_i in HL-60 Cells. Since signalling pathways related to p38 phosphorylation and C/EBPβ activation involve an increase of [Ca^{2+}]_i, the effect of p,p’-DDE (0.019–1.9 μg/mL) on [Ca^{2+}]_i was evaluated. At 1.9 μg/mL of p,p’-DDE and in absence of extracellular Ca^{2+}, [Ca^{2+}]_i increased twofold over baseline (Figures 5(a) and 5(b)). To verify whether the increase in [Ca^{2+}]_i was caused by p,p’-DDE, the Ca^{2+} chelator BAPTA-AM was used. As shown in Figures 5(c) and 5(d), BAPTA-AM completely blocked the p,p’-DDE-induced increase in [Ca^{2+}]_i.
Figure 2: Morphological changes in HL-60 cells induced by p,p'-DDE exposure for 120 h. Representative images of cells with morphological changes (arrows) as evaluated by Wright staining; morphological changes were characterized by a decreased nuclear/cytoplasmic ratio and an irregular nuclear shape. (a) Control cells, (b) cells exposed to 0.019 \( \mu \text{g/mL} \) p,p'-DDE, (c) cells exposed to 0.19 \( \mu \text{g/mL} \) p,p'-DDE, and (d) cells exposed to 1.9 \( \mu \text{g/mL} \) p,p'-DDE. (e) The fraction of cells with morphological changes per 100 live cells after 120 h of treatment. Data from three independent experiments are expressed as the mean ± SEM. ** \( p < 0.01 \) indicates differences from the control group. Data were analysed using one-way ANOVA followed by Dunnett's post hoc test.
Figure 3: Absence of nonspecific esterase and mature cell surface markers in HL-60 cells exposed to \( p,p' \)-DDE. (a) Representative images of nonspecific esterase staining of HL-60 cells exposed to \( p,p' \)-DDE for 120 h. (b) Flow cytometry representative histograms of CD14 and CD16 expression in HL-60 cells exposed for 120 h to \( p,p' \)-DDE. (c) The percentages of cells expressing CD14 or CD16 were detected by flow cytometry analyses. The data represent the mean ± SEM of three different experiments. Data were analysed using one-way ANOVA followed by Dunnett’s post hoc test.
3.6. Effect of p,p′-DDE on PKCα, p38, ROS, and Phospholipase A. Because the activation of PKCα has been shown to be involved in MAPK’s signalling to induce haematopoietic differentiation, the effect of p,p′-DDE on PKCα activation was evaluated. Only exposure to 1.9 µg/mL of p,p′-DDE induced a significant increase in PKCα activation (Figure 6(a)). To determine whether the p,p′-DDE-induced PKCα activation is related to the increase in [Ca2+], PKCα activation was evaluated in cells treated with BAPTA-AM prior to treatment with 1.9 µg/mL of p,p′-DDE. BAPTA-AM blocked the p,p′-DDE-induced increase in [Ca2+], but p,p′-DDE-induced PKCα phosphorylation was not modified by the chelator (Figure 6(b)). To evaluate whether p,p′-DDE is capable of inducing PKCα activation through PLA2 activation or oxidative misbalance, the antioxidant Trolox and a PLA2 inhibitor were used. Both Trolox and the PLA2 inhibitor significantly decreased the PKCα phosphorylation induced by 1.9 µg/mL of p,p′-DDE (Figure 6(b)). The roles of PKCα, ROS, and PLA2 in the p38 phosphorylation were evaluated in cells treated with 1.9 µg/mL of p,p′-DDE for 1h. p,p′-DDE exposure significantly increased p38 phosphorylation when compared to control cells. Inhibitors of PKCα, PLA2, and Trolox significantly decreased the p38 phosphorylation induced by 1.9 µg/mL of p,p′-DDE (Figure 6(c)).

4. Discussion

The presence of p,p′-DDE in bone marrow [5–7, 47] has been correlated with alterations in cell differentiation resulting in haematological disorders [16, 18, 19]. In the present study, the cell viability results suggest that concentrations of p,p′-DDE used were not cytotoxic. At the higher concentration (1.9 µg/mL of p,p′-DDE), a transient decrease in cells seems to occur at 96 h; however cultures compensated the effect and reached the steady state after this time. In fact, because results did not show an effect of 1.9 µg/mL of p,p′-DDE on proliferation at 96 h, we considered that the effect observed in MTT assays at this time is a consequence of a temporal decrease of metabolic capability of cells to reduce of MTT instead of cell death. Morphological changes in promyelocytic HL-60 cells were observed after a five-day exposure to 0.19 and 1.9 µg/mL of p,p′-DDE, suggesting that noncytotoxic concentrations of p,p′-DDE induce incomplete differentiation. These observations agree with the ability of p,p′-DDT (at 20 µM) to induce differentiation in adipocytes 3T3-L1 and preadipocytes 3T3-F442A [40]. They are also consistent with a recent study in human mesenchymal stem cells (MSCs) from bone marrow, which showed that p,p′-DDT exposure enhanced osteogenic and adipogenic differentiation [39]. Our work suggests that p,p′-DDE exposure at the concentrations found in bone marrow of humans can induce intracellular molecular pathways (ROS, PKCα, p38, and C/EBPβ) that are involved in the myeloid differentiation and, consequently, induce morphological differentiation of this human myeloid cell line. The p,p′-DDE-induced morphological changes of HL-60 cells are compatible with early differentiation events of commitment in promyelocytic HL-60 where cells with a regular and prominent nucleus are transformed into smaller cells with an irregularly shaped nucleus [48]. In our study, promyelocytic cells were transformed by p,p′-DDE exposure into cells with a dentated nucleus, which is a change that takes place when promyelocytic HL-60 cells transform into metamyelocyte precursors without becoming fully mature cells. This change
occurred without alterations in cell proliferation, suggesting that \( p,p'\)-DDE exposure induces only partial differentiation: neither the presence of lineage-specific surface markers of monocytes or granulocytes (CD14 and CD16) nor the presence of nonspecific esterases was detected. Previously, it has been shown that another organochlorine pesticide, heptachlor, induced monocyte-macrophage differentiation in a myeloid leukaemia cell line [49]. However, the underlying molecular pathways or mechanisms involved have not been defined. Also, it is noteworthy that exposure to heptachlor is associated with an increased risk of developing leukaemia and non-Hodgkin lymphoma [50, 51]. Similarly, there is data suggesting that DDT and \( p,p'\)-DDE exposure are a risk factor for bone marrow disorders such as aplastic anaemia, AML, and non-Hodgkin lymphoma [13–19].

To evaluate the mechanisms by which \( p,p'\)-DDE induced differentiation in promyelocytic HL-60 cells, the effect of this compound on several molecular targets was studied. We evaluated the effect of \( p,p'\)-DDE on intracellular molecules that are involved in activation of C/EBP\( \beta \).
factor activated during myeloid differentiation [30–32]. Our results demonstrate that \( p,p'-\text{DDE} \) induced an increase in nuclear levels of C/EBP\( \beta \), an effect that was dependent on p38 activation and is consistent with data previously reported [52]. The effect of \( p,p'-\text{DDE} \) on C/EBP\( \beta \) was similar to what occurs in myelopoiesis triggered by external stimuli (such as in response to an infection) and some kinds of proinflammatory diseases such as rheumatoid arthritis [30, 53]. ERK and JNK may also mediate the transcription activity of C/EBP\( \beta \) in different cell types [35, 54], but we found that the \( p,p'-\text{DDE} \)-induced increase in C/EBP\( \beta \) nuclear levels was only dependent on p38. In HL-60 cell differentiation induced by PMA, the activation of PKC results in p38 activation [55, 56]. Our data demonstrate that p38 phosphorylation induced by \( p,p'-\text{DDE} \) is dependent on PKC\( \alpha \) activation. Because it is known that the p38 pathway is activated by oxidative stress stimuli [34, 57] and in HL-60 cells the p38 activity is induced by ROS [58, 59], the relationship between the rise of p38 phosphorylation with ROS production was evaluated using an antioxidant. We found that p38 phosphorylation induced by \( p,p'-\text{DDE} \) was ROS-dependent.

To determine the pathway through which \( p,p'-\text{DDE} \) induces PKC\( \alpha \) activation, we evaluated the impact of \( p,p'-\text{DDE} \) on \([\text{Ca}^{2+}]_i\). Our results demonstrate that exposure to 1.9 \( \mu \text{g/mL} \) of \( p,p'-\text{DDE} \) raises the \([\text{Ca}^{2+}]_i\) level, which is known to occur in other cell types as well [42, 60–62], \([\text{Ca}^{2+}]_i\) chelation partially, but not significantly, inhibited \( p,p'-\text{DDE} \)-induced PKC\( \alpha \) activation. Instead, our data revealed that the activation of PKC\( \alpha \) induced by \( p,p'-\text{DDE} \) is ROS-dependent. Previous studies have reported that \( p,p'-\text{DDE} \) is capable of inducing ROS in blood mononuclear cells at concentrations of 60 and 80 \( \mu \text{g/mL} \) [63]. Because ROS has been associated
with activation of some pathways upstream of PKCα, like PLA2 [64, 65], the role of PLA2 was explored. Our results showed that, in addition to ROS, PKCα activation induced by \( p,p'-\text{DDE} \) was also PLA2-dependent.

It has recently been reported that other organochlorine pesticides, such as Aldrin and \textit{trans}-nonachlor, induced PLA2 and PKCα activity in HL-60 promyelocytes, human THP-1 monocytes, and murine J774A.1 macrophages [44]. It is important to note that PLA2 plays a role in p38 activation induced by \( p,p'-\text{DDE} \) since inhibition of PLA2 prevents PKCα activation which interferes with the p38 phosphorylation induced by \( p,p'-\text{DDE} \). This situation is analogous to that described in monocytes [66].

In summary, this study supports the fact that \( p,p'-\text{DDE} \) exposure in the concentration range found in human bone marrow [5–7] is capable of inducing partial differentiation in HL-60 cells. Our results are consistent with the possibility that PLA2 and ROS trigger signalling pathways involved in PKCα activation, whereas ROS and PKCα induction are the most important stimuli for p38 activation and consequently increase C/EBPβ protein levels in the nucleus of HL-60 cells exposed to \( p,p'-\text{DDE} \) (Figure 7). These findings offer a putative molecular mechanism to partially explain the potential consequences of having \( p,p'-\text{DDE} \) in human bone marrow and suggest that exposure to this pesticide could potentially lead to the development of bone marrow disorders in human. Further studies are necessary to conclusively support this hypothesis.

**Competing Interests**

The authors declare that there is no conflict of interests.

**Acknowledgments**

The authors thank Acosta-Saavedra Leonor C., M.S., for technical assistance. This study was funded by a grant from the Mexican Council for Science and Technology (Conacyt 152491).

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