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Chlorpyrifos inhibits cell proliferation through ERK1/2 phosphorylation in breast cancer cell lines

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HIGHLIGHTS

- Chlorpyrifos (CPF) insecticide is used to control pest worldwide.
- CPF 50 μM inhibited breast cancer cell proliferation by redox imbalance.
- H_2O_2 is the main specie involved in cell proliferation inhibition by CPF.
- H_2O_2 produced by CPF promotes the phosphorylation of ERK1/2 leading to cell death.

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ABSTRACT

It is well known the participation of oxidative stress in the induction and development of different pathologies including cancer, diabetes, neurodegeneration and respiratory disorders among others. It has been reported that oxidative stress may be induced by pesticides and it could be the cause of health alteration mediated by pollutants exposure. Large number of registered products containing chlorpyrifos (CPF) is used to control pest worldwide. We have previously reported that 50 μM CPF induces ROS generation and produces cell cycle arrest followed by cell death. The present investigation was designed to identify the pathway involved in CPF-inhibited cell proliferation in MCF-7 and MDA-MB-231 breast cancer cell lines. In addition, we determined if CPF-induced oxidative stress is related to alterations in antioxidant defense system. Finally we studied the molecular mechanisms underlying in the cell proliferation inhibition produced by the pesticide.

In this study we demonstrate that CPF (50 μM) induces redox imbalance altering the antioxidant defense system in breast cancer cells. Furthermore, we found that the main mechanism involved in the inhibition of cell proliferation induced by CPF is an increment of p-ERK1/2 levels mediated by H_2O_2 in breast cancer cells. As PD98059 could not abolish the increment of ROS induced by CPF, we concluded that ERK1/2 phosphorylation is subsequent to ROS production induced by CPF but not the inverse.

1. Introduction

Agriculture represents one of the most important economic activities, where agricultural workers are exposed to a variety of chemical, physical, and biological hazards. To minimize crop damage and to increase land productivity, the use of pesticides become essential. However, pesticide exposure is recognized as an important environmental risk factor associated with cancer development (Weichenthal et al., 2010; Brophy et al., 2012).

Chlorpyrifos [O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)-phos-phorothioate] (CPF) is one of the most widely used

Abbreviations: CPF, chlorpyrifos; OP, organophosphate insecticides; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD, superoxide dismutase; CAT, catalase; GSH, reduced glutathione; GPX, glutathione peroxidase; TBARS, 2-thiobarbituric acid reactant substances; MDA, malondialdehide; PUFAs, polyunsaturated fatty acids.

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organophosphate insecticides (OP) worldwide and largely applied in Argentina for pest control (Jergentz et al., 2005). There is a great number of registered commercial products containing CPF whose purposes include control for food crops, turf and ornamental plants, indoor pest control and pet collars (Smegal, 2000).

CPF is metabolized in human liver to the active metabolite, chlorpyrifos-oxon, which produces neurotoxicity by inhibiting esterases in the peripheral and central neurons systems. In addition, other targets for CPF have been suggested. CPF (50 μM) induces apoptosis in cultured rat cortical neurons that is regulated by a balance between p38 MAP kinase (p38) and extracellular signal-regulated kinase 1/2 (ERK1/2) over c-Jun N-terminal kinase (JNK), suggesting apoptosis as a toxic endpoint of chlorpyrifos neurotoxicity in the brain, which was described as a mechanism independent of AChE inhibition (Caughlan et al., 2004). Additionally, CPF and fenthion were described as the most potent insecticide activators of PKC in rat brain and liver (Casida and Quistad, 2004). In C6 glioma cells, CPF inhibited DNA synthesis in a concentration-dependent manner and these effects on cell replication were independent of cholinergic stimulation (Garcia et al., 2001). Accordingly to García et al., we have reported that 50 μM CPF affects breast cancer cell replication inducing cell cycle arrest in S-phase by modifying checkpoint proteins through a mechanism that may involve changes in redox balance in the hormone-dependent MCF-7 cell line. Moreover, in hormone-independent breast cancer cells, MDA-MB-231, 50 μM CPF produces an arrest in G2/M phase followed by cell death (Ventura et al., 2012).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are signal-transducing molecules that regulate the activities of a variety of proteins. Cellular redox signaling is mediated by the post-translational modification of proteins in signal-transduction pathways by ROS/RNS or the products derived from their reactions are generated as a part of the normal metabolism of a cell in aerobic organisms. In addition, chemical pollutants are important sources of ROS and RNS in biological systems (Sommer et al., 2002; Valavanidis and Vlahogianni, 2006).

ROS control various biological processes such as cell proliferation, survival, cell cycle arrest and cell differentiation when they are present in physiological amounts. At high or sustained levels, they can react with DNA, proteins and lipids causing genomic instability, protein oxidation and lipid peroxidation. During their evolution, the biological systems have developed adequate enzymatic and non-enzymatic antioxidant mechanisms to protect their cells from oxidative damage. The enzymatic antioxidant mechanism is constituted by superoxide dismutase (SOD), catalase (CAT), and the glutathione (GSH)-dependent system including GSH peroxidase (GPX) and GSH reductase. The non-enzymatic antioxidant mechanism includes GSH, vitamin E, ascorbate, β-carotene and urate (Trachootham et al., 2008).

Eukaryotic cells contain protein kinase cascades to regulate cell metabolism. The mitogen activated protein kinase (MAPK) cascade is a central signaling pathway which includes JNK, p38, ERK1/2 and ERK5 (Mebratu and Tesfaigzi, 2009). Several extracellular stimuli can activate one or more MAPK pathways. Once activated, MAPKs regulate cellular activities including gene expression, mitosis, embryogenesis, cell differentiation, movement, metabolism, and programmed death (Kamata et al., 2005). Although ERK1/2 activation has generally been associated with cell survival and proliferation, a number of studies show that activation of ERK1/2 can mediate cell death depending on the stimuli and cell types involved (Mebratu and Tesfaigzi, 2009; Subramaniam and Unsicker, 2010). Several authors report that ERK1/2 activation is associated with cell death induced by ROS (Torres, 2003; Dong and Ramachandiran, 2004).

The present investigation was designed to identify the pathways involved in CPF-induced cell death in MDA-MB-231 and MCF-7 human breast cancer cell lines. In addition, we determined if CPF-induced oxidative stress is associated with alterations in antioxidant defense system. Finally we studied the molecular mechanisms underlying in the redox unbalance produced by the pesticide.

2. Materials and methods

2.1. Cell culture

Estrogen-dependent MCF-7 and estrogen-independent MDA-MB-231 human breast cancer cell lines were purchased from American Type Culture Collection (ATCC, USA). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 0.3 g L^{-1} glutamine and 0.04 g L^{-1} gentamicin (Gibco BRL, NY, USA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. For the experiments, cells were exposed to CPF (Chem Service, Inc., West Chester, PA, USA) dissolved in ethanol and the final ethanol concentration did not exceed 0.5%. Treatments were added to the cultures in phenol red free-RPMI medium (Gibco BRL, NY, USA) and 10% charcoal-treated FBS. Ethanol 0.5% (vehicle) was added to the control cells.

2.2. Clonogenic assay

3×10^3 MCF-7 and 1.5×10^3 MDA-MB-231 cells were exposed to CPF (0.05, 0.5, 5 and 50 μM) or vehicle for 10 d. When it was required, 5 μM PD98059 (Sigma Chemical Co., MO, USA) or 30 IU mL^{-1} of CAT were added to the culture. Fixed cells were stained with 0.05% violet crystal in 10% ethanol. Clonogenicity was evaluated by counting colonies containing 50 cells or more. Results were expressed as percentage respect to control cells.

2.3. Measurement of ROS and RNS production

Cells were exposed to different treatments as indicated in the legends. Later, the cells were incubated for 30 min at 37 °C with 5 μM DCF-2DA (dichlorodihydrofluorescein diacetate; Sigma Chemical Co., MO, USA) or 5 μM DAF-2DA (4,5-diamino-fluorescein diacetate; Sigma Chemical Co., MO, USA) as a fluorescent probe for ROS and RNS determination, respectively (Dahboul et al., 2012; Lee et al., 2012; Banerjee et al., 2014). Cells were then trypsinized, and suspended in 1 mM phosphate saline buffer, pH 7.4 (PBS). Levels of intracellular ROS and RNS were measured immediately by flow cytometry (CyFlow Pas III, Partec; Görlitz, Deutschland, Germany) and data were analyzed using Cyflogic 1.2.1 software (Perttu Terho & CyFlo Ltd). Results were expressed as percentage respect control cells.

2.4. Measurement of CAT activity

Cells were exposed to CPF (0.05, 0.5, 5 and 50 μM) or vehicle for 24 h, trypsinized and suspended in phosphate buffer (KH_2PO_4/K_2HPO_4 50 mM, pH 7.8). It was followed by sonic disruption and centrifugation at 10000*g* for 10 min at 4 °C. Protein concentration was determined by Bradford assay (Bradford, 1976). CAT activity was measured spectrophotometrically by monitoring the disappearance of H_2O_2 at 240 nm. The reaction mixture for the assay contained 50 mM phosphate buffer (pH 7.8), 25 mM H_2O_2 (MERK, Darmstadt, Germany) and 50 μL of CAT-containing samples, in a total volume of 1.0 mL. One unit of CAT was defined as the disappearance of 1 μmol of H_2O_2/min ($\varepsilon = 43.6\ M^{-1}\ cm^{-1}$). In the experiments where the cells were exposed to different doses of CPF, the results were expressed as percentage respect to the control. To

compare basal levels the results were indicated as International Units of CAT per μg of protein.

2.5. Measurement of SOD activity

Cells were exposed to CPF (0.05, 0.5, 5 and 50 μM) or vehicle for 24 h. Cells were collected by trypsinization and resuspended in TEA-DEA buffer [25 mM triethanolamine (TEA) and 25 mM diethanolamine (DEA), pH 7.4]. Samples were centrifugated at 30 000*g* for 30 min at 4 °C. SOD activity was measured through enzyme competition with NADH oxidation by superoxide anion, following the consumption of NADH at 340 nm (Paoletti et al., 1986). The reaction was performed in a final volume of 1 mL of 100 mM TEA-DEA buffer pH 7.4, containing 0.28 mM NADH, 1.17 mM $MnCl_2$, 2.35 mM EDTA, and 0.95 mM β-mercaptoethanol to trigger the chemical generation of superoxide anion. Control (maximum) NADH oxidization was competed with 20, 50 or 100 μL of supernatant sample to determine the SOD activity. One unit of SOD activity was defined as the amount of SOD leading to a 50% inhibition of NADH chemical oxidation by superoxide anion. SOD units were calculated according to other previous works (Ferrari et al., 2008). In the experiments where the cells were exposed to different doses of CPF, the results were expressed as percentage respect of the control. To compare basal levels the results were indicated as Units of SOD per μg of protein.

2.6. Lipid peroxidation

The formation of lipid oxidation products was evaluated by determination of 2-thiobarbituric acid reactant substances (TBARS). Cells were exposed to CPF (0.05 and 50 μM) of vehicle for 24 h, trypsinizated and resuspended in 50 mM phosphate buffer, pH 7.4. Aliquots were removed to determine the protein content by Bradford assay (Bradford, 1976). After that, 0.3 mL of each sample was mixed with reaction buffer [15% (v/v) trichloroacetic acid, 0.25 N hydrochloric acid and 0.375% (w/v) 2-thiobarbituric acid] and heated for 15 min at 90 °C. The complex formed with 2-thiobarbituric acid was extracted with 3 mL of butanol and quantified fluorometrically (λ_{ex} = 515 nm; λ_{em} = 555 nm). TBARS were expressed as μmol of malondialdehyde (MDA) per gram of protein. MDA standard was prepared from 16.4 μM 1,1,3,3-tetraethoxy propane.

2.7. Western blot

Cells were exposed to different treatments and whole lysate proteins were measured by Bradford assay (Bradford, 1976). Then, proteins (50 μg) were analyzed by SDS–PAGE and immunoassayed as previously described (García et al., 2010). Membranes were probed overnight with primary mouse anti-p-ERK1/2 (1:500, Santa Cruz Biotech, INC. California, USA) or anti Y-nitrosylated-protein (1:1000; Santa Cruz Biotech, INC. California, USA). Rabbit anti-ERK1/2 (1:500, Santa Cruz Biotech, INC. California, USA), mouse anti-β-actin or mouse anti-α-tubulin (1:3000, SIGMA Aldrich Inc., Saint Luis, MO, USA) were used for loading control. Immunoreactivities were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (SIGMA Aldrich Inc., Saint Luis, MO, USA) and visualized by enhanced chemiluminescence (Amersham Biosciences, Arlington Heights, IL, USA). Densitometric analyses were performed using the software ImageJ 1.32 J (NIH, Bethesda, MD, USA).

3. Results

3.1. Effect of CPF on intracellular RNS formation

We have recently reported that CPF elicited ROS production in MDA-MB-231 and MCF-7 cells. To complete our studies, RNS levels were measured after 24 h of the pesticide exposure in both cell lines. As shown in Fig. 1A, an increment of RNS levels was found in MDA-MB-231 cells at 50 μM CPF (23.0 ± 4.3% over control) but not significant changes were detected in MCF-7 cells. 3-nitrotyrosine modification is a post-translational irreversible change caused by NO derived peroxynitrite during pathological conditions, which could alter protein structure and function. Our Western blot analysis showed a slight but not significant increased in the nitration of 30, 35, 37, 55, 65 kDa proteins under oxidative stress induced by CPF in MDA-MB-231 cells. In MCF-7 cells no changes in nitrosylation proteins were induced by the pesticide (Fig. 1B).

3.2. CPF modulates antioxidant enzyme activity

To determine if CPF exposure causes alterations in antioxidant cell defenses, we analyzed the effect of this pesticide on enzymatic antioxidant cell mechanisms. We investigated the CPF action on CAT and SOD enzymatic activities. Both enzymatic activities remained unaltered when the cells were exposed to 0.05 μM CPF (Fig. 2A and C). In contrast, at its highest concentration (50 μM), CPF produced a significant increase in CAT activity in MCF-7 and MDA-MB-231 cells (36.6 ± 9.2% and 99.0 ± 20.3% over control, respectively) as shown in Fig. 2A. In addition, a decrease in SOD activity was observed in MCF-7 cells exposed to 50 μM CPF (57.1 ± 13.8% with respect to the control) but no differences were observed in MDA-MB-231 cells (Fig. 2C). As shown in Fig 2B and D, the basal levels of CAT and SOD activities respectively, were found to be lower in MCF-7 than in MDA-MB-231 cells.

3.3. CPF effect on lipid peroxidation

We studied MDA production as an indicator of lipid peroxidation. As shown in Fig. 3, 50 μM CPF significantly increased the content of lipid breakdown products in MCF-7 cells (50.4 ± 9.7% over control), but not significant effects were observed in MDA-MB-231 cells.

3.4. Effect of H_2O_2 generation CPF-induced on cell proliferation

High levels of ROS were described as responsible of cell apoptosis and necrosis. As we have previously reported that 50 μM CPF increases ROS generation, we investigated the involvement of ROS increment induced by CPF on cell proliferation. We found that the clonogenicity diminution could be reversed by CAT addition (30 IU mL^{-1}) in both cell lines exposed to 50 μM CPF (Fig. 4). These results indicate that the effect of CPF on cell proliferation may be principally adjudicated to H_2O_2 increase in these cells.

3.5. Effect of CPF on ERK1/2 phosphorylation as a pathway for inhibition of cell proliferation

To elucidate the underlying mechanism of cell proliferation inhibition induced by CPF, phosphorylation of ERK1/2 was analyzed. The presence of p-ERK1/2 was evaluated by Western blot analysis with specific antibodies in both cell lines exposed to CPF at 0.05 and 50 μM or vehicle for 5 and 15 min. As shown in Fig. 5A, 50 μM CPF induced a significant increment of ERK1/2 phosphorylation in both MCF-7 and MDA-MB-231 cells (50.0 ± 14.1% and 56.9 ± 4.2% over control, respectively), after 15 min of exposure. We also observed an enhanced ERK1/2 phosphorylation in MDA-MB-231 cells, after 5 min of CPF exposure.

To ascertain if cell growth inhibition was a consequence of ERK1/2 phosphorylation, cells were grown in presence or absence of 5 μM PD98059, a specific MEK1 inhibitor. In MDA-MB-231 cells, PD98059 completely reversed the effect of CPF on cell proliferation indicating that p-ERK1/2 is involved in this action (Fig. 5B). In

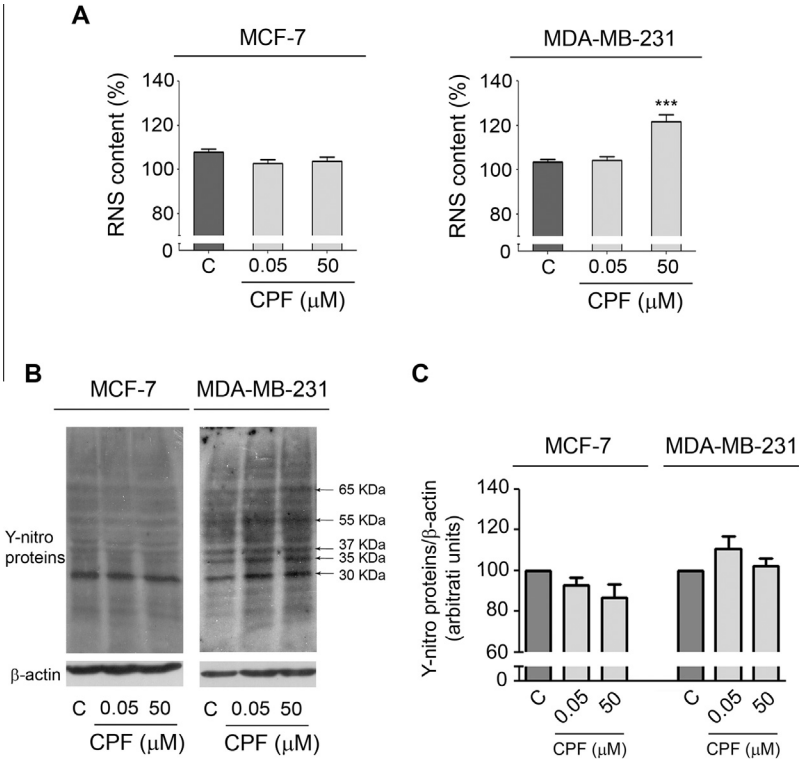

Fig. 1. CPF enhances RNS generation and Y-nitrosylation proteins in MDA-MB-231 cells. (A) MCF-7 and MDA-MB-231 cells were exposed to CPF (0.05 and 50 μM) or vehicle (ETOH 0.5%) for 24 h. Intracellular RNS levels were analyzed by flow cytometry using the fluorescent dye DAF-2DA. The graphs show the mean fluorescence intensity as percentage of the respective control. Data are expressed as means ± SEM of three independent experiments, each performed by duplicates. $^{***}p < 0.001$ vs. *C*. One-way ANOVA followed by Dunnett's Multiple Comparison *post hoc* test. (B) Cells were exposed to CPF (0.05 and 50 μM) or vehicle (ETOH 0.5%) for 24 h. Y-nitrosylation proteins were assayed by Western blot using total cell extracts. Western blots from one representative experiment are shown. (C) Quantification of total Y-nitro proteins (30, 35, 37, 55 and 65 KDa)/β-actin ratios is shown in the graph. Data correspond to two independent experiments, each performed by duplicates. p:ns. One-way ANOVA followed by Dunnett's Multiple Comparison *post hoc* test.

MCF-7 cells, the combined treatment of 5 μM PD98059 and CPF impaired colony formation showing an extreme sensibility to p-ERK1/2 inhibition when these cells are exposed to the pesticide (data not shown).

3.6. Phosphorylation of ERK1/2 is mediated by hydrogen peroxide

It has been demonstrated that ROS can act as a primary messenger modulating signaling cascades leading to various pathological conditions, such as cancer progression or death promoting effects. We hypothesized that CPF induces an increment of H_2O_2 which in turn promotes ERK1/2 phosphorylation conducing to cell proliferation inhibition. When we analyzed the participation of H_2O_2 in ERK1/2 phosphorylation induced by CPF, we found that both CAT and PD98059 abolish ERK1/2 phosphorylation increase induced by 50 μM of CPF, restoring the basal phosphorylation levels in both cell lines. These data illustrate that CPF promote the H_2O_2 increment which is required for ERK1/2 phosphorylation to drive the cells to death (Fig. 6A).

To confirm the responsibility of a rapid increase of ROS as a prior signaling event in p-ERK1/2 induction, we assayed the effect of CPF on intracellular ROS levels after 10 min of exposure in both cell lines (Fig. 6B). Our results indicate that 50 μM CPF induces a rapid increment of ROS content in both MCF-7 (46.4 ± 7.6% over control) and MDA-MB-231 (77.5 ± 7.8% over control) cells which could lead to ERK1/2 phosphorylation.

Finally, we studied if the increment of ERK1/2 phosphorylation induces in turn the increment of ROS generation after 24 h of CPF exposure. As shown in Fig. 6C, PD98059 could not abolish the increment of ROS induced by CPF, indicating that ERK1/2 increment is subsequent to ROS production induced by CPF but not the inverse.

4. Discussion

Chlorpyrifos continues to be one of the most commonly organophosphate pesticide used. We have previously demonstrated that 0.05 μM CPF promotes cell proliferation through ERα in the hormone-dependent breast cancer MCF-7 cells but higher concentrations of the pesticide leads to oxidative stress through an increment of H_2O_2 formation in MCF-7 and MDA-MB-231 breast cancer cells after 24 h of exposure to this pesticide. We had also speculated that another oxidative species were formed by CPF treatment in MDA-MB-231 cells (Ventura et al., 2012). Here, we evaluated the CPF action at different steps of redox balance, such as oxygen and nitrogen reactive species, antioxidant enzymes, lipid peroxidation and the signaling involved in the mechanism that triggers the cell growth inhibition.

To complete our studies RNS were also analyzed. We found that RNS were increased in MDA-MB-231 but not in MCF-7 cells exposed to 50 μM CPF. We have also observed that the pesticide causes a slight increment in protein-nitrosylation content in MDA-MB-231 cells. It could reflect that 50 μM CPF induce oxidative stress in both cell lines, but different reactive species would be generated in MDA-MB-231 compared to MCF-7 cells. We ascertain that H_2O_2 is the main responsible of the inhibition of cell proliferation induced by CPF.

To prevent the damage due to ROS formation, cells possess several antioxidant enzymes such SOD and CAT. SOD activity convert superoxide into hydrogen peroxide and its decomposition to water

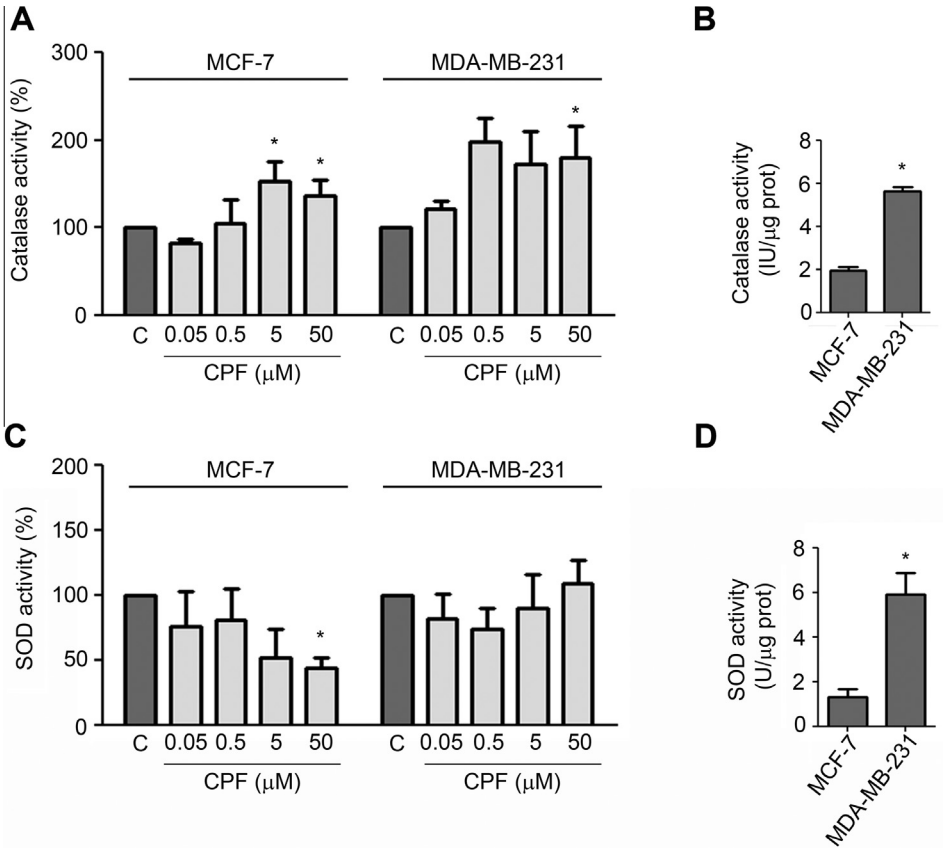

Fig. 2. CPF modifies antioxidant enzyme activities in breast cancer cells. CAT and SOD activities were evaluated in MCF-7 and MDA-MB-231 cells exposed to CPF (0.05, 0.5, 5 and 50 μM) or vehicle (ETOH 0.5%) for 24 h. Graphs show (A) CAT activity and (C) SOD activity as percentage of values obtained with each cell line control. Data are expressed as means ± SEM of three independent experiments, each performed by duplicates. $^{*}p < 0.05$ vs. *C*. One-way ANOVA followed by Dunnett's Multiple Comparison *post hoc* test. Basal CAT (B) and SOD activities (D) were evaluated in no exposed MCF-7 and MDA-MB-231 cells. Results were expressed as International Units of CAT activity per μg of protein (IU/μg of protein) and Units of SOD activity per μg of protein (U/μg of protein) respectively. $^{*}p < 0.05$. Unpaired two tailed *T* Test.

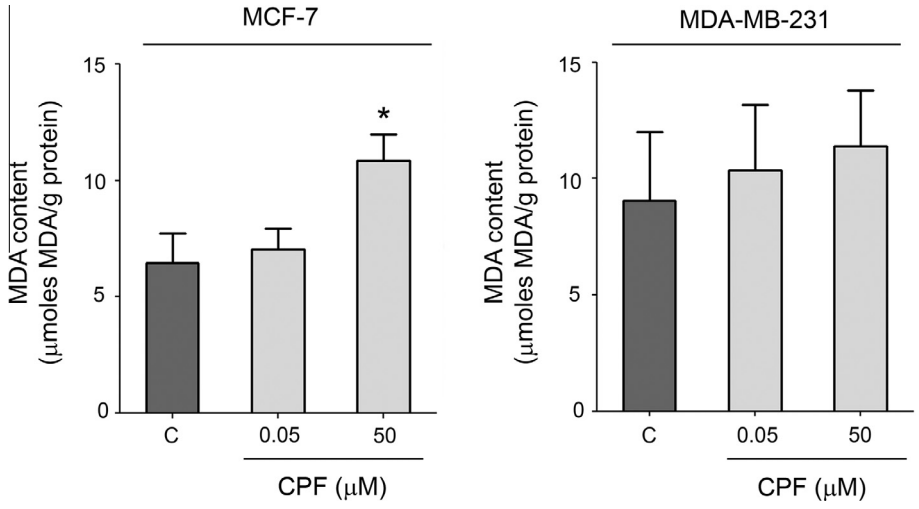

Fig. 3. Lipid peroxidation is increased by 50 μM CPF in MCF-7 cells. Cells were exposed to CPF (0.05 and 50 μM) or vehicle (ETOH 0.5%) for 24 h. Lipid peroxidation was evaluated by TBARS assay and quantified fluorometrically (515 nm, excitation; 555 nm, emission). Results were expressed as μmols of MDA per gram of protein (MDA/g of protein). Data represent the means ± SEM of three independent experiments, each performed by duplicates. $^{*}p < 0.05$ vs. *C*. One-way ANOVA followed by Dunnett's Multiple Comparison *post hoc* test.

and oxygen is further catalyzed by CAT. CPF modified the activities of antioxidant proteins in both cell lines. CAT activity was enhanced by 50 μM CPF in MCF-7 and MDA-MB-231 cells. It was reported that the finely and dynamically controlled antioxidant system acts to support the maintenance of a redox cellular state (Hsieh et al., 2010). It has been reported that CPF is able to increase ROS generation and the activities of antioxidant defense enzymes in *Drosophila melanogaster* exposed to the pesticide. These authors have observed that an increase of the enzymatic activities in the exposed organisms may be an attempt by them to counterbalance

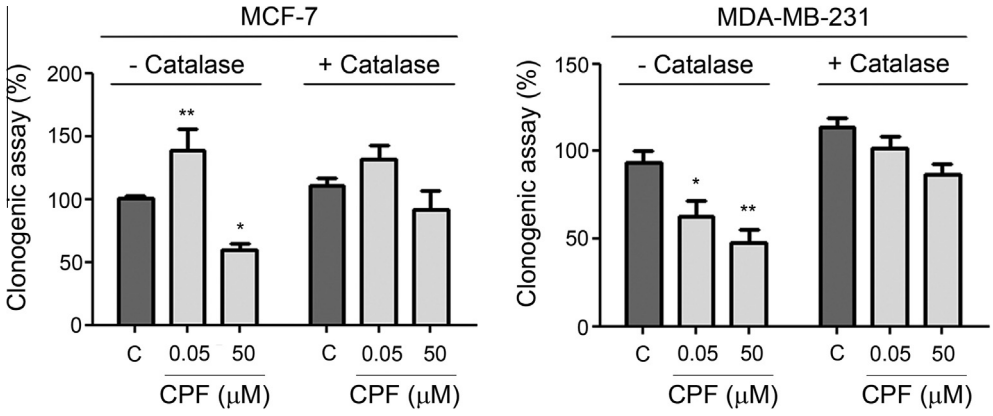

Fig. 4. H_2O_2 generation is involved in CPF-induced cell proliferation inhibition. Cells were exposed to CPF (0.05 and 50 μM) or vehicle (ETOH 0.5%) for 10 d in presence and absence of 30 IU mL^{-1} of CAT. Proliferation was evaluated by counting colonies with 50 cells or more. For each cell line, results are expressed as percentage respect its respective control. Data represent the means ± SEM of three independent experiments, each performed by duplicates. $^{*}p < 0.05$ vs. *C*; $^{**}p < 0.01$ vs. *C*. One-way ANOVA followed by Dunnett's Multiple Comparison *post hoc* test.

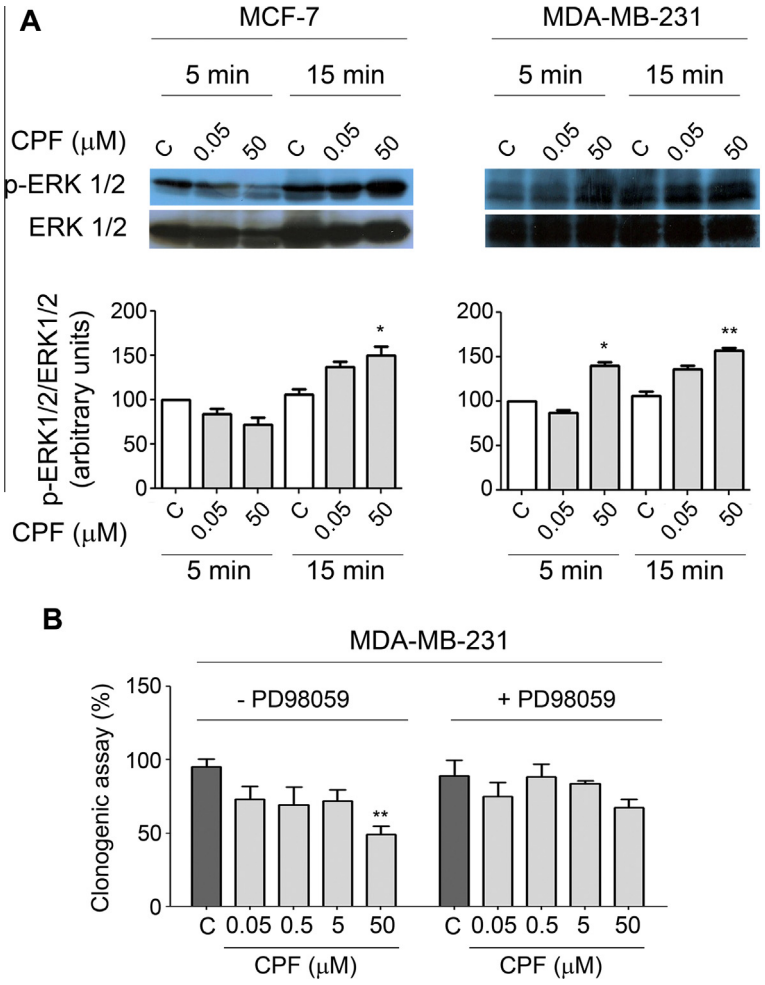

Fig. 5. ERK1/2 phosphorylation is involved in proliferation decrease induced by CPF 50 μM. (A) Starved cells were exposed to CPF (0.05 and 50 μM) or vehicle for 5 and 15 min. ERK1/2 phosphorylation was assayed by Western blot using total cell extracts. Western blots results from one representative experiment are shown in the upper panels. Quantification of p-ERK1/2/ERK1/2 ratios are shown in the lower panels. $^{*}p < 0.05$ vs. corresponding *C*; $^{**}p < 0.01$ vs. corresponding *C*. One-way ANOVA followed by Dunnett's Multiple Comparison *post hoc* test. (B) MDA-MB-231 cells were exposed to CPF (0.05, 0.5, 5 and 50 μM) or vehicle (ETOH 0.5%) for 10 d in presence and absence of 5 μM PD98059. Proliferation was evaluated by counting colonies with 50 cells or more. Data are expressed as means ± SEM of two independent experiments, each performed by duplicates $^{**}p < 0.01$ vs. *C*. One-way ANOVA followed by Dunnett's Multiple Comparison *post hoc* test.

the damage induced by ROS (Gupta et al., 2010). In contrast, SOD activity was only diminished in MCF-7 but not in MDA-MB-231 cells exposed to CPF 50 μM. This apparent discrepancy could be explained by the difference in basal levels of SOD found in both cell lines. In this regard, SOD catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide and it has been proved

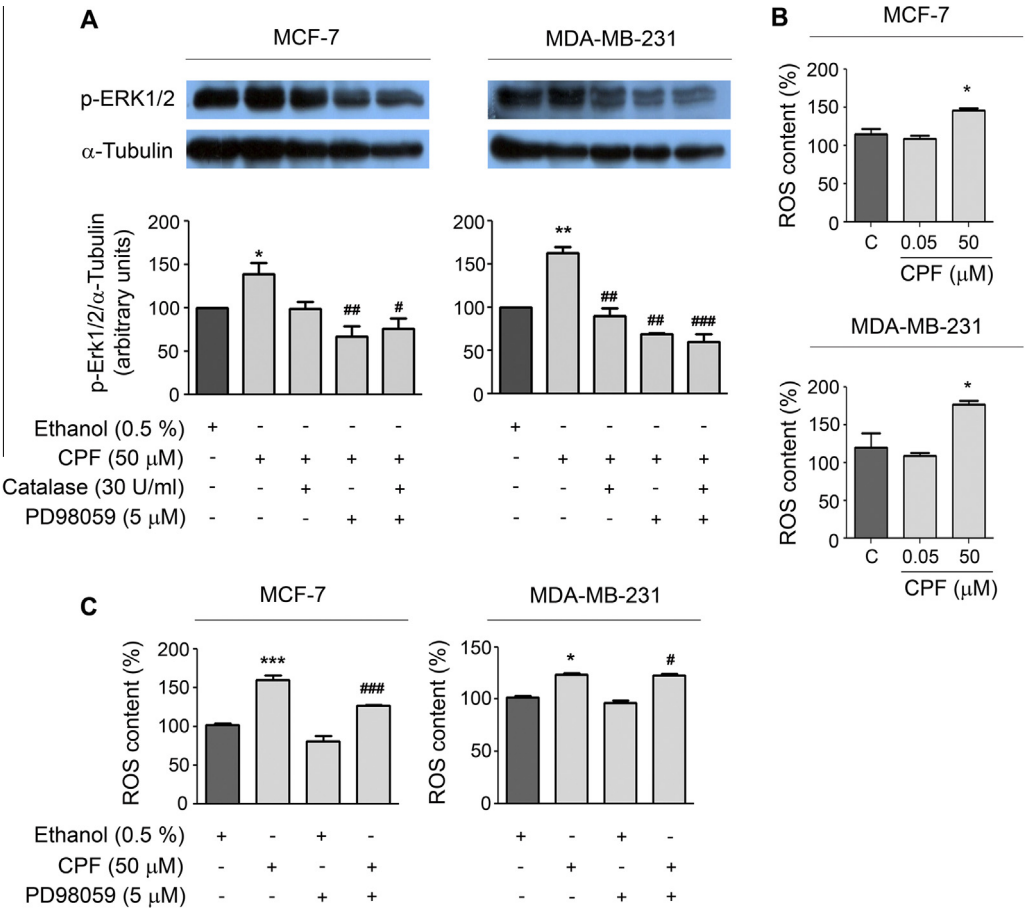

Fig. 6. H_2O_2 generation is involved in CPF-induced ERK1/2 phosphorylation. (A) Starved cells were exposed to vehicle or 50 μM CPF in presence and absence of 30 IU mL^{-1} of CAT or 5 μM PD98059 for 15 min. ERK1/2 phosphorylation was assayed by Western blot using total cell extracts. Western blots from one representative experiment are shown in the upper panels. Quantification of p-ERK1/2/α-tubulin ratios is shown in the lower panels. Data are expressed as the mean ± SD of two independent experiments. $^{*}p < 0.05$ vs. C; $^{**}p < 0.01$ vs. C; $^{\#}p < 0.05$ vs. 50 μM CPF; $^{\#\#}p < 0.01$ vs. 50 μM CPF; $^{\#\#\#}p < 0.001$ vs. 50 μM CPF. One-way ANOVA followed by Tukey *post hoc* test. (B) Cells were exposed to CPF (0.05 and 50 μM) or vehicle during 10 min. Intracellular ROS was analyzed by flow cytometry using the fluorescent dye DCF-2DA. The graphs show the mean fluorescence intensity as percentage of the respective control. Results are expressed as mean ± SEM of two independent experiments, each one performed by triplicate. $^{*}p < 0.05$ vs. C. One Way ANOVA and Dunnett's Multiple Comparison *post hoc* test. (C) Cells were exposed to vehicle and 50 μM CPF in presence and absence of 5 μM PD98059 for 24 h. Intracellular ROS was analyzed by flow cytometry using the fluorescent dye DCF-2DA. The graphs show the mean fluorescence intensity as percentage of the respective control. Results are expressed as mean ± SEM of two independent experiments, each one performed by triplicate. $^{*}p < 0.05$ vs. C; $^{***}p < 0.001$ vs. C; $^{\#}p < 0.05$ vs. C + PD98059; $^{\#\#\#}p < 0.001$ vs. C + PD98059. One Way ANOVA and Tukey *post hoc* test.

that high levels of H_2O_2 are capable to inhibit SOD activity as this enzyme is sensitive to its own product (Weisiger and Fridovich, 2013). In view of our results and taken into account that SOD basal levels in MDA-MB-231 cells were found higher than those in MCF-7 cells, it is possible that the increment of ROS produced in MDA-MB-231 cells could not be enough to inhibit SOD activity in these cells. SOD activity was also found diminished in other systems like PC12 neuronal cells where CPF-induced oxidative stress was associated with alterations in enzymes related to endogenous antioxidant defense systems such as HO-1, CuZnSOD and MnSOD (Lee et al., 2012).

Lipid peroxidation is a free radical-driven reaction which causes membrane damage by reaction of oxygen with polyunsaturated fatty acids (PUFAs) (Barrera, 2012). We have found an augment in the content of MDA when MCF-7 cells were exposed to CPF at the dose which was capable to increase ROS generation. The increment of MDA content has been correlated to protein modifications and possibly membrane damage in the exposed cells which could then derivate the cells to death (Ziech et al., 2010). In our experiments, we detected that MDA-MB-231 cells show higher antioxidant capacity and can restore the redox balance more efficiently than MCF-7 cells. This discrepancy may explain the increment in MDA production that was observed in MCF-7 cells only.

In addition, it was described that H_2O_2 activates protein kinases such as ERK1/2 (Zhou et al., 2007), phosphoinositide 3-kinase/serine-threonine kinase (PI3 K/Akt) (Jiao et al., 2010), protein kinase B (PKB) (Mehdi et al., 2007) and protein tyrosine phosphatases (PTPs) (Wu, 2006). Because these pathways regulate cellular migration, proliferation, survival and death responses, their aberrant activation has been suggested to be a potential mechanism of ROS-induced carcinogenesis (Klaunig et al., 2010). In our experiments, we found a rapid increment of p-ERK1/2 when the cells were exposed to 50 μM CPF. Furthermore, we showed that this augment was accompanied by a rapid increment of ROS after 10 min of exposure to 50 μM CPF in both lines. We have previously reported that 50 μM CPF inhibits clonogenic capacity in breast cancer cells. Then, we proved that p-ERK1/2 is involved in cell growth inhibition using PD98059, a potent and selective inhibitor of MEK-1, which could restore the cell expansion inhibited by the pesticide.

With the aim of evaluating if p-ERK1/2 was induced by the increment of H_2O_2 we analyzed the effect of CAT on ERK1/2 phosphorylation induced by CPF. As this enzyme restored p-ERK1/2 levels in MCF-7 and MDA-MB-231 cells, we conclude that the main mechanism involved in the inhibition of cell proliferation induced by CPF is an increment of p-ERK1/2 levels mediated by H_2O_2 in breast cancer cells. In view of our results, we claim that the incre-

ment of ROS is upstream of ERK1/2 phosphorylation. As ERK1/2 phosphorylation could in turn affect ROS generation induced by CPF and a cross-regulation between the two is possible, we have studied the increment of ROS in presence of PD98059 after 24 h of CPF exposure. The increment of ROS was non-dependent of p-ERK1/2 showing that 50 μM CPF inhibits the clonogenic capacity of breast cancer cells and this inhibition may be adjudicated to the increment of ROS production which in turn promotes the phosphorylation of ERK1/2 driving the cells to death. However, other mechanism could be promoted by the increment of ROS which could contribute to cancer promotion. We demonstrated that a significant number of cells die after CPF exposure while an important group of cells is able to survive after treatment. In view that the increased ROS in cancer cells may affect certain redox-sensitive molecules and lead to cellular proliferation or differentiation, alteration in sensitivity to anticancer agents, promotion of mutations and genetic instability, it is possible that carcinogenesis process could be generated in the cells that stay alive.

US EPA has not yet recognized evidences of CPF-induced carcinogenicity in animals. However, a rising number of investigations indicate that CPF may be carcinogenic in human and rodents. An association between CPF use and lung cancer incidence was found in an agriculture health study (Lee et al., 2004). A relationship between diseases affecting public health and environmental exposures, particularly pesticides increasingly continues to strengthen. There are a significant number of studies that inform about the association between agrochemicals and cancer risk. In this sense it is necessary to find the way of reducing the use of pesticide and find alternatives for agriculture avoiding the use of agrochemicals.

In conclusion, in this study we demonstrated for the first time, that CPF (50 μM) induces redox imbalance in breast cancer cells. Furthermore, we found that the main reactive specie generated by CPF is H_2O_2 which promotes ERK1/2 phosphorylation. This mechanism triggers inhibition of cell proliferation in both dependent and independent-hormone MCF-7 and MDA-MB-231 cells.

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