Dichlorvos Exposure Results in Activation Induced Apoptotic Cell Death in Primary Rat Microglia

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Dichlorvos-Induced Cell Cycle Arrest and DNA Damage Repair Activation in Primary Rat Microglial Cells

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Dichlorvos, an organophosphate (OP), is known to cause oxidative stress in the central nervous system (CNS). Previously we have shown that dichlorvos treatment promoted the levels of proinflammatory molecules and ultimately induced apoptotic cell death in primary microglial cells. Here we studied the effect of dichlorvos on crucial cell cycle regulatory proteins and the DNA damage sensor ataxia-telangiectasia mutated (ATM). We found a significant increase in p53 and its downstream target, p21, levels in dichlorvos-treated microglial cells compared with control cells. Moreover, dichlorvos exposure promoted the levels of different cell cycle regulatory proteins. These results along with flow cytometry results suggested that primary microglial cells were arrested at G1 and G2/M phase after dichlorvos exposure. We have shown in a previous study that dichlorvos can induce DNA damage in microglia; here we found that microglial cells also tried to repair this damage by inducing a DNA repair enzyme, i.e., ATM. We observed a significant increase in the levels of ATM after dichlorvos treatment compared with control.

Key words: Dichlorvos; microglia; cell cycle; p53; p21; ATM

Microglia are resident macrophages, which remain quiescent in the normal central nervous system (CNS). They are activated in response to various pathological conditions and remove damaged cells by phagocytosis (Gehrmann and Banati, 1995). Recently we have shown that these cells become activated and start producing proinflammatory molecules after dichlorvos exposure (Sunkaria et al., 2012). A growing body of evidence indicates that excessive expression of these activated microglia-secreted factors is deleterious to neurons (Boje and Arora, 1992; McGuire et al., 2001). However, in addition to acting as debris scavengers, activated microglia can also secrete neurotrophic factors and growth factors, suggesting that they have beneficial roles in CNS repair. Therefore, it is important to control their detrimental functions selectively (Kreutzberg, 1996).

In a previous study, we have shown that dichlorvos exposure can induce significant DNA damage in microglial cells (Sunkaria et al., 2012). Dichlorvos has also been found to induce DNA damage in human lymphocytes (Atherton et al., 2009) and might be responsible for micronucleus formation in Chinese hamster cells (Oshiro et al., 1991). Aschner et al. (1999) have suggested the possible involvement of nitric oxide (NO) in organophosphate (OP)-mediated neurotoxicity. Activated microglia are known to produce high levels of NO, which rapidly react with O_2– to produce peroxynitrite anion (Banati et al., 1993; Brosnan et al., 1994). The NO causes intracellular damage mainly by oxidizing DNA and nitrosylating proteins (Kawanishi et al., 2006).

The normal cellular response to DNA damage involves activation of checkpoint pathways that impose the delay of cell cycle progression and control DNA repair and replication (Khanna et al., 1998). In the past decade, the precise mechanisms regulating cell cycle machinery, especially in nonneural cells, have been revealed (Bollen and Beullens, 2002; Sherr and Roberts, 1995). Progression through the cell cycle is controlled by sequential expression of cyclins, cyclin-dependent kinases (CDKs), CDK inhibitors (CDKIs), phosphatases, and so on.

After DNA damage, ATM (a serine/threonine protein kinase) undergoes rapid autophosphorylation and dimer dissociation, leading to its activation (Bakkenist and Kastan, 2003). Among other targets, activated ATM phosphorylates p53 on serine 15 (Banin et al., 1998; Khanna et al., 1998) and indirectly, via Chk2, on serine 20 (Chehab et al., 2000). Both events contribute to p53 nuclear accumulation and transcriptional induction of p21, an inhibitor of the cyclin-dependent kinase 2/cyclin E complex required for cell cycle progression from G1 to S phase (Canman et al., 1994; el-Deiry et al., 1994;

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TABLE I. Primer Sequences, PCR Conditions, and Sizes of PCR Products

Gene	Forward sequence	Reverse sequence	Tm (°C)	Product size (bp)
p53	GCTTCGAGATGTTCCGAGAG	AGGATGCAGAGGCTGTCAGT	57.8	380
p21	TCAGTGGACCAGAAGGGAAC	GGTCCCCATCCCAGATAAGT	57.1	197
ATM	GGCTACCAAGACCGGACATA	TCCAAATGTCATGGCTTTCA	58.2	194
Chk2	TGGCAAGGAATGGATAGGAG	GTGAGAGGTCGTGCAACTGA	57.8	197
Cdc25A	TGTACGGGAACGAGATAGGC	TCTTGGTGCGGAACTTCTTT	57.4	182

Bartek and Lukas, 2001). The functional link between Chk2 and p53 in the DNA damage has been further substantiated in recent studies showing that Hdmx, a negative regulator of p53, is directly phosphorylated by Chk2 and that this event accelerates Hdmx degradation (Chen et al., 2005; LeBron et al., 2006). DNA damage is also linked to the G1/S checkpoint via the p53-p21 and Cdc25A-CDK2 pathways. Whereas the p21 link has been well established, the involvment of the Cdc25A pathway in this context remains to be elucidated completely.

Cellular checkpoints are signaling pathways evolved in eukaryotes to maintain genomic integrity. Several reports have shown that checkpoint activation by genotoxins such as OPs results in cell cycle arrest and repair of the damage, senescence or apoptosis (Mattiuzzo et al., 2006; Carlson and Ehrich, 2008). It is reported that dichlorvos showed stronger apoptosis-inducing ability than chlorpyrifos at lower doses in human natural killer (NK) cells (Li et al., 2007). Our previous study has shown that dichlorvos can induce significant DNA damage and apoptosis in microglial cells (Sunkaria et al., 2012). Therefore, in the present study, we wanted to check the sequence of events happening just before microglia become committed for apoptotic cell death, with a main emphasis on cell cycle regulatory molecules.

MATERIALS AND METHODS

Reagents and Antibodies

Minimum essential medium (MEM; SH30008.02) was purchased from Hyclone Thermo Scientific (Logan, UT). Fetal bovine serum (FBS; 10082-139) and 0.25% trypsin were purchased from Invitrogen (Carlsbad, CA), and 96- and six-well tissue culture plates, 25 cm^2 T-flasks and cell scrapers were purchased from Greiner Bio-One (St. Gallen, Switzerland). Syringe filters (0.2 μl) were purchased from Millipore (Bedford, MA). Griess reagent (G4410) and dichlorvos (45441) were procured from Sigma-Aldrich (St. Louis, MO). ATM (mouse monoclonal; sc-23921), p53 (rabbit polyclonal; sc-6243), p21 (mouse monoclonal; sc-6246), cyclin A (goat polyclonal; sc-31086), Chk2 (rabbit polyclonal; sc-9064) and Cdc25A (rabbit polyclonal; sc-7157) primary antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA), and secondary antibodies were purchased from Bangalore Genei (Bangalore, India). All other chemicals used in this study were of tissue culture grade.

Microglial Cell Culture

For all experiments, Wistar rats were used, which were bred and kept under constant conditions (12 hr light/12 hr dark cycle) in the central animal house of our Institute. Neonatal pups of 1–2 days were sacrificed as per the guidelines of our Institutional Animal Ethical Committee. All efforts were made to minimize the number of animals used and their suffering. Microglial cells were obtained from the mixed brain cell culture of newborn pups as previously described, with slight modifications (Sunkaria et al., 2012).

Experimental Design

The study was divided into two groups: 1) Control group, primary microglial cells cultured and maintained in minimum essential medium (MEM) supplemented with 10% FBS for 3 DIV; 2) dichlorvos-treated group, primary microglial cells cultured and maintained in minimum essential medium (MEM) supplemented with 10% FBS for 3 DIV and then treated with 10 μM (one-third of IC50) of dichlorvos for 24 hr.

Cell Cycle Analysis

For each experiment, 5×10^6 harvested cells were washed in ice-cold phosphate-buffered saline (PBS; pH 7.4), fixed in ice-cold 70% ethanol, and stored at 4°C. Cells were then washed with PBS, treated with 0.5 mg/ml RNase (Sigma) at 37°C for 15 min, and finally stained with propidium iodide (Sigma; 50 μg/ml) in PBS. Cell cycle analysis was performed with a Becton-Dickinson (San Jose, CA) fluorescence-activated cell analyzer. The samples were kept in the dark for 2 hr at 4°C before further analysis, and 10,000 cells were counted for each determination. For detailed analysis, the three cell cycle compartments (G0/G1, S, and G2/M phases) were distinguished, and the percentage of cells was quantified with CELLQuest software (Becton-Dickinson).

Semiquantitative PCR

Microglial cells (2×10^6 cells/well) were cultured in six-well tissue culture plates. Total RNA was extracted from control and dichlorvos-treated microglial cells with a Total RNA Extraction Kit (Taurus Scientific). Extracts were assayed to determine the quality and concentration of the RNA using a spectrophotometer. Extracts were stored at −20°C. Isolated RNA was then digested by DNase (Promega Germany) to destroy contaminating DNA, and cDNA was synthesized with RevertAidH Minus M-muLV Reverse Transcriptase (Fermentas Germany). cDNA (10 ng) was subjected to reverse transcriptase-PCR amplification. For primer sequences, PCR conditions, and sizes of PCR products see Table I.

Immunoblot Analysis

The protein was isolated from microglial control as well as dichlorvos-treated (24 hr) cells. Samples containing 75 μg protein were boiled in Laemmli buffer for 5 min and sub-

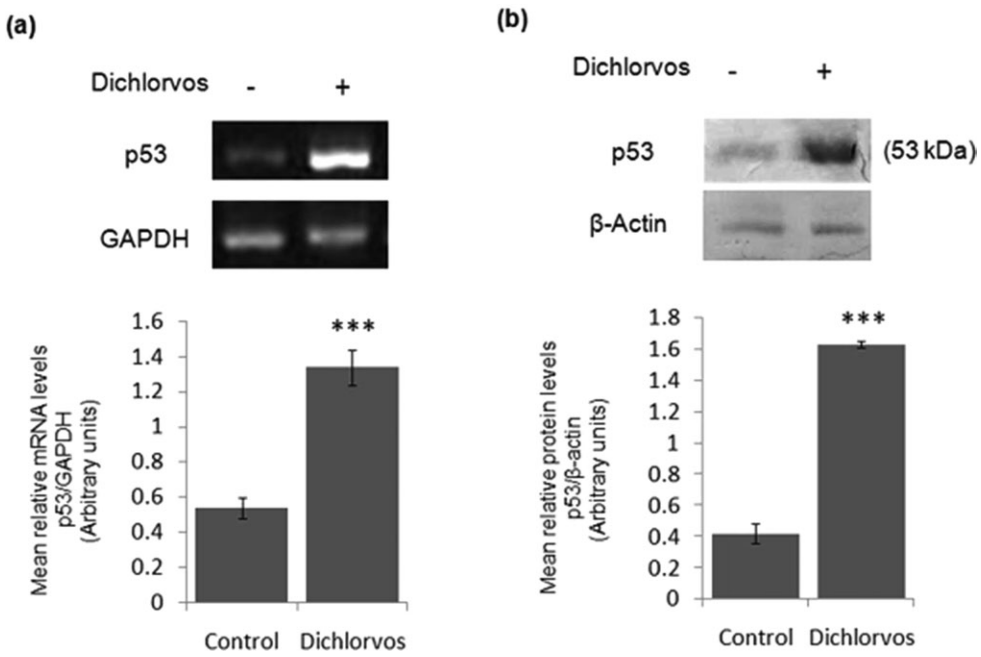

Fig. 1. Effect of dichlorvos exposure on p53 expression. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37°C for 3 DIV. **a**: Semiquantitative PCR results show a significant increase in p53 mRNA levels in the presence of dichlorvos compared with control cells. **b**: Western blot analysis shows a significant increase in p53 protein levels in the presence of dichlorvos compared with control cells. Data are expressed as mean ± SEM of three independent experiments. $^{***}P < 0.001$ vs. control group.

jected to electrophoresis (12% SDS-PAGE), followed by transfer to nitrocellulose membrane. The blots were blocked with 5% nonfat dry milk for 5 hr, and the membranes were then incubated with primary ATM (1:500)/p53/p21 (1:500)/cyclin A (1:1,000)/Chk2 (1:500)/Cdc25A (1:500)/β-actin (1:100) at room temperature for 3 hr. After incubation, the nitrocellulose membrane was washed with PBS plus 0.1% Tween-20 for 30 min with 5-min intervals. Membrane was again incubated for 1 hr at 37°C with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000). After 1 hr of incubation, the blots were again washed with PBS plus 0.1% Tween-20 for 30 min with 5 min intervals. Immunoreactive proteins were visualized with diaminobenzidine (DAB). The densitometry analysis of the protein bands was carried out using AlphaEase FC software to compare the relative expression of proteins.

Protein Estimation

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistical Analysis

For all experiments, data were analyzed from at least three independent experiments, each with at least duplicate determinations. Student's *t*-test was applied to compare the statistical difference between control and dichlorvos-treated cells. Statistical analysis of the data was performed in SigmaStat 3.5 software. $P \leq 0.05$ was considered statistically significant. Error bars represent SEM.

RESULTS

Dichlorvos Induces p53 Expression in Primary Microglial Cells

Accumulation of the tumor suppressor p53 is known as a negative regulator of the cell cycle and an established target of NO. Therefore, our first objective was to check the expression of p53 in microglial cells after dichlorvos exposure. Western blot analysis displayed very negligible expression of p53 in controls. However, semiquantitative PCR and Western blot analysis showed significant increase in the expression levels of p53 (~2.5-fold) after treating with dichlorvos (10 μM) for 24 hr (Fig. 1). Results are expressed as mean of three independent experiments.

Dichlorvos Induces p21 Expression in Primary Microglial Cells

Because p53 is known to drive expression of the cell cycle inhibitor p21, we wanted to check the levels of p21 in response to dichlorvos exposure. We found significant increase in the p21 mRNA (~2-fold) and protein (~2-fold) expression after 24 hr of treatment (Fig. 2). Results are expressed as mean of three independent experiments.

Dichlorvos Elicits G2/M Arrest in Primary Microglial Cells

In the next experiment, we explored the change in cell cycle distribution of microglial cells when exposed to dichlorvos. The microglial cells were exposed to

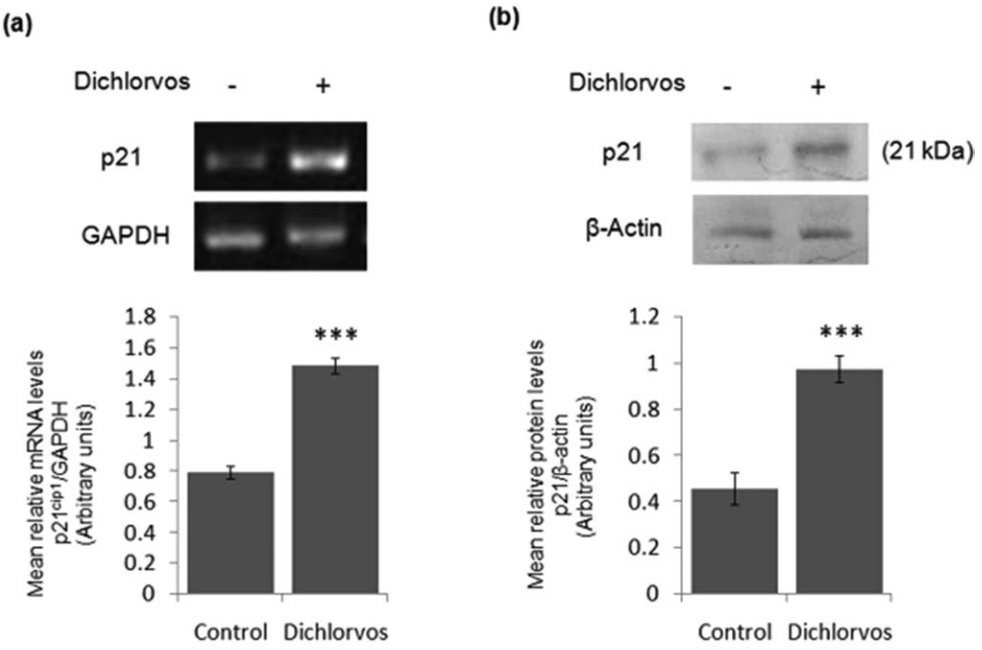

Fig. 2. Effect of dichlorvos exposure on p21 expression. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37°C for 3 DIV. **a**: Semiquantitative PCR results show a significant increase in p21 mRNA levels in the presence of dichlorvos compared with control cells. **b**: Western blot analysis shows a significant increase in p21 protein levels in the presence of dichlorvos compared with control cells. Data are expressed as mean ± SEM of three independent experiments. $***P < 0.001$ vs. control group.

dichlorvos for 24 hr and evaluated by propidium iodide-based cell cycle analysis. We observed that dichlorvos exposure resulted in an almost 137.51% increase in the G2/M microglial cell population after 24 hr compared with control cells. However, approximately 7.12% increase was observed in the G0/G1 population and 62.61% decrease was observed in the S phase population of microglial cells, whereas the sub-G1 population showed a 41.82% increase after dichlorvos treatment (Fig. 3). Results are expressed as mean of three independent experiments.

Dichlorvos Mediates Induction of DNA Damage Repair

ATM is a serine/threonine protein kinase that is recruited and activated by DNA double-strand breaks. We have shown in our previous study that dichlorvos can induce DNA fragmentation in microglial cells. Here we wanted to check whether one of the main DNA damage sensor and repair enzymes, i.e., ATM, becomes activated in response to the damage observed in microglia. Significant upregulation (~2-fold) was observed in the levels of ATM mRNA and protein when treated with dichlorvos (Fig. 4). Results are expressed as mean of three independent experiments.

Dichlorvos Modulates Expression of Cell Cycle Regulatory Proteins

To confirm the flow cytometry results, we checked the expression levels of different cell cycle regulators. Semiquantitative PCR (~2.5-fold) and Western blot (~2-fold) results showed significant increases in the Chk2 levels when microglia were exposed to dichlorvos (Fig. 5). We also observed significant increases in the Cdc25A mRNA (~2-fold) and protein (~2-fold) expression after 24 hr of treatment compared with control (Fig. 6). Results are expressed as mean of three independent experiments.

DISCUSSION

Very few reports describing the role of OPs in CNS are available in the literature. In our previous study, we have shown that, in response to dichlorvos exposure, microglial cells underwent apoptotic cell death, and the major players involved in this were reactive nitrogen species and proinflammatory cytokines (Sunkaria et al., 2012). The present study further increases our understanding of the effects of OPs in the CNS by revealing the molecular events occurring in microglia, prior to undergoing apoptotic cell death. OPs are known to be acetylcholinesterase inhibitors; however, several studies have suggested that they are also able to damage DNA (Eroglu, 2009; Sunkaria et al., 2012). The cellular response to genotoxic stress is initiated by the activation of the ATM and ATR (ATM and Rad-3 related) protein kinases. These kinases phosphorylate various target proteins, including p53, the checkpoint kinases Chk1 and Chk2, and p21. These proteins along with many others act to slow the cell cycle progression, to enhance

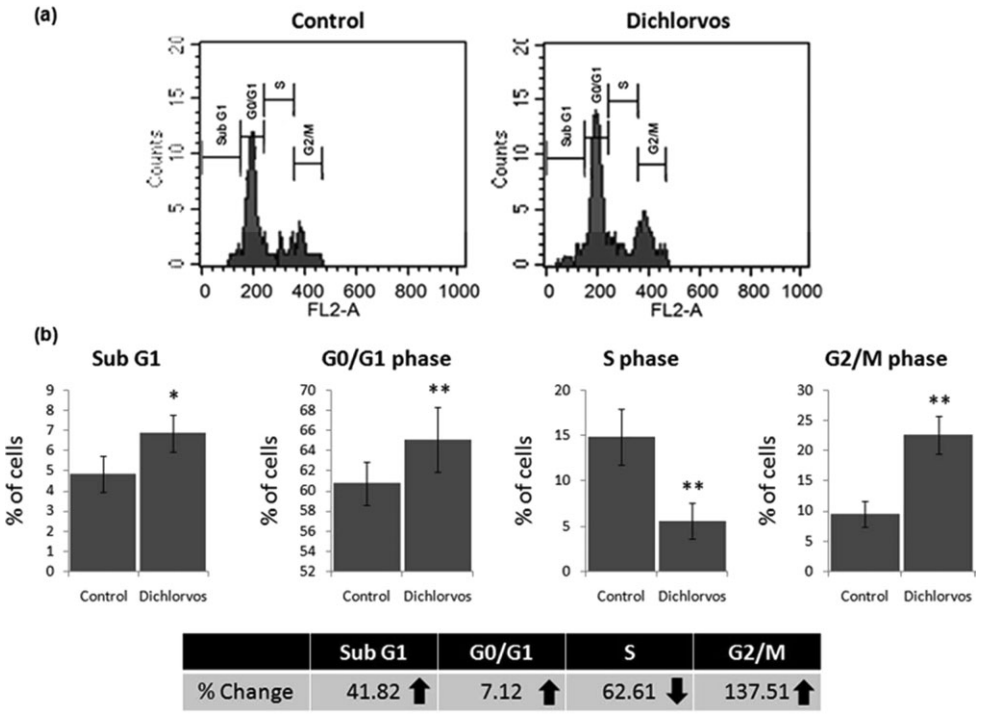

Fig. 3. Changes in cell cycle distribution after dichlorvos exposure. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37°C for 3 DIV. **a**: Flow cytometry results show the distribution of microglial cells in different phases of cell cycle when treated with dichlorvos for 24 hr. **b**: Bar graphs show percentage of cells in each phase of cell cycle after dichlorvos exposure. Table depicts percentage change in each phase. Data are expressed as mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

the DNA repair capacity of cells, or to direct cells to apoptosis.

Dichlorvos has been shown to exert cytotoxic effects in rat hepatocytes (Yamano, 1996) as well as a rat tracheal epithelial cell line (Lin et al., 1988). DNA strand breakage caused by dichlorvos is correlated with its DNA alkylating property in cultured Chinese hamster cells (Green et al., 1974). Dichlorvos was also found to induce DNA damage in human lymphocytes (Atherton et al., 2009) and micronucleus in CHO cells (Oshiro et al., 1991). Based on this, we hypothesized that, in response to dichlorvos exposure, DNA damage signaling pathways should also be activated, which would result in p53 induction and cell cycle arrest or apoptosis, if repair does not occur. Therefore, we checked the expression levels of master regulator of cell cycle, i.e., p53 in microglial cells after dichlorvos exposure. We observed significant increase in the expression of p53 both at transcription and at translational levels after 24 hr of treatment. This would suggest that 10 μM dichlorvos caused an increase in p53 levels as a result of the DNA damage in microglial cells. These results are consistent with previous reports showing induction of p53 levels in different cells after pesticide exposure (Yang and Tiffany-Castiglioni, 2008; Saquib et al., 2011).

It is established that p53 may initiate cell-cycle arrest or apoptosis, with the former being at least in part mediated by p53-dependent transcriptional activation of p21, a potent inhibitor of cell cycle kinases (Ho et al., 1996; Gartel and Tyner 1999). Several observations suggest that the failure to express sufficient levels of p21 converts the normal cell-cycle arrest into apoptotic cell death (Stewart et al., 1999). Therefore, to understand the fate of microglial cells, it was mandatory to check the levels of p21 after the dichlorvos exposure. In accordance with previous reports, we observed a significant increase in the levels of p21 in dichlorvos-treated cells compared with control cells. Several reports have suggested that p21 plays an essential role in growth arrest after DNA damage (Brugarolas et al., 1995), and its overexpression leads to G1- and G2- (Niculescu et al., 1998) or S-phase arrest (Ogryzko et al., 1997). In another study, OPs were shown to increase the levels of p21 and other DNA damage-responsive genes *in vitro* (Hreljac et al., 2008). These reports support our results and suggest that dichlorvos exposure can induce the expression of p21 in microglial cells.

Signal transduction upon DNA damage involves the sequential activation of protein kinases and dynamic association with other interactors or adaptors, together amplifying the signal elicited by even a single DNA lesion. In response to DNA double-strand breaks (DSBs), ATM, the protein kinase defective in ataxia telangiectasia (McKinnon, 2004), plays a fundamental role as the first activator of the damage response (Lavin et al., 2005) by phosphorylating a wide range of target

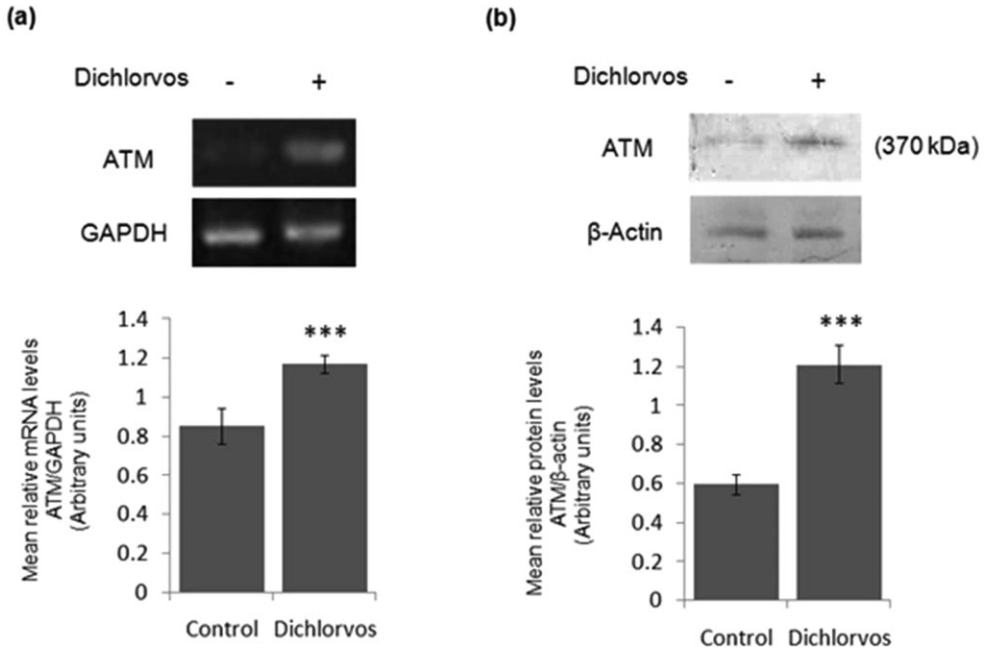

Fig. 4. Effect of dichlorvos exposure on ATM expression. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37°C for 3 DIV. **a**: Semiquantitative PCR results show a significant increase in ATM mRNA levels in the presence of dichlorvos compared with control cells. **b**: Western blot analysis shows a significant increase in ATM protein levels in the presence of dichlorvos compared with control cells. Data are expressed as mean ± SEM of three independent experiments. ***$P < 0.001$ vs. control group.

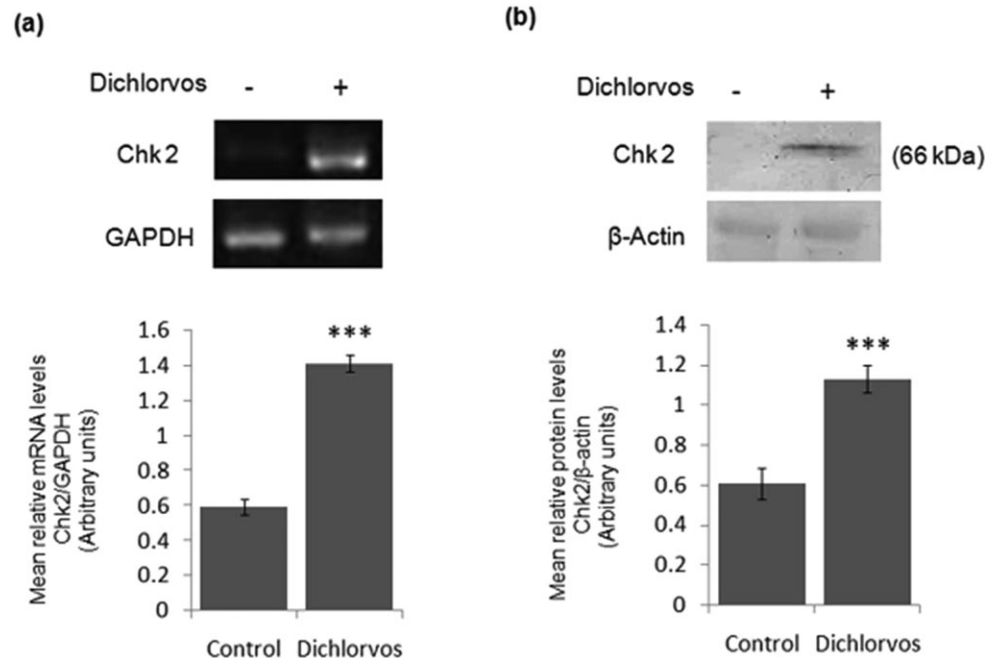

Fig. 5. Effect of dichlorvos exposure on Chk2 expression. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37°C for 3 DIV. **a**: Semiquantitative PCR results show a significant increase in Chk2 mRNA levels in the presence of dichlorvos compared with control cells. **b**: Western blot analysis shows a significant increase in Chk2 protein levels in the presence of dichlorvos compared with control cells. Data are expressed as mean ± SEM of three independent experiments. ***$P < 0.001$ vs. control group.

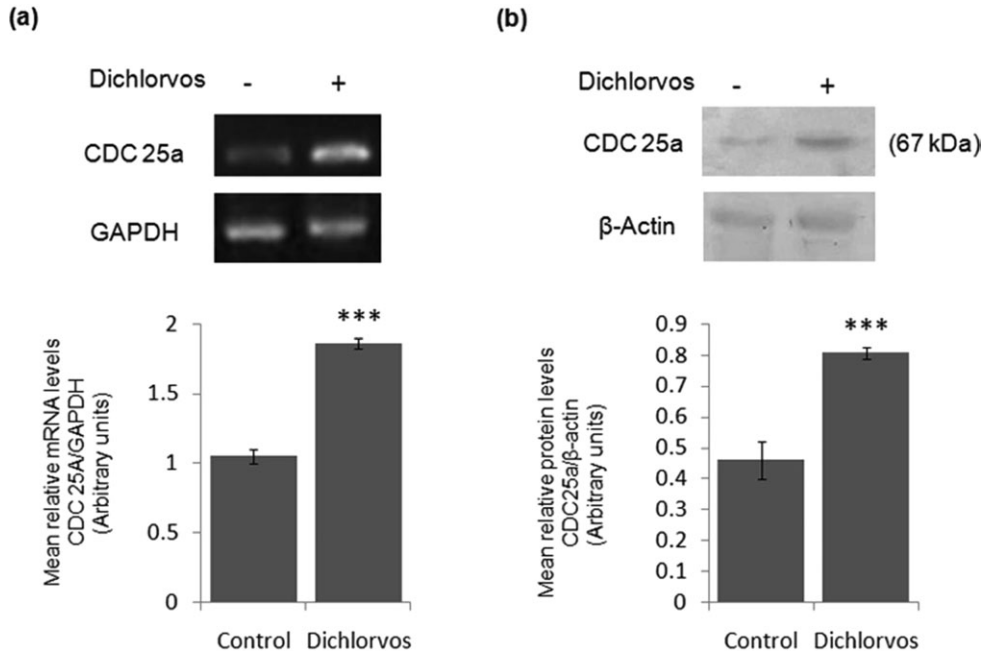

Fig. 6. Effect of dichlorvos exposure on Cdc25A expression. Primary microglial cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum and incubated in 5% CO_2 at 37°C for 3 DIV. **a**: Semiquantitative PCR results show a significant increase in Cdc25A mRNA levels in the presence of dichlorvos compared with control cells. **b**: Western blot analysis shows a significant increase in Cdc25A protein levels in the presence of dichlorvos compared with control cells. Data are expressed as mean ± SEM of three independent experiments. $^{***}P < 0.001$ vs. control group.

proteins. Human Chk2 is a kinase directly activated by phosphorylation on threonine 68 (T68) by ATM following DNA damage (Ahn et al., 2002). Activated Chk2 propagates the damage signal through the phosphorylation of several targets involved in cell cycle phase progression or apoptosis (Bartek and Lukas, 2001). We found a significant increase in the mRNA as well as protein levels of ATM and Chk2 in microglial cells when exposed to dichlorvos. These findings are in association with previous reports showing activation of ATM and its downstream effectors in response to various genotoxicants (Gastaldo et al., 2007). Once activated, Chk2 can phosphorylate several key substrates, including Cdc25A, p53, Brca1, the promyleocytic leukemia protein (PML), and E2F-1, which is required to mediate cell cycle arrest, DNA repair, and apoptosis (Bartek and Lukas, 2001).

Cdc25A, one of the three members of the Cdc25 dual-specificity phosphatase family, plays a critical role in the control of the cell cycle and in the checkpoint response to DNA damage. It has been found that, by phosphorylating and targeting Cdc25A for degradation, Chk2 induces arrest at G1, S, and G2/M phases (Matsuoka et al., 1998; Falck et al., 2001). We assessed the Cdc25A expression after dichlorvos exposure and observed a significant increase in its mRNA as well as protein levels compared with control. Our results are in association with previous studies demonstrating the role of Cdc25A in cell cycle arrest (Falck et al., 2001). Flow cytometric analysis also supports our Western blot results and shows a significant increase in the G1 and G2/M population of microglial cells in response to dichlorvos exposure compared with control. These results are supported by another study in which human lymphoblastoid AHH-1 cells were treated with dichlorvos and marked accumulation of cells in the G1 and G2/M phase of the cell cycle was observed (Mattiuzzo et al., 2006). In addition to our previous study, these results suggest that, in response to dichlorvos exposure, microglia may try to check the damage by arresting the growth at different cell cycle checkpoints and activating the repair machinery, but, if the damage is beyond repair, they undergo apoptotic cell death.

CONCLUSIONS

To maintain genome integrity, eukaryotic cells have evolved signalling pathways that are activated in response to genotoxic damage. These checkpoint pathways halt the cell cycle to provide extra time for DNA repair or, if the damage cannot be repaired, to induce apoptosis. Here we have elucidated the molecular events that take place in microglial cells after dichlorvos exposure, just before commitment to apoptosis. Expression of various cell cycle regulatory genes was analyzed after dichlorvos exposure, and a significant increase in the number of microglial cells that were arrested in G1 and G2/M phase was found. We also observed significant

increases in the ATM expression in response to dichlorvos exposure, which suggests that microglia first try to repair the damage caused by dichlorvos, but, if the damage is beyond repair, the microglia will undergo apoptosis.

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